Clin Chem Lab Med 2008;46(11):1641–1647 © 2008 by Walter de Gruyter • Berlin • New York. DOI 10.1515/CCLM.2008.308

Electrochemical detection of blood alcohol concentration using a disposable biosensor based on screen-printed electrode modified with Nafion and gold nanoparticles

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Abstract

Background: Blood alcohol determination plays an important role in laboratory medicine and forensic medicine. Nowadays, many methods are being used for alcohol measurement, but these methods are time-consuming and complex to perform laborious sample pre-treatment. The disposable amperometric biosensor, due to its portability, low cost and potential for fabrication, should be readily applicable for blood alcohol determination.
Methods: The biosensor was fabricated by immobilizing alcohol dehydrogenase and nicotinamide adenine dinucleotide coated by Nafion combined with gold nanoparticles onto the surface of screen-printed electrode modified with Meldola's blue. Evaluations of biosensor performance were performed according to the relevant National Committee for Clinical Laboratory Standards standard.
Results: The biosensor response for serum alcohol presents good linearity, precision, stability, accuracy, and specificity.
Conclusions: The biosensor exhibits the capability of detecting blood alcohol concentration in the clinical laboratory and in forensic medicine, unnecessarily performing laborious sample pre-treatment.
Clin Chem Lab Med 2008;46:1641–7.

Keywords: blood alcohol concentration; disposable biosensor; electrochemical detection; gold nanoparticles; screen-printed electrode.

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Received March 14, 2008; accepted July 8, 2008

Introduction

Blood alcohol determination plays an important role in laboratory medicine and forensic medicine for several reasons. First, drunk driving is a very serious problem in the modern world, which results in many fatal accidents every year (1, 2). Blood alcohol concentration values served as the ''gold standard'' in which drivers were recognized as drunk driving by police officers (3, 4). It is also necessary for clinical laboratories to detect acute alcoholism and some alcohol abuse-related syndromes. Nowadays, many methods are being used for alcohol measurement, such as spectrometric and chromatographic analysis or breathalyzer, where the alcohol concentration or refractivity is detected (5–8). However, these methods are time-consuming and complex to perform laborious sample pre-treatment. In addition, expensive analytical apparatuses are necessary. Thus, there is an increasing requirement for rapid, accurate and inexpensive methods for blood alcohol determination. The disposable biosensor, due to its portability, low cost and potential for fabrication, should be readily applicable for blood alcohol determination. Several alcohol biosensors have been reported to control the fermentation process in the food and beverages industries, based on immobilization of alcohol oxidase (AOD) (9–11) or alcohol dehydrogenase (ADH) (12–14). However, these biosensors are either of low selectivity for alcohol or of high likelihood of interferences. Meldola's blue (MB) as an electron transfer mediator can catalyze the oxidation of NADH to NAD^+ at a working potential of 0.0 V (vs. saturated calomel reference electrode, SCE), thus avoiding interferences due to the presence of oxidizable substances in real blood samples (15–17). However, it has been found that MB could not be absorbed stably on the electrode (18). To improve the stability of MB, some methods of immobilizing MB on the electrode have been used, but the preparation methods are complicated (15, 16). This paper reports that MB incorporated into carbon ink is printed onto a screen-printed electrode (SPE).

It is one of the most important considerations for preparing a biosensor to easily immobilize the enzymes onto the electrode and chronically maintain the enzymes activity. In the conventional cross-linking method, glutaraldehyde is directly contacted with the enzymes immobilized onto the electrode, which would bring about the loss of enzymes activity. In addition, enzymes immobilized directly on the electrode could easily fall from the biosensor, resulting in

lower stability and life cycle of the biosensor. Recently, there has been an increasing interest in using Nafion as a protective and selective coating material to support the immobilization of the enzymes, because it has favorable characteristics, such as high chemical stability and good biocompatibility (19–21). All types of nanoparticles, due to their high specific surface area, biocompatibility, electrical conductivity and their easy fabrication, have been widely used to fabricate the amperometric biosensor. It was reported that gold nanoparticles (GNPs) could act as electron transfer tunnels, facilitating the electron transfer, and could immobilize the enzymes without reducing their biological activity (22–24).

Screen printing technology is a versatile tool for the inexpensive, easy and highly reproducible production of disposable biosensors. Up to now, since the disposable biosensor based on SPE could resolve such critical problems as fast response and portability, they are applied in laboratory diagnosis (25–27). In this work, ADH and NAD^+ coated by Nafion combined with GNPs were immobilized onto the working area of MB modified SPE to construct the disposable amperometric biosensor. Evaluations of biosensor performance were performed according to the relevant National Committee for Clinical Laboratory Standards (NCCLS) standard. The biosensor presents good linearity, precision, stability, accuracy, and specificity. The biosensor was applied for measuring serum alcohol and satisfactory results were obtained.

Materials and methods

Reagents

ADH (EC 1.1.1.1), NAD^+, Nafion117 solution, MB, and chloroauric acid ($HAuCl_4$) were purchased from Sigma-Aldrich (Shanghai, China). Carbon ink, silver ink, and UV adhesives were purchased from Tai Tung Hong (Shen Zhen, China). All other chemicals were of analytical grade. Absolute ethanol was added to the pooled serum to prepare the serum alcohol solution with different concentrations. In addition, all of the solutions were prepared with ultrapure water from Millipore Milli-Q system (Millipore, Billerica, USA).

Apparatus

A μAutolab electrochemical analyzer (Ecochemie, Utrecht, Netherlands), a GC-9A gas chromatograph (Shimadzu, Kyoto, Japan), and a manual screen-printer (ShengDa, ZheJiang, China) were used.

Preparation of GNPs

GNPs were prepared by adding sodium citrate solution to a boiling $HAuCl_4$ aqueous solution, as described previously (28). The solution was stored in brown glass bottles at 4°C in a refrigerator. The average particle diameter was 22 nm, as determined by transmission electron microscopy.

Preparation of MB modified SPE

The SPEs were prepared according to the literature (17, 29). The polyester was selected as a support of the biosensor.

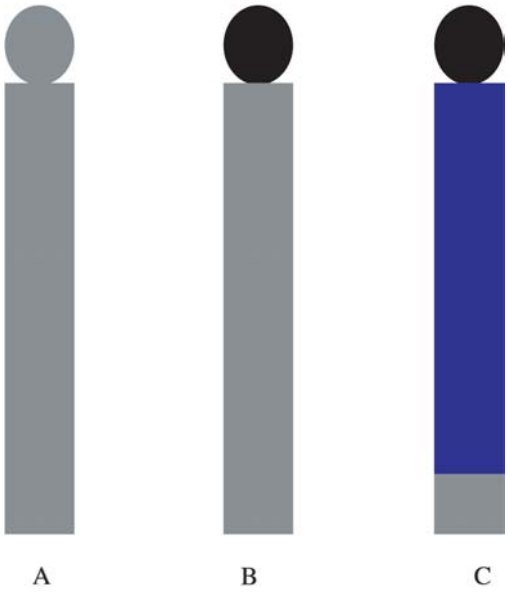

Figure 1 Production steps of the SPE.
(A) Printing of silver ink; (B) printing of carbon ink incorporating 2% (w/w) MB; (C) printing of UV adhesives.

The three production steps of the transducer are shown schematically in Figure 1. The biosensor comprises a 3-mm diameter working area, an insulation layer and an electrical contact site. The biosensors were printed onto the polyester sheet. The different layers including silver ink, carbon ink, and UV adhesives were printed one after the other. After printing silver and carbon ink, the polyester sheet was heated for 2 h in an oven at 80°C to drive off the solvents from the applied ink. After printing UV adhesives, ultraviolet curing of UV adhesives was followed.

Preparation of alcohol biosensor

The MB modified SPE was ultrasonically cleaned with absolute ethanol and ultrapure water for 10 min, respectively, and dried at room temperature. Enzymes solution was obtained by dissolving 4 mg of ADH and 6 mg of NAD^+ in 200 μL of 0.1 mol/L (pH 8.0) phosphate buffer solution (PBS), then 100 μL of enzymes solution and 100 μL of 5% (w/w) Nafion solution were mixed with vigorous stirring. Then, 100 μL of GNP solution was added to this mixed solution with vigorous stirring. Following this, 10 μL of the freshly prepared mixed solution was dropped onto the working area of the SPE and allowed to dry in air at room temperature for 12 h. The alcohol biosensor was prepared and kept dry in a refrigerator at 4°C before use.

Analytical methods

All electrochemical measurements were carried out in a conventional three-electrode cell which was composed of SCE, a platinum wire counter electrode, and a modified working electrode. Chromatographic analysis for blood alcohol concentration was performed according to the literature (30).

Sample collection

Blood alcohol samples were obtained from Chongqing Institute of Forensic Medicine and the First Affiliated Hospital of Chongqing Medical University. After the samples were centrifuged, the clear supernatants were diluted appropriately

and used without previous filtration or any other pretreatment.

Results and discussion

Electrochemical behavior of MB-enzyme-Nafion-GNPs electrode

Cyclic voltammograms of three different modified SPEs in 0.1 mol/L PBS were recorded and are shown in Figure 2. Voltammograms A, B and C correspond to an MB electrode, MB-enzymes-Nafion-GNPs electrode, and MB-enzymes-Nafion electrode, respectively. As shown in Figure 2a, the anodic peak current (i_{pa}) and cathodic peak current (i_{pc}) of the MB electrode are the highest in these peak currents obtained for three differently modified SPEs. A possible explanation is that electrons can be directly transferred between MB and electrodes. In contrast, when the enzyme is entrapped in the Nafion film without the presence of GNPs, both i_{pa} and i_{pc} decreased (Figure 2c). It is most likely that the Nafion film can slow the electron transfer rate. However, both i_{pa} and i_{pc} of MB-enzymes-Nafion-GNPs electrode are higher than that of MB-enzymes-Nafion electrode, as shown in Figure 2b. A possible explanation is that the GNPs, due to their high specific surface area and good electrical conductivity, could act as electron transfer tunnels, which may facilitate the electron transfer between enzymes and electrodes.

Current response of biosensors to alcohol

Figure 3 presents the alcohol biosensor mechanism of response. When alcohol is oxidized to acetaldehyde, NAD^+ is reduced to NADH under the catalysis of ADH and NAD^+ immobilized on the electrode. Meanwhile, MB could again electrocatalyze the oxidation of NADH to NAD^+, and electrons would be quickly transferred between enzymes and the modified working electrode so that the increase in the anodic current could be detected amperometrically.

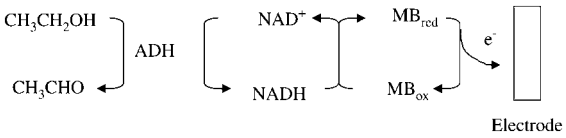

Figure 3 Reaction scheme of alcohol biosensor.
Med_{red}, reduced mediator; Med_{ox}, oxidated mediator.

Cyclic voltammograms of the biosensor based on MB-enzymes-Nafion-GNPs modified SPE for the absence (Figure 4a) and the presence (Figure 4b) of 1 mmol/L alcohol in 0.1 mol/L PBS (pH 8.0) at a scan rate of 10 mV/s are shown in Figure 4. There is an increase in the anodic current and a decrease in the cathodic current simultaneously, which shows that the catalytic reaction occurs on the biosensor through the mediated mechanism. It is most likely that the complex film composed of Nafion and GNPs could prevent enzymes from falling from the biosensor. This means that the biosensor based on MB-enzymes-Nafion-GNPs modified SPE could remarkably keep enzyme activity and stability.

Optimization of the operating conditions

For optimal working potential, 0.0 V vs. SCE was chosen, as described previously (15, 16), because such a potential could reduce the interference of easily oxidizable substances in real blood samples and obtain higher current intensity.

The effect of pH on the biosensor response for alcohol was investigated during the pH range of 6.0 to 8.5 in 0.1 mol/L PBS with 1 mmol/L serum alcohol. As shown in Figure 5, the current intensity increased with the solution pH increasing from 6.0 to 8.0, reaching a

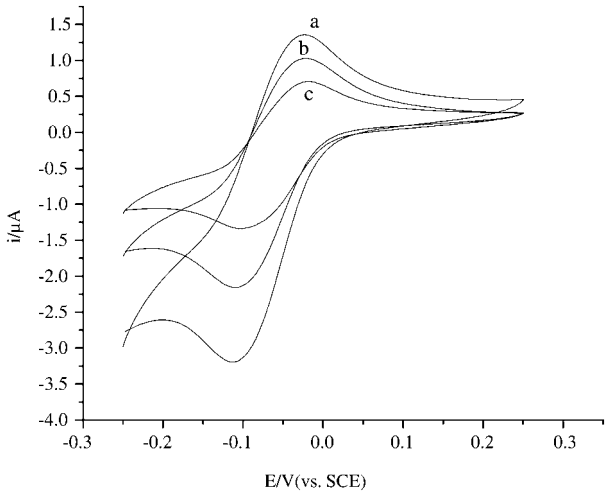

Figure 2 Cyclic voltammograms.
Cyclic voltammograms of (a) MB electrode; (b) MB-enzymes-Nafion-GNPs electrode; (c) MB-enzymes-Nafion electrode in 0.1 mol/L PBS (pH 8.0). Scan voltage: –0.25 to 0.25 V (vs. SCE). Scan rate: 10 mV/s. Temperature: 25°C.

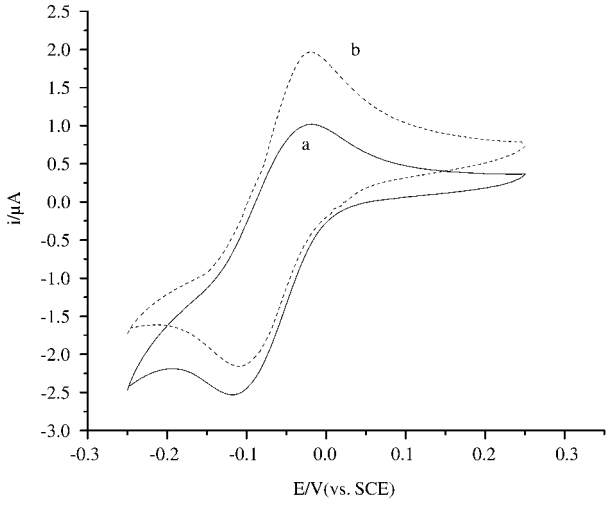

Figure 4 Cyclic voltammograms.
Cyclic voltammograms of the biosensor based on MB-enzymes-Nafion-GNPs modified SPE for the absence (a) and the presence (b) of 1 mmol/L alcohol in 0.1 mol/L PBS (pH 8.0). Scan voltage: –0.25 to 0.25 V (vs. SCE). Scan rate: 10 mV/s. Temperature: 25°C.

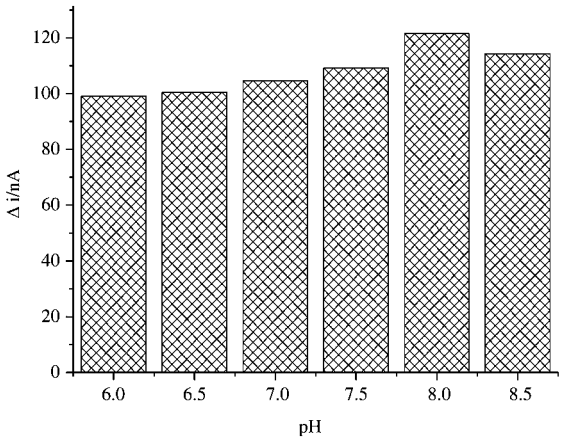

Figure 5 Effect of pH on the biosensor response for alcohol, obtained in 0.1 mol/L PBS with 1 mmol/L serum alcohol at the working potential of 0.0 V (vs. SCE) at 25°C.

maximum current at pH 8.0. It is most likely that some necessary groups of enzymes have different dissociating power in different pH conditions and those could easily combine with substrate in a certain pH condition. Accordingly, this pH range reflects the optimal conditions for both the enzymatic and electrochemical reactions in the biosensor.

The different buffer solutions with the same pH and concentration also influence the biosensor response for serum alcohol. As shown in Figure 6, the maximum current intensity is observed in PBS. The possible explanation is the complex formation between NADH and phosphate, like an adduct, making the NADH oxidation easier (31). Based on these results, PBS is chosen as the optimal buffer solution.

The effect of temperature on the biosensor response for serum alcohol is demonstrated for the range between 15°C and 40°C. As shown in Figure 7, the response current increases with temperature. However, according to the properties of enzymes that higher temperatures would bring about the partial denaturation of the enzymes, further increases in temperature would result in a decrease of the response

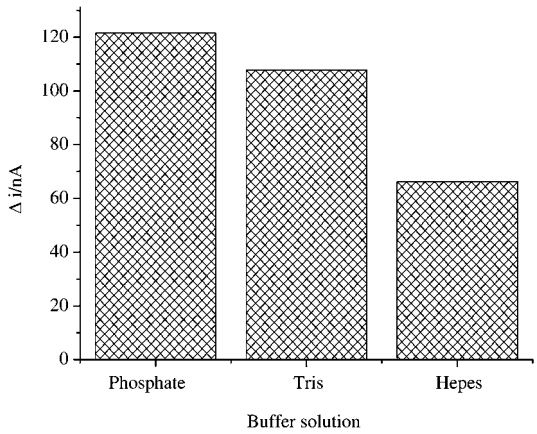

Figure 6 Effect of buffer solution on the biosensor response for alcohol, obtained in 0.1 mol/L (pH 8.0) buffer solution with 1 mmol/L serum alcohol at the working potential of 0.0 V (vs. SCE) at 25°C.

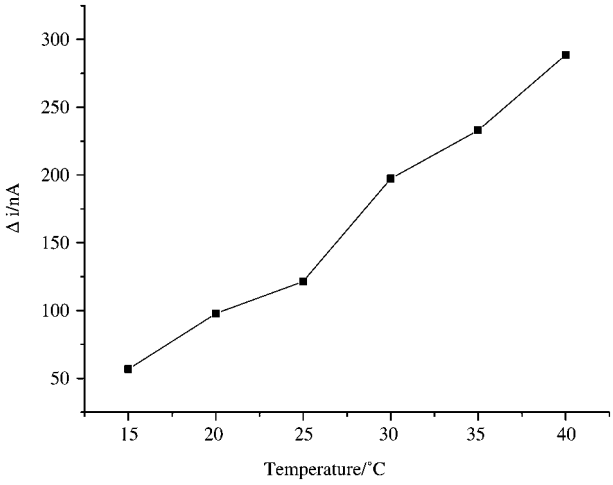

Figure 7 Effect of temperature between 15°C and 40°C on the biosensor response for alcohol, obtained in 0.1 mol/L PBS (pH 8.0) with 1 mmol/L serum alcohol at the working potential of 0.0 V (vs. SCE).

current. Accordingly, 25°C was selected as the operating temperature.

Response time

Figure 8 shows chronoamperometric response plots obtained in 0.1 mol/L PBS at the working potential of 0.0 V (vs. SCE) at 25°C. The biosensor response time is very short, reaching 95% of the steady-state current within 40 s. The biosensor response time is excellent considering that the mediator and GNPs both could quickly transfer the electrons between the enzymes and electrode.

Linear range

Evaluation of the linearity of the biosensor response for serum alcohol was performed according to the NCCLS document EP6-A (32). Figure 9 shows a linear calibration curve of the steady-state current vs. serum

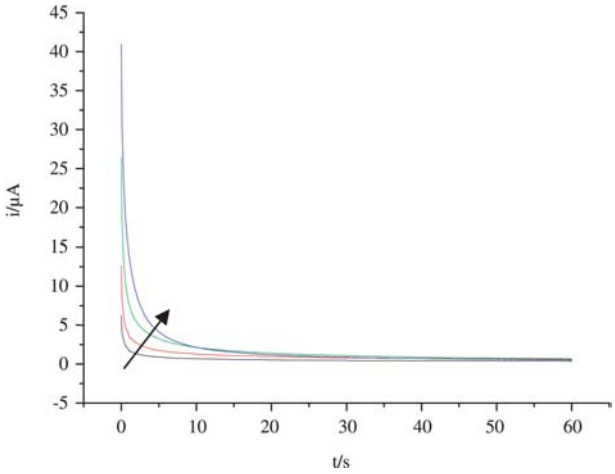

Figure 8 Chronoamperometric response plots of the biosensor obtained in 0.1 mol/L PBS (pH 8.0) at the working potential of 0.0 V (vs. SCE) at 25°C. Serum alcohol concentration from bottom to top: 2, 4, 6, and 8 mmol/L.

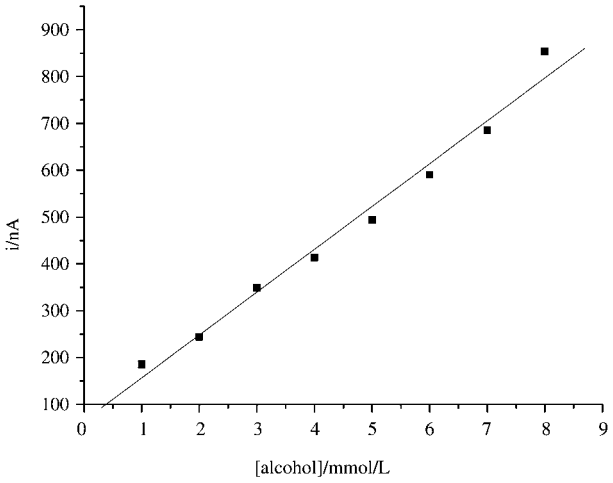

Figure 9 Linear calibration curve of the steady-state current vs. serum alcohol concentration.

alcohol concentration. The analytical curve was calibrated by the linear regression equation: i(nA) = 65.2 + 91.5C (mmol/L), r = 0.991. The detection limit of the biosensor was estimated to be 1.6×10^{-5} mol/L serum alcohol at a signal-to-noise ratio of 3. The linear response range of the biosensor to serum alcohol could be extended at least to 8 mmol/L. The apparent Michaelis-Menten constant (K'_m) could be estimated by the electrochemical version of the Lineweaver-Burk equation, $I_k - I_m - K'_m \times I_k / [S]$ (33), where I_k is the electrocatalytic current after the addition of the substrate, [S] is the bulk concentration of the substrate, and I_m is the maximum current measured under saturate condition. A K'_m value was calculated to be 8.3 mmol/L, which is smaller than that reported in the literature (16), showing that the immobilization procedure could not result in a significant change in enzyme activity and the biosensor presents higher affinity to serum alcohol.

Precision and stability

Evaluation of precision performance of biosensor response for serum alcohol was performed according to the NCCLS document EP5-A (34). The two different serum alcohol concentrations (1 and 5 mmol/L) were detected using the identical biosensor for 10 successive assays within 1 day. The between-run precision was the most important factor in the practical application of disposable biosensor. In total, 10 electrodes were randomly selected from 10 batches of SPEs to fabricate the alcohol biosensors independently. In total, 1 and 5 mmol/L alcohol concentrations were detected using the 10 different biosensors, respectively. The results are shown in Table 1. After being stored at 4°C for 10, 20, and 30 days, the response current for 1 mmol/L serum alcohol decreased by 3.1%, 4.6%, and 7.7% of the initial response, respectively. The above results indicate good precision and stability of the biosensor.

Interference tests

The electroactive substances commonly present in real blood samples, such as methanol and glucose, could cause problems in accurate determination of alcohol. Interference tests were performed according to the NCCLS document EP7-A (35). The results are shown in Table 2. Through paired-difference statistical analysis, methanol, glucose, lactic acid and ascor-

Table 1 Precision tests of biosensor response for serum alcohol.

Serum alcohol concentration, mmol/L	Within-run (n = 10), mmol/L	RSD, %	Between-run (n = 10), mmol/L	RSD, %
1	0.91 ± 0.05	5.49	0.86 ± 0.06	6.98
5	4.87 ± 0.07	1.44	4.82 ± 0.12	2.49

RSD, relative standard deviation.

Table 2 Interference tests of biosensor response for serum alcohol (n = 3).

Sample, mmol/L	Added concentration, mmol/L	Found concentration, mmol/L	Interference concentration, mmol/L
Serum alcohol	0	1	0
Methanol	5	3.82 ± 0.04	2.82
	3	2.36 ± 0.03	1.36
	1	1.23 ± 0.03	0.23
Glucose	5	1.87 ± 0.06	0.87
	3	1.46 ± 0.05	0.46
	1	1.14 ± 0.04	0.14
Lactic acid	5	1.94 ± 0.05	0.94
	3	1.57 ± 0.03	0.57
	1	1.18 ± 0.06	0.18
Ascorbic acid	5	1.73 ± 0.04	0.73
	3	1.35 ± 0.06	0.35
	1	1.12 ± 0.03	0.12

Table 3 Determination results of alcohol concentration in serum samples (n=3).

Found in diluted serum, mmol/L[a]	Added, mmol/L	Total, mmol/L[b]	Recovery, %
0.58	0.2	0.75±0.03	96.3±3
	0.4	0.99±0.05	101.3±5
	0.6	1.17±0.03	99.7±3
0.86	0.2	1.08±0.04	101.6±4
	0.4	1.22±0.06	96.8±6
	0.6	1.47±0.05	100.9±5

[a]Alcohol concentration determined by gas chromatography. [b]Alcohol concentration determined by alcohol biosensor.

bic acid all could not influence the biosensor response for serum alcohol. Absence of sample interference could be related to the low working potential and the specificity of ADH.

Recovery tests

Recovery tests were performed according to the NCCLS document EP15-A (36). Two real serum samples were firstly analyzed by a GC-9A gas chromatography. Then, the samples were assayed with the biosensor. Table 3 shows the concentration found and the recoveries obtained by the biosensor. The biosensor presents good recovery values. The results show that the biosensor demonstrates highly accurate and selective serum alcohol.

Method comparison

Method comparison was performed according to the NCCLS document EP9-A2 (37). A total of 10 serum samples were used for a method comparison study between gas chromatography and the biosensor. As shown in Figure 10, the data show good correlation between the results obtained with the biosensor and results obtained by gas chromatography. The analytical curve is calibrated by the linear correlation equation: Y=0.9X–0.01, r=0.996.

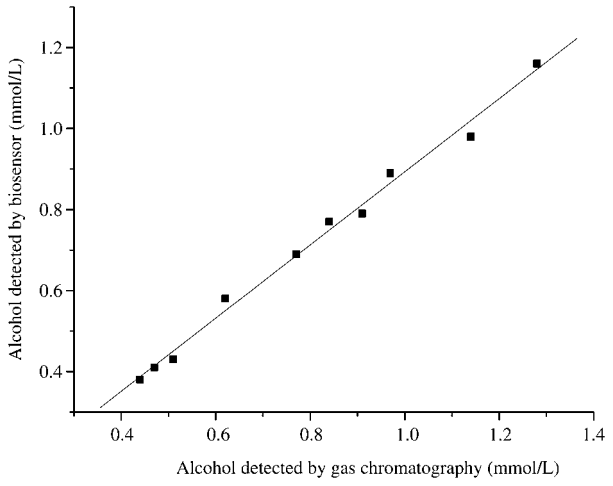

Figure 10 Method comparison of serum alcohol results obtained with gas chromatography and the biosensor.

Conclusions

In this paper, an amperometric disposable blood alcohol biosensor has been reported, which is prepared by immobilizing ADH and NAD^+ coated by Nafion combined with GNPs onto the surface of MB modified SPE. The experiments described above exhibit the capability of this biosensor of detecting blood alcohol concentration in laboratory medicine and forensic medicine, unnecessarily performing laborious sample pre-treatment. In continuation of this work, the screen-printed three-electrode system for blood alcohol determination will be investigated and described in the near future.

Acknowledgements

This work was supported by the Innovative Foundation of Chongqing Medical University (No. CX200532), the Excellent Doctor Foundation of Chongqing Medical University, China.

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