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Contributed Paper

Performance of a Gold Electrode Modified with a Nickel Oxy-hydroxide Film for Amperometric Determination of Ethyl Glucuronide, a Biomarker for Alcohol Consumption

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ABSTRACT

Ethyl glucuronide (EtG) is a specific and sensitive biomarker of alcohol consumption. It can be detected in urine up to 80 h after the elimination of alcohol from the body. Electrochemical detection based on a gold electrode modified with a nickel oxy-hydroxide film was developed for EtG. This method was simple and produced a rapid result that had an excellent linearity for the determination of EtG, 0.04-30.0 mg L^{-1} ($R^2 = 0.996$). The limit of detection (LOD) was 0.04 mg L^{-1} and the limit of quantification (LOQ) was 0.10 mg L^{-1}. The recoveries of the spiked EtG in urine blank samples were in the range of 78±8-119±3%.

Keywords: ethyl glucuronide, biomarker, electrochemical detection, alcohol drinking, nickel modified gold electrode

1. INTRODUCTION

Alcohol is perhaps the most widely consumed licit drug in the world, but its harmful use results in approximately 2.5 million deaths each year [1]. Alcohol abuse is a serious social problem and a major factor for death and injury. Almost all offenders convicted of impaired driving, intra-family violence, child custody cases for family courts, accidents in workplace (pilots in air plane accidents, surgeon) or home and more than a half of the sexual assault victims had been drinking alcohol. Therefore, determination of the amount of alcohol in the blood or the rapid detection of an alcohol marker is

necessary because it can be used as scientific evidence to arrest and prosecute offenders.

In humans, alcohol is eliminated from the body by 2 routes. Route 1, about 90-95% of the total elimination of ethanol is mainly metabolized through its enzymic oxidation to acetaldehyde by alcohol dehydrogenase in the liver followed by further oxidation by aldehyde dehydrogenase to acetic acid [2-3]. Minor amounts are excreted by the kidneys (0.5-2.0%), lungs (1.6-6.0%) and skin (max. 0.5%) [4-5]. Route 2, is the detoxifying pathway, in which about 0.5-1.5% of the total ethanol elimination involves biotransformation to ethyl glucuronide (ethyl-β-D-6-glucosiduronic acid, EtG), ethyl sulfate (EtS), fatty acid ethyl esters (FAEE), and phosphatidylethanol (PEth) [6-8] and excreted in the urine. The detection of alcohol in the body is possible only for a relatively short time after consumption (less than 12 h) [4, 9]. The interpretation of the alcohol level in the cases when the samples cannot be collected immediately or in the case of a dead body is very difficult. For this reason much research has been focused on the finding of a suitable alcohol marker(s) that can be detected several days after ethanol itself has been eliminated. EtG, a direct metabolite of alcohol, has been found to be the most interesting marker for alcohol consumption since it has a much longer half life time than ethanol and a high sensitivity (ability to correctly identify all individuals who have consumed alcohol) and specificity (ability to correctly identify all individuals who have not consumed alcohol) [4, 8]. It can be detected up to 36 h in serum and up to 80 h in urine [10].

Several analytical methods have been developed and published for EtG detection such as gas chromatography/mass spectrometry (GC/MS) [4], high performance liquid chromatography/mass spectrometry (HPLC/MS) [11], liquid chromatography (tandem) mass spectrometry (LC/MS/MS) [4], high performance liquid chromatography with pulsed electrochemical detection (HPLC/PED) [12]. Although these techniques provide for a highly sensitive detection and accurate identification the instruments required are expensive, detection is time-consuming, needs skilled operators and often requires sample preparation prior to analysis. An interesting alternative could be electrochemical detection due to this method being highly sensitive, provides fast response, has a simple operating procedure, a short analysis time and uses relatively inexpensive equipment [13-14].

Pulsed electrochemical detection coupled with HPLC analysis has earlier been applied to detect EtG in post-mortem urine specimens using an Au rotating disk electrode, which involved multi-step potential-time waveforms and the system is relatively expensive [12]. Therefore, an approach based on a simpler electrochemical detection system would be well received. A variety of metallic materials (Au, Ni, Cu) have been extensively use as working electrodes [13]. Among them gold electrode is selected this is because of their versatile potential window, low background current, low cost and chemical inertness.

The detection of EtG with bare gold electrode was first investigated but no oxidation peak of EtG was observed. So, we have interesting the modifications that provided routes to improve the electrocatalytic properties and mediate fast electron transfer between analyte and the electrode. The Ni oxy-hydroxide film catalyst has been selected because the nickel hydroxide exhibit excellent electrocatalytic behavior in alkaline medium, high surface area that can easily prepared by a simple procedure and cost-effective.

Therefore, in this report, a gold electrode

modified with a Ni oxy-hydroxide film was employed for the amperometric detection of EtG in a flow injection system. Various parameters were optimized to obtain the highest efficiency of the developed system. Analytical performances such as the linear dynamic range, limit of detection, stability, and electrode-to-electrode reproducibility were evaluated. The developed method was also tested with urine samples.

2. MATERIALS AND METHODS

2.1 Materials

Ethyl-β-D-glucuronide (EtG, >95% purity) was from Athena Environmental Sciences, Inc. (AthenaES™) (Baltimore, USA), methyl-β-D-glucuronide sodium salt (MetG, >99% purity), uric acid and ascorbic acid were from Sigma-Aldrich (St. Louis, MO, USA). Sodium hydroxide, sulfuric acid and nitric acid were from LAB-SCAN Analytical Science (Labscan Asia Co., Ltd., Bangkok, Thailand). Potassium chloride was from RANKEM (RFCL Limited, New Delhi, India). Sodium hydrogen carbonate, sodium carbonate and hydrogen peroxide were from Merck (Damstadt, Germany). Nickel nitrate was from APS Finechem (NSW, Australia). *D*-(+)-glucose anhydrous was from Fluka (Stienheim, France). All other chemicals and reagents were of analytical grade and all solutions were prepared with deionized water that had been treated by a reverse osmosis system and purified with a Maxima ultrapure water instrument to obtain the resistivity of 18.2 MW-cm (ELGA, England).

2.2 Apparatus

A flow injection system for the determination of EtG is shown in Figure 1. It consisted of a peristaltic pump (Minipuls 3, Gilson, France) to drive the carrier solution (0.2 M NaOH containing 0.5 M KCl electrolyte), an injection valve (6 port valve, Valco Instruments Co. Inc., USA) to control the flow direction and the injected sample volume, a flow cell (dead volume of 10 μL) to hold the electrodes. A three-electrode system that consisted of a gold working electrode modified with a Ni oxy-hydroxide film, a custom made Ag/AgCl reference electrode, and a stainless steel counter electrode (diameter 0.5 mm). They were connected to a potentiostat (potentiostat EA161, eDAQ, Australia) that was connected to a computer.

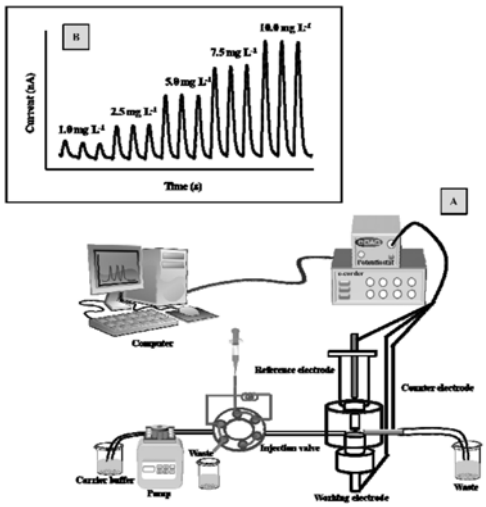

Figure 1. (A) The flow injection system used for EtG detection. (B) Amperograms of EtG in the concentration range 1.0-10.0 mg L^{-1}.

2.3 Preparation of The Gold Electrode Modified with a Ni Oxy-hydroxide Film

Prior to use, each gold electrode (diameter 3.0 mm, 99.99% purity) was cleaned by soaking in concentrated nitric acid (70% w/w) for 10 seconds. The gold electrodes were first polished using alumina slurries with particle diameters of 5.0, 1.0 and 0.3 μm, respectively and washed with deionized water and ethanol. They were then electrochemically cleaned in freshly prepared 0.5 M H_2SO_4 to remove organic compounds on the electrode surface by cycling the potential between +0.10 and +1.50 V *vs.* the Ag/AgCl reference electrode (Metrohm, Switzerland) at a scan rate of 100 mV s^{-1} for 30 scans. A Ni oxy-hydroxide film was electrodeposited onto the gold electrode surface by a cycling voltage of between 0.00 and +1.00 V *vs.* the Ag/AgCl reference electrode at a scan rate of 50 mV s^{-1} in 100 μM $Ni(NO_3)_2$ solution buffered at a pH of 10.00 with $NaHCO_3$/Na_2CO_3 (0.10 M) in a batch system. Ni oxy-hydroxide films of different thicknesses were prepared by varying the number of the electrodeposition scans [15] between 50 to 250 cycles. The modified gold electrode was then washed thoroughly with deionized water and conditioned in a flow system by continuously cycling the potential between -0.25 and +0.55 V for 400 cycles at a scan rate of 50 mV s^{-1} in the carrier solution.

2.4 Optimization of The Flow Injection Amperometric System

EtG (carbohydrate analogue) is a polar aliphatic compound with hydroxyl groups that were active in an electrocatalytic reaction and this made it suitable to be detected by a gold electrode modified with a Ni oxy-hydroxide film in a flow injection amperometric system. Since EtG is very expensive and MetG, is much cheaper, with a similar structure and chemical reactivity as the EtG, the optimization of the parameters in the flow injection amperometric system was first carried out using MetG. After the optimum conditions were obtained, EtG was tested by the system to reconfirm the optimum parameters.

The parameters that might affect the responses of the flow injection amperometric system were studied, *i.e.* the number of scans in the electrodeposition step during formation of the modified electrode, the applied potential, the carrier flow rate, the sample volume and the concentration of the carrier solution. The initial conditions of the system used before optimizations were 50 scan cycles for the electrodeposition of the Ni oxy-hydroxide film, an applied potential of +0.48 V, 0.1 M NaOH as the carrier solution with a flow rate of 0.1 mL min^{-1} and a 100 μL sample volume. Optimizations were carried out by changing one parameter and keeping the others constant. Each of these parameters was investigated by analyzing a series of MetG standard solutions in the concentration range of from 1.0 to 10.0 mg L^{-1}. The sensitivity (slope of the calibration curve) obtained at each condition was compared and the optimum condition was considered to be the one that provided a high sensitivity and a short analysis time.

2.5 Analytical Performance

Under the optimum conditions, the performances of the developed flow injection amperometric system for detection of the EtG were investigated and validated as follows.

2.5.1 Linear dynamic range, limit of detection and limit of quantification

The linearity of the EtG detection was evaluated by injecting standard solutions of EtG in concentrations that ranged from 0.01 to 40.0 mg L^{-1} into the flow injection

amperometric system (three replicates for each concentration). A calibration curve was plotted between the peak height and the concentration of EtG and the linearity of the developed system was obtained from the concentration range that provided the coefficient of determination (R^2) equal or greater than 0.99 [16]. The limit of detection (LOD) and the limit of quantification (LOQ) were based on the signal to noise ratio (S/N) approach [17-19]. A S/N of 3:1 and 10:1 were employed for the estimation of the LOD and LOQ, respectively [18-19].

2.5.2 Operational stability

The stability of the gold electrode modified with a Ni oxy-hydroxide film, set in a flow injection amperometric system was studied by consecutively injecting 5.0 mg L^{-1} of the EtG standard solution in the carrier solution into the flow injection amperometric system. The peak height obtained from each injection was converted into a percentage response in which the peak height from the first injection was set at 100%. The operational stability was determined from the number of injections that still provided a responses that was within ±10% (t_{L10}) of the one obtained from the first injection [20].

2.5.3 Electrode-to-electrode reproducibility

The electrode-to-electrode reproducibility was investigated by fabricating six gold electrodes modified with a Ni oxy-hydroxide film, modified at different time but using the same conditions. Each electrode was used to analyse EtG standard solutions in the concentration range of from 1.0 to 10.0 mg L^{-1}. The sensitivities of each electrode were compared using a two-way ANOVA (analysis of variance) to indicate whether they differed significantly.

2.6 Recovery of EtG from Urine Samples

Three blank urine samples collected from three nondrinker volunteers in the laboratory were analyzed and this study had the approval of the ethics committee of the Faculty of Medicine, Prince of Songkla University, Hat Yai, Thailand. Samples were stored at 4°C until they were analysed. The samples were centrifuged at 3000 rpm for 10 min after being filtered through a 0.2 µm syringe filter. Matrix effect was first investigated by dividing each blank urine sample into 6 portions, five were spiked with EtG and diluted 100 times with the carrier solution to obtain final concentrations of 0.1, 0.5, 1.0, 5.0 and 10.0 mg L^{-1} and the last portion was used as a blank (unspiked sample). These spiked and unspiked urine samples were analyzed under the optimum conditions. The slope of the spiked calibration curve was compared with that of the standard calibration curve using two-ways ANOVA. If the matrix interference was present, both slopes would be different [21]. Therefore, the quantitative analysis of the analyte should be calculated from the matrix matched calibration curve. Recoveries were tested and calculated for each sample to evaluate the accuracy of the developed system [22] at five concentration levels in the linear range of the method, *i.e.* 0.1, 0.5, 1.0, 5.0 and 10.0 mg L^{-1} of EtG concentration (five replicates of each concentration).

3. RESULTS AND DISCUSSION

3.1 Voltammetric Behavior of EtG on The Gold Electrode, Modified with a Ni Oxy-Hydroxide Film

The electrochemical behavior of EtG on the gold electrode modified with a Ni oxy-hydroxide film was characterized by cyclic voltammetry in 0.1 M NaOH at a scan rate of 50 mV s^{-1} in the flow system. The cyclic voltammograms in the presence and absence

of 10.0 mg L^1 EtG solution are shown in Figure 2(A). The dashed line shows the redox pair of $Ni(OH)_2/Ni(O)OH$. In a negative scan, the reduction peak (c_1) was related to the reduction of Ni(O)OH to $Ni(OH)_2$ and the oxidation peak (a_1) was related to conversion of the $Ni(OH)_2$ to Ni(O)OH in a positive scan [14]. After addition of EtG in the solution (solid line), the Ni(O)OH on the electrode surface rapidly oxidized the EtG into a product (which expected to be 6-ethoxy-3, 4-dihydroxy-5-oxo-tetrahydro-2H-pyran-2-carboxylic acid). At the same time, the consumption of the Ni(O)OH species and the production of $Ni(OH)_2$ species resulted in a decrease of the reduction peak (c_2) and an increased of the oxidation peak (a_2). It indicated that EtG is oxidized by Ni(O) OH which acts as an electro catalyst. The oxidation potential range from this cyclic voltammogram was later studied for the optimal applied potential in the amperometric flow injection system (see 3.3.1).

From Figure 2(B), MetG and EtG displayed similar profiles, with an increase of the anodic oxidation response at +0.48 V. These cyclic voltammograms indicated that the MetG can be used as an effective analogue for EtG.

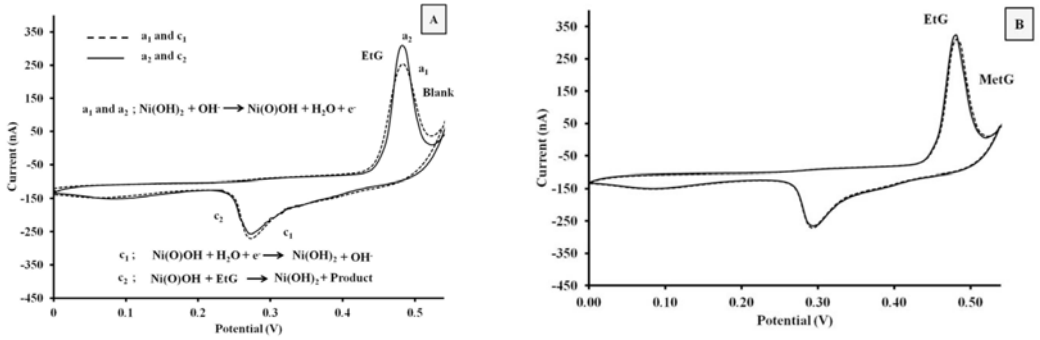

Figure 2. (A) Cyclic voltammogram in the absence (dashed line) and presence (solid line) of 10.0 mg L^{-1} EtG in 0.1 M NaOH at the gold electrode modified with a Ni oxy-hydroxide film. (B) Cyclic voltammogram of EtG (solid line) MetG (deshed line) in 0.1 M NaOH at a scan rate of 50 mV s^{-1}.

3.2 Electrodeposition of Ni Oxy Hydroxide Film on The Gold Electrode

The thickness of the Ni oxy-hydroxide film on the gold electrode can influence the current response to MetG. The film thickness was controlled by the number of scans used for the electrodeposition of the Ni oxy-hydroxide. Each of the modified electrodes, prepared with 50, 100, 150, 180, 190, 200, 210 and 250 cycles, was set into the flow injection amperometric system to detect a series of MetG standard solution. The sensitivity increased from 7.9±0.4 nA $(mg\ L^{-1})^{-1}$ at 50 cycles to 44±2 nA $(mg\ L^{-1})^{-1}$ at 200 cycles and after more scans the sensitivity decreased. These results indicated that at less than 200 scan cycles the films were too thin and had reduced catalytically active sites so the current was small. In contrast at 210 and 250 cycles the film became too thick and the sensitivity decreased due to there being a greater barrier for the transfer of electron in the thick film [23-24]. Therefore, 200 scan cycles were employed for the deposition of the Ni oxy-hydroxide film on the gold electrode.

3.3 Optimization of The Flow Injection Amperometric System

3.3.1 Applied potential

In the flow system, a cyclic voltammogram of the oxidation of $Ni(OH)_2$ to Ni(O)OH in the presence of MetG started at +0.45 V and ended at +0.52 V (Figure 2(B)). The applied potential was therefore investigated between +0.46 and +0.50 V with a 0.01 V interval. The sensitivity increased from 13±1 nA $(mg\ L^{-1})^{-1}$ to 15±2 nA $(mg\ L^{-1})^{-1}$ when the potential was increased from +0.46 V to +0.48 V and became constant with any further increase of the applied potential. Hence, +0.48 V was chosen as the optimum applied potential.

3.3.2 Flow rate of the carrier solution

The flow rate of the carrier solution was tested at 0.2, 0.3, 0.4, 0.5 and 0.6 mL min^{-1}. The sensitivity increased from 12±1 nA $(mg\ L^{-1})^{-1}$ at 0.2 mL min^{-1} to 20±2 nA $(mg\ L^{-1})^{-1}$ at 0.4 mL min^{-1}. This is because the increased flow rate reduced the dispersion of the sample plug, thus, the increase in peak height. However, too fast a flow rate would not allow enough contact time between the target analyte and the electrode surface so the sensitivity decreased. Therefore, a flow rate of 0.4 mL min^{-1} was chosen.

3.3.3 Sample volume

The effect of the sample volume was investigated at 100, 200, 300 and 400 µL. The sensitivity increased from 15±2 nA $(mg\ L^{-1})^{-1}$ to 31±3 nA $(mg\ L^{-1})^{-1}$ with an increase of the volume from 100 µL to 300 µL and became constant. Thus, 300 µL was chosen for further studies.

3.3.4 Concentration of the carrier solution

An alkaline medium solution is required for the electrocatalytic activity of several transition metals for the oxidation of EtG and MetG. Therefore, the effect of the NaOH concentration in an 0.5 M KCl electrolyte solution on the peak current of MetG standard solution was studied from 0.1 M to 0.4 M. The sensitivity increased from 42±3 nA $(mg\ L^{-1})^{-1}$ at 0.1 M to 51±2 nA $(mg\ L^{-1})^{-1}$ at 0.2 M and then decreased at higher concentration of NaOH. This may be due to the decomposition of MetG when the concentration of NaOH became too high [25]. Therefore, 0.2 M NaOH in 0.5 M KCl as the electrolyte was used as the carrier solution for further studies.

3.4 Analytical Performance

The analytical performances of the developed system were evaluated using the optimum conditions; 200 scan cycles for the electrodeposition of Ni oxy-hydroxide film, an applied potential of +0.48 V, a flow rate 0.4 mL min^{-1}, a 300 µL sample volume and 0.2 M NaOH containing a 0.5 M KCl electrolyte. Responses to the EtG and MetG were first compared in the concentration range of 1.0-10.0 mg L^{-1}. The EtG provided a higher sensitivity (86±3 nA $(mg\ L^{-1})^{-1}$) than the MetG (51±2 nA $(mg\ L^{-1})^{-1}$), and similar results have been obtained by Kaushik and co-worker [12].

3.4.1 Linear dynamic range, limit of detection and limit of quantification

The gold electrode modified with the Ni oxy-hydroxide film in an amperometric flow injection system exhibited a good linear response to EtG in the concentration range of 0.04-30.0 mg L^{-1} (three replications for each concentration) with a linear equation of $y(nA) = (93±2)x(mg\ L^{-1}) + (46±18)$ and a coefficient of determination (R^2) of 0.996. The limit of detection (LOD) ($S/N \geq 3$) and the limit of quantification (LOQ) ($S/N \geq 10$) were 0.04 and 0.10 mg L^{-1} of EtG, respectively.

3.4.2 Operational stability

The operational stability of the modified electrode was studied by consecutively injecting 5.0 mg L^{-1} of EtG standard solution into the flow injection amperometric system. The percentage responses against the number of injections are shown in Figure 3. The modified electrode can be used for 42 injections where all the responses were within ±10% (t_{L10}) of the signal obtained from the first injection [20] with an average response of 97±3%. This injection number was high enough to finish several experiments using only one modified electrode.

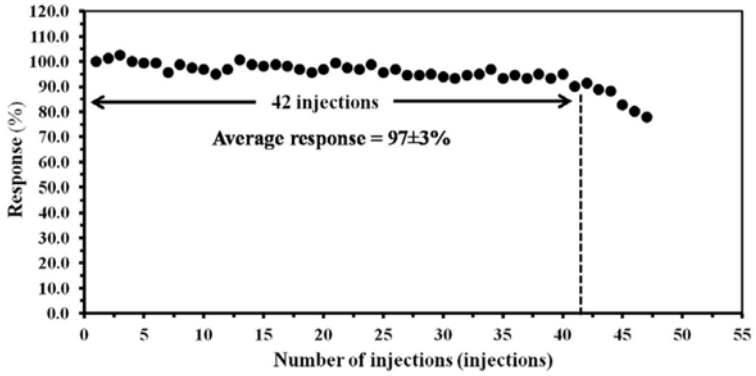

Figure 3. Operational stability of the gold electrode modified with a Ni oxy-hydroxide film for the analysis of 5.0 mg L^{-1} EtG in a flow injection amperometric system in a carrier solution.

3.4.3 Electrode-to-electrode reproducibility

Six electrodes were prepared at different time and modified with Ni oxy-hydroxide film under the same conditions as described in section 2.3. Each electrode was used to determine EtG in the concentrations range from 1.0 to 10.0 mg L^{-1}. The peak heights were used to plot the calibration curves and their sensitivity was compared using a two ways ANOVA (analysis of variance). The sensitivities of the six modified electrodes were not significantly different ($P > 0.05$). An RSD of the sensitivity of the six electrode was 1.5% which indicated that the method used to prepare the gold electrodes modified with the Ni oxy-hydroxide film provided good electrode-to-electrode reproducibility.

3.5 Recovery of EtG from Urine Samples

To test the application of the gold electrode modified with Ni oxy-hydroxide in a real sample, three urine samples were obtained from normal healthy persons. They were initially diluted 10 times with the carrier solution and tested with the developed system to see the background effect. High current responses were obtained even though there should be no EtG in the samples. This was most likely due to the effect of interfering compounds in the urine. To systematically test for the matrix interference the urine samples were spiked with five different concentrations of EtG. They were diluted 100 times with carrier solution, to help reduce the matrix, before injecting into the system. The slope of this matrix matched calibration curve and the slope of the standard curve was compared by two-way ANOVA. The slopes of these two curves were significantly different ($P < 0.05$) which clearly indicated that the matrix had some influence on the analysis. Using the matrix matched calibration curve

the accuracy of the method was then evaluated by calculating the percentage recovery of a known amount of EtG spiked into the urine blank samples. The recoveries were in the range of 78-119% with an RSD= 1-13% (Table 1) which indicated a good accuracy of the developed system as the results corresponded well with the acceptable criteria of 70-120% recommended by the AOAC at the analyte concentration of ppb and ppm level [26].

Table 1. Determination of ethyl glucuronide in the injected sample using the proposed method.

Sample no.	Added amount (mg L^{-1})	Found (mg L^{-1})	Recovery (%)
1	0.1	0.1±0.01	102±13
	0.5	0.6±0.01	119±3
	1.0	0.8±0.07	83±7
	5.0	5.1±0.06	101±1
	10.0	10±0.26	100±3
2	0.1	0.08±0.01	78±8
	0.5	0.5±0.05	108±11
	1.0	1.1±0.04	107±4
	5.0	4.8±0.03	97±1
	10.0	10.1±0.11	101±1
3	0.1	0.1±0.01	98±9
	0.5	0.4±0.02	79±4
	1.0	1.1±0.06	114±6
	5.0	5.0±0.03	100±1
	10.0	10.0±0.03	100±1

Possible interfering compounds in urine sample including ascorbic acid, uric acid, glucose and urea were tested and found that amperometric responses could also be obtained from these compounds. Although the gold electrode modified with the Ni oxy-hydroxide film can not selectively determine EtG, this relatively simple detection system could still be very useful. For example, it can be coupled to a system that helps to separate the different compounds such as a capillary electrophoresis technique. Capillary electrophoresis has been employed by Křivánková and co-workers to detect EtG by indirect UV detection. However, the indirect UV detector provided a narrow linear range (0.079-0.89 mg L^{-1}) and a high detection limit (0.079 mg L^{-1}) [27], compared to our method with a wider linear range of 0.04-30.0 mg L^{-1} and a lower detection limit of 0.04 mg L^{-1}. Thus, the proposed detection method when coupled with the capillary electrophoresis would help to improve the sensitivity, selectivity and detection limit of EtG. With the separation ability of the capillary electrophoresis this method would also be potentially useful for the detection of various other organic solutes in urine samples such as uric acid, ascorbic acid, urea and glucose, which would be very useful in forensic applications and for identification of clinical disease.

4. CONCLUSIONS

The electrochemical detection based on a gold electrode modified with a Ni oxy-

hydroxide film can be applied for the determination of ethyl glucuronide by a flow injection amperometric system. Under the optimum conditions, the flow injection amperometric system provided a good linearity for the detection of EtG in the range of 0.04 to 30.0 mg L^{-1} with a sensitivity of 93±2 nA (mg $L^{-1})^{-1}$ and R^2 = 0.996. LOD and LOQ were 0.04 and 0.10 mg L^{-1}, respectively. The developed gold electrode modified with the Ni oxy-hydroxide film provided good stability (up to 42 injections), good electrode-to-electrode reproducibility (RSD 1.5%), and good recoveries. Future developments of this simple and highly sensitive electrochemical detection system in conjunction with capillary electrophoresis, to help separate the various compounds, for the detection in biological sample would be potentially useful for application in clinical and forensic toxicology.

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