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# Immobilized enzymatic fluorescence capillary biosensor for determination of sulfated bile acid in urine

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ABSTRACT

The authors have proposed an immobilized enzymatic fluorescence capillary biosensor (SBAs-IE-FCBS) for the determination of sulfated bile acids (SBAs). The reaction principle of the biosensor is that under the catalysis of the bile acid sulfate sulfatase (BSS) and β-hydroxysteroid dehydrogenase (β-HSD) immobilized on inner surface of a medical capillary, SBAs desulfates to 3β-hydroxyl bile acids, then the latter reacts with nicotinamide adenine dinucleotide ($NAD^+$), and is converted into 3-ketosteroid; meanwhile, $NAD^+$ is converted to reduced nicotinamide adenine dinucleotide (NADH). NADH continuously reacts with 1-methoxy-5-methylphenazinium methyl sulfate (1-MPMS) and is converted into $NAD^+$ circularly and 1-$MPMSH_2$. Finally resazurin is reduced into resorufin by 1-$MPMSH_2$, the formed resorufin ($\lambda_{ex}/\lambda_{em}$: 540 nm/580 nm) is used for quantifying the concentration of SBAs.

Optimized conditions being suitable with the biosensor are as follows: the concentrations of BSS and β-HSD used for the immobilization all are 5 kU $L^{-1}$; the concentrations of 1-MPMS and resazurin all are 25 μmol $L^{-1}$; the concentrations of Tris–HCl buffer and $NAD^+$ are 100 and 400 μmol $L^{-1}$, respectively; total volume of the enzyme, reagent and sample is only 18 μL per time for determining; the reaction temperature is 37 °C; the reaction time is 15 min. The concentration of SBAs is directly proportional to the fluorescence intensity of the biosensor measured from 0.5 to 5.0 μmol $L^{-1}$. The relative standard deviation is less than 3.4%, and the detection limit was 0.16 μmol $L^{-1}$. The recoveries are in the range 95.5–106%. This SBA-IE-FCBS can be used for quantifying SBAs in urine to diagnose and judge hepatobiliary diseases, etc.



## 1. Introduction

Bile acids are finally metabolized product of cholesterol in liver system and also are bases in choler. Under normal physiological cases, 99% of bile acids exist in intestinal and hepatic circle systems, and hardly enter into body circle system. But when hepatobiliary systems were damnified, the content of bile acids entering into body circle system will obviously increase. Meanwhile, bile acids will take place sulfation reaction and form sulfated bile acids (SBAs) (Palmer, 1967). Duo to the water-solubility of SBAs distinctly increases than that of the bile acids, they are very easily eliminated from urine through nephridium (Makino et al., 1973; Takikawa et al., 1984). Hereby, in the light of the SBAs concentration in urines, we can diagnose the function of the hepatobiliary system whether or not working order (Makino et al., 1975; Goto et al., 1978). Well then it is very essential to develop a simple, sensitive, accurate, micro-quantitative and low-cost method for determining SBAs in urine.

At present in Japan, the content of SBAs in urine has become a new index for estimating the function of liver and gall system and for diagnosing of liver cancer and hepatocirrhosis (Obatake et al., 2002). Normally, SBAs mainly exist in enterohepatic circulation system, and the concentration of SBAs is about 7 μmol $L^{-1}$ in urine (Muraji et al., 2003).

In foretime methods for determining SBAs, mostly there were various high performance liquid chromatographies (Goto et al., 1980, 1981, 1987). These methods require complicated sample pretreatment such as extraction, solvolysis and derivation, and have some disadvantages such as needing expensive equipment and large sample volume, and time consuming, etc. So they cannot be adapted to actual clinic assay. Besides, other methods were also reported for the determination of SBAs, such as monoclonal antibody-based enzyme-linked immunosorbent assay (Kobayashi et al., 2000), amperometric method (Koide et al., 2007) and elec-

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trospray ionization mass spectrometry (Shinka et al., 2007), etc. These methods possess some good characteristics, but they still need further perfection and simplification.

Tazuke et al. (1992), utilizing a novel bile acid sulfate sulfatase (BSS), has proposed an enzyme-colorimetric assay to determine the concentration of SBAs in urine and strided forward practicability of SBAs (Tazuke et al., 1997). But their method used a lot of expensive enzymes and other reagents, and the test cost is higher.

From 1997, based on flow injection analysis (FIA) (Ruzicka and Hansen, 1988; Li and Gao, 2002) and immobilized enzyme technology, Gao (2000) also has researched on determination approach of SBAs in urine, and developed an automatic chemiluminescence (Gao et al., 1997a,b) and spectrophotometric (Gao et al., 2001) methods, and successfully used for rapid determination of SBAs in urine.

Fluorescence capillary analysis (FCA) (Li and Gao, 2005) is a new method, based on simple spectrofluorimetry and medical capillary. The maximal merit of FCA is that it can realize microanalysis in precondition of micro-dosage of expensive enzymes or reagents, and can promote the micromation of traditional spectrofluorimeters (Li and Gao, 2007). The FCA replaces the common fluorescent cell with the capillary and can save about 99% enzymes or reagents.

Based on FCA, we have successfully developed immobilized enzyme capillary bioreactors for the determination of alcohol in wines (Li and Gao, 2005, 2007) and lactic acid in blood (Li et al., 2008), and immobilized probe biosensors marked by using Cy-5 reagent for the determination of DNA (Gao et al., 2006). In the research, to quantify trace-level SBAs in urine, based on immobilized enzyme and FCA technology, we have developed a novel SBAs biosensor (SBAs-IE-FCBS) and established a determination method of SBAs.

## 2. Experimental

### 2.1. Analytical principle

The determination principle of SBA-IE-FCBS is as follows: SBAs are desulfated under catalysis of BSS to form 3β-hydroxyl bile acids. The latter reacts with nicotinamide adenine dinucleotide ($NAD^+$) in existence of 3β-hydroxysteroid dehydrogenase (3β-HSD), and is converted to 3-ketosteroid; meanwhile, $NAD^+$ is converted to reduced nicotinamide adenine dinucleotide (NADH). And then NADH reacts with 1-methoxy-5-methylphenazinium methyl sulfate (1-MPMS) to generate $NAD^+$ and 1-$MPMSH_2$, which makes resazurin reduce into resorufin. SBAs can be quantified by the fluorescence intensity emitted from resorufin ($\lambda_{ex}/\lambda_{em}$: 540 nm/580 nm).

There are more than 10 different kinds of SBA in human urine (Goto et al., 1987), but we only determine the concentration of total SBA (SBAs) in urine. Therefore, we must select a sort of representative SBA from SBAs. Besides, the BSS enzyme all has catalysis to hydrolyzing reaction of any SBA in urine, but its catalysis is very lower to the hydrolyzing reaction of glycolithocholate sulfate (GLCA-S) in SBAs (Tazuke et al., 1992), so we also adopted GLCA-S as the representative matter of SBAs for our study according to the reference.

### 2.2. Reagents and materials

All chemicals used were analytical reagent grade without further purification. 3β-HSD (EC 1.1.1.51) and GLCA-S were from Sigma–Aldrich Japan K.K. (Tokyo, Japan); BSS (EC 2.8.2.14) was from Marukin Chuyu Co., Ltd. (Tokyo, Japan); $NAD^+$ and NADH were purchased from Oriental Yeast Co. (Tokyo, Japan); Resazurin and 1-MPMS were purchased from Wako Pure Chemical Industries Ltd. (Tokyo, Japan); 3-aminopropyltriethoxysilane (APTES), Tris and gultaraldehyde (50%, v/v) were purchased from Chengdu Chemicals Ltd. (Chengdu, China). Urine samples after overnight fasting were collected from some volunteers.

The apparatuses to be used in the research include a F-4500 type spectrofluorimeter made by Hitachi Co. (Tokyo, Japan), a HM-30V pH meter of TOA Electronics Ltd. (Tokyo, Japan) and an AUW120D type electric balance (measuring precision: 0.01 mg) made by Shimadzu Co. (Kyoto, Japan). Besides, we made a homemade capillary holder critically matched with the dark chamber of the spectrofluorimeter above mentioned. The capillaries used (ID: 0.9 mm) were purchased from Drummond Scientific Co. (Pennsylvania, USA).

### 2.3. Preparation of reagent solutions

Ultra purified water (conductivity: 0.065 $\mu S\,cm^{-1}$) was used for the preparation of the following solutions. Collected urine samples were fresh from researchers of our group and patients of West China Hospital of Sichuan University, after overnight fasting.

#### 2.3.1. GLCA-S stock solution (0.5 mmol $L^{-1}$)

The solution was prepared by dissolving 13.90 mg of GLCA-S in a 50-mL calibrated flask and completing the volume to the mark with water, and then stored in refrigerator at 4 °C. Various concentrations of GLCA-S were served as the standard solution of SBAs.

#### 2.3.2. $NAD^+$ solution (2.5 mmol $L^{-1}$)

8.30 mg $NAD^+$ was dissolved in water and diluted to 5.0 mL by employing 0.1 mol $L^{-1}$ of Tris–HCl buffer solution (pH 8.8).

#### 2.3.3. BSS/β-HSD stock solution (10 kU $L^{-1}$)

50 U of BSS and β-HSD was dissolved in water and diluted to 5.0 mL with Tris–HCl buffer solution (pH 8.0), respectively, and then both of them were stored in refrigerator at 4 °C until used.

#### 2.3.4. 1-MPMS solution (1.0 mmol $L^{-1}$)

7.70 mg of 1-MPMS was weighed, dissolved in 25 mL of 100 mmol $L^{-1}$ Tris–HCl buffer solution.

#### 2.3.5. Resazurin solution (1.0 mmol $L^{-1}$)

6.30 mg of resazurin was dissolved in 25 mL of 0.1 mol $L^{-1}$ Tris–HCl buffer solution.

#### 2.3.6. Tris–HCl buffer solution (0.1 mol $L^{-1}$)

6.570 g of Tris was weighed in a 500-mL beaker, dissolved with 400 mL water, and adjusted its pH to 8.0 with concentrated HCl solution (37%) and pH meter. Finally, it was transferred in 500 mL of calibrated flask and diluted to the mark.

#### 2.3.7. The phosphate buffer solutions with different pH (pH 8.0/7.5/7.0)

Preparations of buffer solutions with pH 8.0, 7.5 and 7.0 were completed by mixing 0.1 mol $L^{-1}$ of $Na_2HPO_4$ and 0.1 mol $L^{-1}$ of $KH_2PO_4$ solutions according to different proportion, respectively.

#### 2.3.8. 1.0% (v/v) APTES solution

0.50 mL of APTES was taken in a 50-mL measuring cylinder and diluted to the 50 mL with *n*-hexane reagent.

#### 2.3.9. Fluorescent reaction solution

It was prepared by mixing 500 μmol $L^{-1}$ of $NAD^+$, 25 μmol $L^{-1}$ of 1-MPMS, 25 μmol $L^{-1}$ of resazurin and 0.1 mol $L^{-1}$ of Tris–HCl buffer (pH 8.0).

### 2.4. Preparation of SBAs capillary bioreactor (SBAs-IE-FCBS)

#### 2.4.1. Amidogen modifying on the capillary

The capillaries are marinated in 0.1 mol $L^{-1}$ of NaOH for 2 h and washed by using 75% (v/v) ethanol solution, and then are flushed with ultra purified water. These capillaries were put in a thermostat to dry 2 h at 40 °C, and the pretreatment of the capillaries was finished. Whereafter, the capillaries pretreated are marinated in 1.0% of APTES for 5 h at 65 °C to make APTES covalently combine with hydroxyl group on inner surface of the capillaries. After that procedure, again these capillaries are rinsed by the ethanol solution, and are dried with a hair drier. Finally, the capillaries are marinated in 2.5% (w/v) of gultaraldehyde solution as a cross-linker for 1 h on the vacuum condition at 65 °C, to make gultaraldehyde covalence combination with APTES.

#### 2.4.2. Enzymatic immobilization on the capillary

5 kU $L^{-1}$ of BSS and β-HSD solution prepared by PBS (pH 7.5), containing 2.0 g $L^{-1}$ of bovine serum albumin, is sucked into the capillaries and kept for 12 h at 4 °C. The purpose is to make the coupling of amido of BSS and β-HSD and aldehydic group of gultaraldehyde and the immobilization of BSS and β-HSD on the capillaries.

#### 2.4.3. Closure of active groups modified on the capillary

The capillaries are marinated in 0.5 mmol $L^{-1}$ of Tris-buffer for about 2 h, to block out otiose aldehydic groups. In this way, the immobilized enzyme capillary bioreactors for SBAs (SBAs-IE-FCBS) are formed. When not in use, these SBAs-IE-FCBS are maintained in 0.05 mol $L^{-1}$ of PBS (pH 7.5) and stored in refrigerator at 4 °C. Under this condition, they could be used for at least 6 months.

#### 2.4.4. Determination procedure

A SBA-IE-FCBS and a special capillary holder with a hollow ($\phi$ 0.9 mm) as well as a fluorescent instrument constitute this SBAs measure system. The size of the capillary holder is the same with standard fluorescent cell. The holder can fit into some commercial fluorimeter if it can use a standard fluorescent cell. Before the determination, the holder is inserted into the light path of the fluorophotometer to replace common fluorescent cell.

Determination procedure is shown in Fig. 1. First, after the urine sample or standard solution (50 μL) and the fluorescent reaction solution (30 μL) in a test tube are mixed, and the reaction mixture solution beforehand reacts for 10 min at 37 °C to eliminate the effect of the interfere materials in the urine samples. Whereafter, the solution in the tube is sucked into the biosensor (18 μL), and it is vertically inserted in top hollow of the holder. When the excitation beam (540 nm) enters into an incident slit (width and length: 0.8 mm × 20 mm) on lateral of the holder and passes through the biosensor in it, the resorufin formed in the biosensor is excited and then it emits fluorescence (580 nm), and enters into a monochromatic filter and reaches the detector. Initial fluorescence intensity generated in the biosensor is detected immediately as a blank value ($F_1$). Then, keeping on the reaction for 15 min, its final fluorescence intensity is detected as a measure value ($F_2$). The fluorescence difference ($\Delta F = F_2 - F_1$) is proportional to the concentration of BSAs to be determined in the biosensor.

## 3. Results and discussion

### 3.1. Optimization of BSS concentration for immobilization on the biosensor

In the reaction system of SBAs-IE-FCBS, because the first step of hydrolyzing reaction was catalyzed by using BSS, it was a key enzyme and had an important effect on the reaction rate and sensitivity of the reaction system. So the optimization was conducted to the concentration of BSS in the biosensor. Under experimental conditions of 500 μmol $L^{-1}$ of $NAD^+$, 50 μmol $L^{-1}$ of 1-MPMS, 50 μmol $L^{-1}$ of resazurin, 5 kU $L^{-1}$ of β-HSD and 18 μL of the reaction solution, the effect of the BSS concentration on the sensitivity was investigated by using a set of SBAs-IE-FCBS, containing different concentrations (1, 2.5, 5, 7.5 and 10 kU $L^{-1}$) of BSS. 1.0 and 2.0 μmol $L^{-1}$ of GLCA-S as the sample were tested at 37 °C after reacting for 10 min, respectively. The results obtained were shown in Fig. 2. When the BSS concentration was 5.0 kU $L^{-1}$, the fluorescence intensity attains the maximum. Consequently, the concentration of BSS was selected 5 kU $L^{-1}$ to be immobilized in the biosensor.

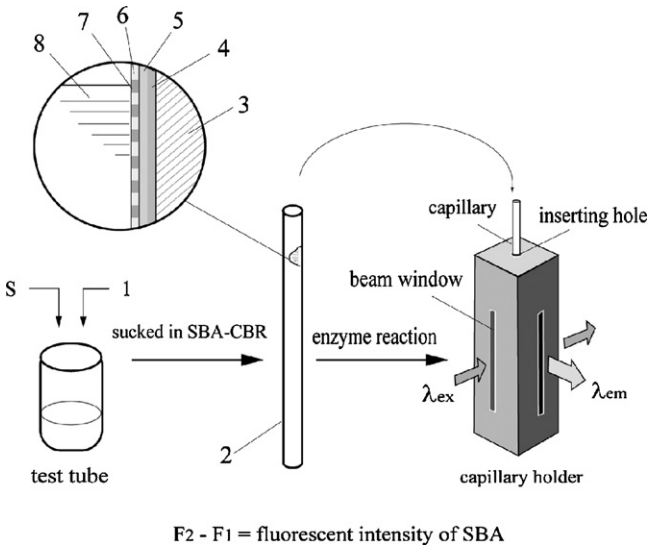


**Fig. 1.** Illustration of SBAs-IE-FCBS for SBAs determining. S: sample; 1: fluorescence reaction solution ($NAD^+$/1-MPMS/resazurin); 2: SBA-IE-FCBS; 3: capillary wall; 4: APTES; 5: gultaraldehyde; 6: BSS; 7: β-HSD; 8: reaction mixture solution; $F_1$: blank value; $F_2$: measure value.

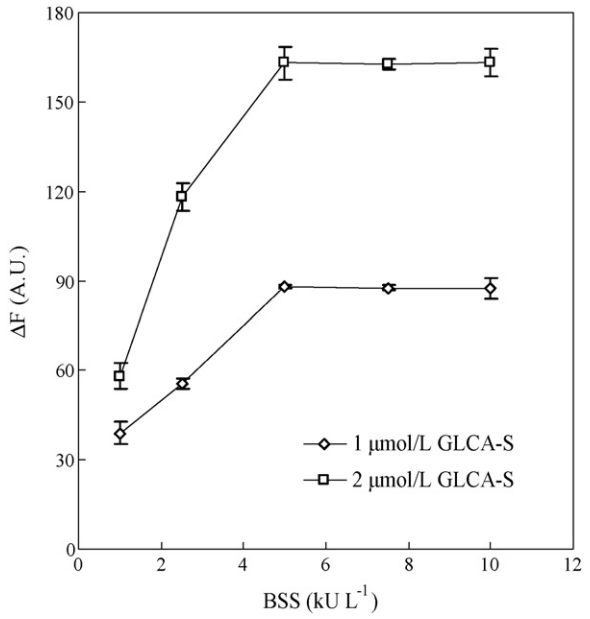


**Fig. 2.** Optimization of the BSS concentration for immobilization on the biosensor.

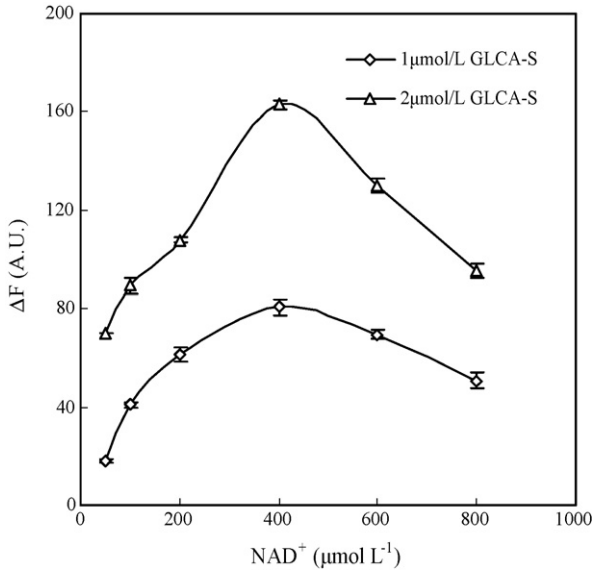


**Fig. 3.** Effect of the $NAD^+$ concentration on the biosensor.

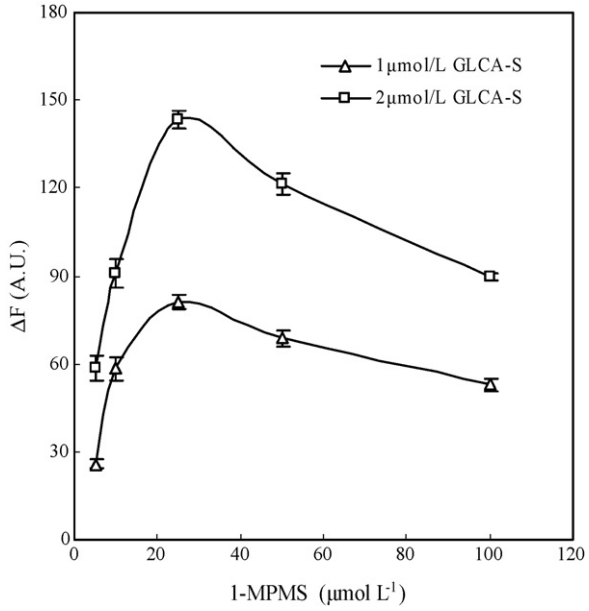


**Fig. 4.** Effect of the 1-MPMS concentration on the biosensor.

### 3.2. Optimization of β-HSD concentration for the immobilization on the biosensor

In this experiment, different concentrations (1.0, 2.5, 5.0, 7.5 and 10.0 kU $L^{-1}$) of β-HSD were immobilized in five biosensors to examine effects of the concentration of β-HSD to be used for the immobilization on the fluorescence intensity. The concentration of BSS was fixed at 5.0 kU $L^{-1}$, 1.0 and 2.0 μmol $L^{-1}$ of GLCA-S were employed as test samples. Other experimental conditions were as in Section 3.1. The results obtained show that the fluorescence intensity gradually increases with increasing the concentration of β-HSD in the range 1.0–5.0 kU $L^{-1}$, and arrive constant after 5.0 kU $L^{-1}$. So the concentration of β-HSD used for the immobilization was selected to be 5.0 kU $L^{-1}$.

### 3.3. Effect of $NAD^+$ concentration on the biosensor

Under catalysis of β-HSD, $NAD^+$ as a cofactor reacts with 3β-hydroxyl bile acids formed by SBAs and produces NADH having fluorescence. The concentration of SBAs can be indirectly quantified by detecting its fluorescence intensity. Consequently, high or low of $NAD^+$ concentration will affect on this enzymatic reaction system of determining SBAs.

In the testing, 5.0 kU $L^{-1}$ of BSS and β-HSD were used for the immobilization on the biosensor, the concentration of GLCA-S was selected 1.0 and 2.0 μmol $L^{-1}$, respectively; the reaction time was 15 min, influence of the concentration of $NAD^+$ on the response of the biosensor was investigated in the range 50.0–800.0 μmol $L^{-1}$ at 37 °C. The result was shown in Fig. 3. It was observed that the fluorescence intensity of the biosensor was increased continuously with the $NAD^+$ concentration up to 400.0 μmol $L^{-1}$. At the concentration over this value, reversibly the fluorescent signal decreases with increasing concentration. The reason may be that the high concentration of $NAD^+$ affects on the activation of β-HSD enzyme or produces the concentration quenching. Hence 400.0 μmol $L^{-1}$ of $NAD^+$ was chosen for further experiments.

### 3.4. Effect of 1-MPMS concentration on the biosensor

The experimental conditions were as follows: 100 mmol $L^{-1}$ of Tris–HCl buffer solution (pH 8.0), 50.0 μmol $L^{-1}$ of resazurin, 400.0 μmol $L^{-1}$ of $NAD^+$, 5.0 kU $L^{-1}$ of BSS and β-HSD for the immobilization, reaction temperature was 37 °C and reaction time was 10 min. The effect of the concentration of 1-MPMS on the fluorescence intensity was examined in the range 5.0–100.0 μmol $L^{-1}$. Fig. 4 indicates that the biosensor's fluorescence intensity increases with increasing the concentration of 1-MPMS and reaches the maximum at 25.0 μmol $L^{-1}$, but it becomes to decrease over 25.0 μmol $L^{-1}$. Therefore, 25.0 μmol $L^{-1}$ of the 1-MPMS concentration will be selected for the biosensor.

### 3.5. Effect of resazurin concentration on the biosensor

The experimental conditions were the same as Section 3.4, except that the 1-MPMS concentration used was 25.0 μmol $L^{-1}$. To investigate the effect of the resazurin concentration in the range 1.0–50.0 μmol $L^{-1}$ on the biosensor, the fluorescence intensity was measured by using 1.0 and 2.0 μmol $L^{-1}$ of GLCA-S as test samples. The obtained results shown that the fluorescence intensity increases with the increase of the resazurin concentration in the range 1.0–10.0 μmol $L^{-1}$ and then it decreases with the concentration increasing after 10.0 μmol $L^{-1}$. Therefore, the resazurin concentration was selected 10.0 μmol $L^{-1}$.

### 3.6. Effect of pH on the biosensor

Acidity in fluorescent reaction system containing enzymes is a very important factor because it can influence on the catalytic rate of enzymes and quantum yield of fluorescence, so the optimization was made to it. The experimental conditions were the same as Section 3.5, except that the resazurin concentration used was 10.0 μmol $L^{-1}$.

Different fluorescence reaction solution ($NAD^+$/1-MPMS/resazurin) having different pH values (7.0, 7.5, 8.0 8.5, and 9.0) were prepared by using Tris–HCl buffer solutions. And then the effect of pH for the fluorescent system on the biosensor was examined by using 0.5 and 1.0 μmol $L^{-1}$ of GLCA-S as testing samples, respectively. Detail operation procedure was the same with Section 2.4. From obtained results, it could be seen that the fluorescence intensity reached the maximum when pH value

was 8.0. So this pH value was selected by us for the fluorescence reaction solution.

### 3.7. Effect of the concentration of Tris–HCl buffer on the biosensor

The experimental conditions were the same as Section 3.6, except that pH used was 8.0. Due to the buffer solution to be added in the enzymatic reaction system shall change ionic intensity of the solution and affect the fluorescence intensity of the system, the concentrations of the Tris–HCl buffer solution (10.0–200.0 μmol $L^{-1}$) were investigated by using 1.0 and 2.0 μmol $L^{-1}$ of GLCA-S as samples, respectively. Obtained results indicate that the fluorescence intensity increases with the increase of the concentration of the Tris–HCl buffer solution till 100.0 μmol $L^{-1}$, then it decreases gradually. Consequently, next experiments were performed by using 100.0 μmol $L^{-1}$ of Tris–HCl buffer solution.

### 3.8. Effect of the reaction temperature on the biosensor

Experimental conditions were as in Section 3.7, except that the concentration of the Tris–HCl buffer was 100.0 mmol $L^{-1}$. The effect of reaction temperature on the sensitivity of the biosensor was examined by using 1.0 and 2.0 μmol $L^{-1}$ of GLCA-S as test samples. The fluorescence intensity of the reaction mixture solution in the biosensor was detected at 30, 34, 37, 40 and 45 °C, respectively. The results shown that the fluorescence intensity increases with raising the reaction temperature and arrives at maximal peak value at 37 °C. But the fluorescence intensity become to decreasing trend when the temperature is over 37 °C. This is mainly due to influence of the temperature on catalytic reaction of the enzymes. On one hand the raising of the temperature quickens reactive rate, on the other hand, it also can cause denaturation of the enzymes. Accordingly, the reaction temperature of the biosensor was selected to be 37 °C.

### 3.9. Effect of reaction time on the biosensor

In this experiment, the reaction temperature was 37 °C, other experimental conditions were as in Section 3.7. 1.0 and 2.0 μmol $L^{-1}$ of the GLCA-S were used as test samples. Changing reaction time from 0 to 25 min, the effect of the elapsed time on the fluorescence intensity was investigated at 37 °C. The results obtained show that the fluorescence intensity increases gradually with the reaction proceeding from 0 to 15 min; over 15 min, the fluorescence intensity becomes constant. Therefore, the optimal reaction time was selected to be 15 min.

### 3.10. Interference test

Urea (50.0–1000.0 mmol $L^{-1}$), ascorbic acid (0.25–2.0 mmol $L^{-1}$), uric acid (0.25–2.5 mmol $L^{-1}$), glucose (50.0–200.0 mg $L^{-1}$) and NaCl (100.0–500.0 mg $L^{-1}$) individually were added into several GLCA-S solutions (2.5 μmol $L^{-1}$), to simulate some substances coexisting in urine samples. And then by using these mixture solutions, it was conducted investigation to the effect of the SBA-IE-FCBS. The experimental results show that added substances do not interfere to our SBA sensor.

### 3.11. Investigation of pre-reaction time

In urine samples, there are other interferences of unknown coexistence substance except that above mentioned as Section 3.10. Therefore, pre-reaction time of actual urine samples was examined. 100.0 μL of $NAD^+$ (500.0 μmol $L^{-1}$), 25.0 μL of 1-MPMS (25.0 μmol $L^{-1}$) and 25.0 μL of resazurin (25.0 μmol $L^{-1}$) were added into 250.0 μL of the urine sample, respectively, the changing trend of blank fluorescence intensity was investigated. The obtained result shows that the blank fluorescence intensity of the urine increases with increasing the reaction time from 0 to 10 min; after 10 min it becomes constant. This reason is that the unknown coexistence substance in the urine sample reacts with mixing reagents added and reaches end point after 10 min. As a result, the pre-reaction time used for determining urine sample may take 10 min to eliminate some unknown interference.

### 3.12. The stability of the biosensor

To investigate the storage stability of the SBAs-IE-FCBS, the fluorescence intensity values repetitiously were detected by using the same biosensor thrice per week for 1 month. 2.0 μmol $L^{-1}$ of the GLCA-S standard was used as samples. Immobilized concentrations of BSS and $NAD^+$ used for the biosensor all were 5.0 kU $L^{-1}$. Obtained results shown that the mean value of the fluorescence intensities was $165.4 \pm 2.4$ and its relative standard deviation was <1.4% ($n = 12$). Namely, the stability of the biosensor was very satisfactory.

### 3.13. Comparison of sensitivity between the biosensor and liquid enzyme methods

After mixing 25.0 μL of the standard solution and 15.0 μL of the fluorescent reaction solution (containing 400.0 μmol $L^{-1}$ of $NAD^+$, 25.0 μmol $L^{-1}$ of 1-MPMS and resazurin) in the test tube and pre-reacting at 37 °C, the reaction mixture solution was sucked into the biosensor containing immobilized BSS and β-HSD, the blank value of the fluorescence intensity ($F_1$) was detected immediately. And then the fluorescence intensity ($F_2$) again was detected when the biosensor keeps on the reaction for 15 min.

According to the operation procedure above-mentioned, a series of the GLCA-S standard solutions were detected by using two methods of the SBAs-IE-FCBS and a liquid enzyme FCA (SBAs-LE-FCA), respectively. Obtained experimental results shown that the sensitivity of the SBAs-IE-FCBS ($\Delta F = 71.286 C_{SBA} + 1.8095$, $r = 0.9991$) was heightened one multiples than that of the SBAs-LE-FCA without the enzymatic immobilization ($\Delta F = 38.055 C_{SBA} + 5.6223$, $r = 0.9989$) under respective optimized conditions.

## 4. Determination of sample and recovery with SBA-IE-FCBS

### 4.1. Determination of urine samples

Based on the optimal conditions above-mentioned and the determination procedure in Section 2.4, various concentrations of the GLCA-S standard solution were determined. The result obtained shows that there is a good linear relationship between the fluorescence intensity ($\Delta F$) emitted by the biosensor and the GLCA-S concentration in the range 0.5–5.0 μmol $L^{-1}$.

Subsequently, three fresh urine samples were chosen as test samples. To eliminate residue in the urine, before the determination the three samples were centrifuged at 3000 rpm for 15 min. Other operation procedure using the SBAs-IE-FCBS was the same with Section 2.4. The results obtained were shown in Table 1.

To investigate the accuracy of results obtained by using the SBAs-IE-FCBS, according to the diluting ratio (1 + 9) of the standard and sample solutions, different concentrations (25.0–100.0 μmol $L^{-1}$) of the GLCA-S standard solutions were added into urine samples, respectively, a recovery test was conducted. The results were shown in Table 2. It can be seen that the recovery values of the SBAs-IE-FCBS were in the range 95.5–106%, which can be considered to be satisfactory.

**Table 1**
Determination of actual urine samples by using the SBA-IE-FCBS ($n = 3$)

| Sample no. | Dilution multiple | $F_1$ | $F_2$ | $\Delta F$ | SBAs ($\mu$mol L$^{-1}$) | Mean value SBAs ($\mu$mol L$^{-1}$) | Regression equation |
|---|---|---|---|---|---|---|---|
| 1 | – | 687.3 | 737.1 | 49.8 | 2.62 | 2.72 ± 0.11 | $\Delta F = 68.286C_{SBA} + 5.1365$ |
| | | 690.1 | 743.5 | 53.4 | 2.83 | | |
| | | 686.0 | 737.5 | 51.5 | 2.72 | | |
| 2 | 4 | 652.4 | 805.5 | 153.1 | 8.73 | 8.83 ± 0.20 | $\Delta F = 68.281C_{SBA} + 4.1302$ |
| | | 651.1 | 809.8 | 158.7 | 9.05 | | |
| | | 651.8 | 804.4 | 152.6 | 8.70 | | |
| 3 | 4 | 638.4 | 735 | 96.6 | 5.09 | 4.96 ± 0.12 | $\Delta F = 72.938C_{SBA} + 3.7619$ |
| | | 640.1 | 732.5 | 92.4 | 4.86 | | |
| | | 640.8 | 734.2 | 93.4 | 4.92 | | |

**Table 2**
Recovery of SBAs in urine samples obtained by using SBA-IE-FCBS ($n = 3$)

| Sample no. | SBAs concentration ($\mu$mol L$^{-1}$) | Added standard ($\mu$mol L$^{-1}$) | Dilution multiple | $\Delta F$[a] | Found value[b] ($\mu$mol L$^{-1}$) | Recover standard ($\mu$mol L$^{-1}$) | Recovery (%) |
|---|---|---|---|---|---|---|---|
| 1 | 2.45(2.72 × 9/10) | 2.50 | 4 | 90.3 | 4.99 | 2.54 | 101.6 |
| | | 10.0 | 10 | 91.7 | 12.65 | 10.20 | 102.0 |
| 2 | 7.94(8.83 × 9/10) | 2.50 | 8 | 92.3 | 9.35 | 2.40 | 95.5 |
| | | 10.0 | 10 | 129.6 | 17.43 | 10.43 | 105.0 |
| 3 | 4.46(4.96 × 9/10) | 2.50 | 4 | 129.2 | 6.88 | 2.42 | 96.8 |
| | | 10.0 | 10 | 111.4 | 14.76 | 10.30 | 103.0 |

[a] Nos. 1, 2 and 3, respectively used the equations: $\Delta F = 68.286C_{SBA} + 5.1365$, $\Delta F = 68.281C_{SBA} + 4.1302$ and $\Delta F = 72.938C_{SBA} + 3.7619$.
[b] $C_{SBA} = [(\Delta F - b)/k] \times$ dilution multiple. Recovery = [(found value − SBA concentration)/added standard] × 100.

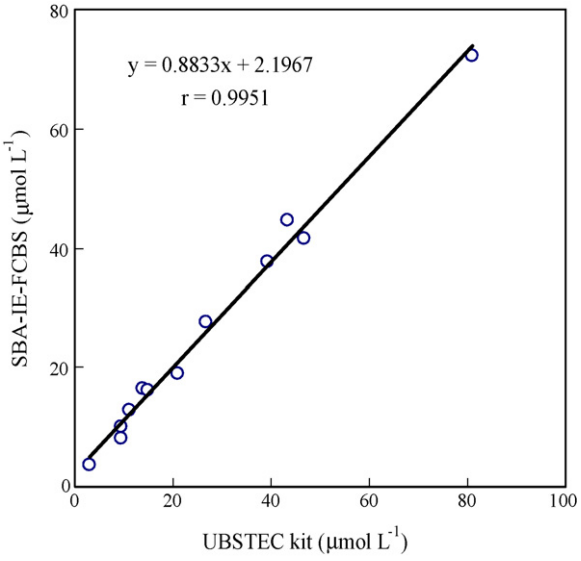


**Fig. 5.** Relativity between our biosensor and UBSTEC kit.

### 4.2. Reproducibility and detection limit of the biosensor

To verify the precision of the SBAs-IE-FCBS, it was made the determination of its reproducibility by using the lower (0.5 $\mu$mol L$^{-1}$), medium (1.0 $\mu$mol L$^{-1}$) and higher (2.0 $\mu$mol L$^{-1}$) concentrations of GLCA-S for eleven repeating times, respectively. The mean values of the fluorescence intensity and the standard deviation ($\sigma$) obtained by determining 0.5, 1.0 and 2.0 $\mu$mol L$^{-1}$ of GLCA-S were 50.8 ± 1.7, 103.0 ± 3.0 and 179.8 ± 3.9, respectively. Results show that the relative standard deviation is in the range 3.3–2.2%. It can be seen that the reproducibility of this biosensor was satisfactory. Besides, by calculating the detection limit value ($C_L$) of the SBAs-IE-FCBS was obtained 0.16 $\mu$mol L$^{-1}$ ($C_L = 3\sigma/k = 3 \times 3.9/71.286 = 0.16$ $\mu$mol L$^{-1}$).

### 4.3. Comparison with UBASTEC kit

In order to evaluate the credibility of SBAs-IE-FCBS, urine samples were used as testing samples, and contrast experiment was conducted with a commercial UBASTEC kit used for determining SBAs. The detection of the UBASTEC kit was made by a 721-A type spectrophotometer at 540 nm. The results were shown in Fig. 5. It can be seen that the data obtained between both methods do not show obvious differences.

## 5. Conclusions

Based on the fluorescent reaction system comprising BSS/β-HSD/NAD$^+$/1-MPMS/resazurin, a novel and high sensitive SBAs-IE-FCBS was successfully developed for the determination of SBAs. Optimized test conditions are as follows: the concentrations of BSS and β-HSD used for the immobilization are all 5 kU L$^{-1}$; the concentrations of 1-MPMS and resazurin are all 25 $\mu$mol L$^{-1}$; the concentrations of the Tris–HCl buffer and NAD$^+$ are 100 and 400 $\mu$mol L$^{-1}$, respectively; the reaction temperature is 37 °C; the reaction time is 15 min. The determination range of the biosensor is from 0.5 to 5.0 $\mu$mol L$^{-1}$. The relative standard deviation is less than 3.4%, and the detection limit was 0.16 $\mu$mol L$^{-1}$. The recoveries are in the range 95.5–106%.

By using the biosensor, the dosage of the fluorescent reaction solution per time is only 1/400 of traditional fluorometric method. Accordingly, the analytical cost can decrease to a great extent. Because the capillary volume used as reaction container is only 18 μL, the SBAs-IE-FCA method has realized trace analysis to a micro-volume sample. The operation procedure of the method and the apparatus adopted are simple, so it can be easily popularized. The method will be applicable for clinical test.

Following this preliminary report, we further attempt to estimate clinical samples of patients suffering from various hepatobiliary diseases with the biosensor.

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