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Determination of metformin hydrochloride using precolumn derivatization with acetaldehyde and capillary electrophoresis coupled with electrochemiluminescence

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ABSTRACT: A novel method was developed using capillary electrophoresis (CE) coupled with tris(2,2′-bipyridyl)ruthenium(II) electrogenerated chemiluminescence (ECL) for highly sensitive detection of metformin hydrochloride (MH) derivatizatized with acetaldehyde. The precolumn derivatization of MH with acetaldehyde was performed in phosphate buffer solution (0.3 mol/L, pH 7.5) at room temperature for 120 min. The effects of acetaldehyde concentration, buffer pH, electrokinetic voltage and injection time were investigated. Under optimized detection conditions, the MH ECL detection sensitivity was more than 120 times that without derivatization. The linear concentration range for MH was 0.001–15.00 μg/mL (with a correlation coefficient of 0.9992). The detection limit was 0.31 ng/mL with a signal:noise ratio of 3. The recoveries of MH in human urine were in the range 98.50–99.72%. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: capillary electrophoresis; electrochemiluminescence; metformin hydrochloride; acetaldehyde; derivatization

Introduction

Metformin hydrochloride (MH) (Fig. 1) is a biguanide antidiabetic agent widely used in the treatment of non-insulin-dependent (type 2) diabetes. In patients with type 2 diabetes it effectively lowers HbA1c values, positively affects lipid profiles, and improves vascular and haemodynamic indices (1). It is often recommended for obese patients, due to its effects on weight reduction and normalization of serum lipids.

A number of analytical methods have been reported for MH determination in both pharmaceutical formulations and biological samples. These have included spectrophotometric detection (2–4), voltammetry (5,6), liquid chromatography–tandem mass spectrometry and high performance liquid chromatography tandem mass spectrometry (7–9), capillary electrophoresis (10,11), chemiluminescence (12) and near-infrared reflectance (13). However, methods such as UV/Vis spectrophotometry and HPLC generally suffer from low sensitivity, and methods such as liquid chromatography–mass spectrometry (LC–MS) require expensive and complicated apparatus.

Capillary electrophoresis (CE) is a widely used separation method that is highly efficient, has a relatively short analysis time and requires only small sample volumes. In recent years, $Ru(bpy)_3^{2+}$-based electrochemiluminescence (ECL) has become an important detection method because it is simple and economical and has low background noise, high sensitivity, good selectivity and wide dynamic linear range (14,15). Recently, the combination of ECL with CE has been used as a powerful tool for the determination of compounds such as thrombin (16), DNA (17), amino acids (18), various drugs (19–28) and NADH (29). Most of these analytes contain tertiary amines, which provide good ECL intensities in reaction with $[Ru(bpy)_3]^{3+}$ without prior derivatization. It has been reported (30) that the detection sensitivity of amines follows the order tertiary > secondary > primary. Analytes containing primary amines have very low ECL intensity. Consequently, only a few studies have used $[Ru(bpy)_3]^{2+}$-based ECL detection for compounds containing primary amine groups; exceptions include selenomethionine (18), octylamine and alanine (31).

The detection sensitivity of drugs containing primary or secondary amines can usually be enhanced by changing their structures or derivatizing with different reagents to form tertiary amines. In this work, a novel and sensitive method for MH was developed based on precolumn derivatization and CE coupled with $[Ru(bpy)_3]^{2+}$-ECL detection. When MH was derivatized with acetaldehyde, a strong ECL intensity was detected in the presence of $[Ru(bpy)_3]^{2+}$. Acetaldehyde did not interfere with the determination of MH, as it has no ECL signal. The detection sensitivity of the proposed method is higher than those reported previously (2–12).

Experimental

Reagents and chemicals

MH (purity > 99.9%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Tris(2,2′-bipyridyl)ruthenium(II) chloride hexahydrate

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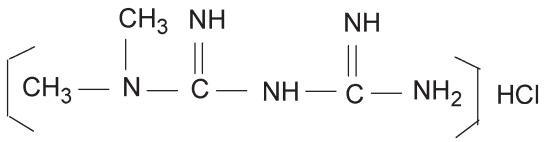

Figure 1. Molecular structure of metformin hydrochloride.

was purchased from Alfa Aesar (a Johnson Matthey Company, Ward Hill, MA, USA). β-Cyclodextrin (β-CD) (recrystallized before use) and acetaldehyde were purchased from Shanghai Chemical Factory. Buffer systems were prepared with sodium dihydrogen phosphate, disodium hydrogen phosphate and sodium borate (Hunan Reagent Company, Hunan, China). All chemicals and reagents were of analytical grade, unless otherwise specified, and used without further purification. All solutions were prepared with double-distilled water (DDW) and stored in the refrigerator at 4°C. Before use, all solutions were filtered through 0.45 μm membrane filters (Shanghai Xinya Purification Material Factory, Shanghai, China) and sonicated for 3 min to degas.

Apparatus and procedures

All separations and analyses were performed on a Model MPI-B CE–ECL Analyser System (Xi'an Remax Tech. Ltd, Xi'an, China). The CE–ECL system consisted of a high-voltage power supplier for separation and injection, a potential control system, a chemiluminescence detection system and a data processor. The output ECL intensity was amplified and recorded using the MPI-B software. The chemiluminescence detection system was composed of a three-electrode system: 200 μm diameter Pt disk working electrode, Pt wire auxiliary electrode and Ag/AgCl reference electrode. The surface of the working electrode was polished with 0.3 μm alumina powder and cleaned with water in an ultrasonic cleaner before use. A reactivation process to eliminate the oxide layer on the Pt electrode was performed by scanning the applied potential on the Pt disk from –0.5 to 0.0 V (vs. Ag/AgCl) for 10 cycles. The performance of the working electrode was stable for at least 2 months after the electrochemical treatment (32). An uncoated fused silica capillary (40 cm × 75 μm i.d., 375 μm o.d.) was used for electrophoresis separation (Yongnian Optical Fiber Factory, Hebei, China). The capillary was filled with 1 mol/L NaOH for 30 min before use and was subsequently flushed with DDW for 30 min, using a syringe. Prior to each run, the capillary was flushed with DDW and the corresponding buffer for about 15 min.

Approximately 350 μL $[Ru(bpy)_3]^{2+}$ (5 mmol/L) in phosphate buffer (50 mmol/L, pH 7.5) was added to the cell for CE–ECL detection. The $[Ru(bpy)_3]^{2+}$–phosphate solution was replaced every 3 h during the experiments to avoid errors in ECL measurement due to possible changes in $[Ru(bpy)_3]^{2+}$ concentration (21). In all experiments, samples were introduced from the anodic end of the capillary by electrokinetic injection at 10 kV for 8 s, and separated in the capillary at 8 kV. Detection potential was fixed at 1.25 V. The running buffer, pH 10.5, contained 7.5 mmol/L phosphate, 7.5 mmol/L borate, 2 mmol/L β-CD and 6 mmol/L Na_2SO_4. The lower layer of the cell was a piece of optic glass through which the photons were captured by a photomultiplier tube (PMT), which was operated at –800 V. After the baseline ECL signal had stabilized, electromigration injection was used for sample introduction and the electropherogram was recorded.

Precolumn derivatization of MH

MH standard (400 μL, 12.5 μg/mL) was mixed with 100 μL acetaldehyde (0.040%), and then 150 μL 1.3 mol/L phosphate buffer solution (PBS), pH 7.5, was added. The final concentrations of PBS and acetaldehyde were 0.3 mol/L and 1.4 mmol/L, respectively. The mixture was allowed to stand for 120 min at room temperature. The resulting mixture was analysed by CE–ECL.

Sample preparation

A male volunteer (age 52 years, weight 65 kg) abstained from any medications during the week preceding the study. After discharge of overnight urine, the participant did not consume any food or drink in the morning apart from 100 mL water. The urine collected before dosing was employed as a blank. The volunteer took MH (250 mg) with 100 mL water, and urine samples were then collected after 3 h. To eliminate the influence of ionic strength in the sample and obtain a clear electrophoretic profile, a modified Rurak's extraction procedure (33) was performed before electrophoresis. For extraction, 200 μL of either the blank urine or urine samples collected after MH ingestion were pipetted into clean centrifugation tubes. Ethyl acetate (2 mL) and 1 mol/L NaOH (5 μL) were added to the tubes. The solutions were shaken for 5 min, centrifuged (10 min at 3000 r/min) and the upper layer was then transferred into a clean tube. The ethyl acetate layer was evaporated to dryness under a stream of dry nitrogen at 80°C. The inner wall of the tube was then rinsed with 200 μL methanol, and the methanol evaporated off in order to concentrate the sample. The dry residue was dissolved in 200 μL water, and the solution was derivatized according to the section on Precolumn derivatization of MH, above, prior to analysis by CE–ECL.

Results and discussion

ECL curves for cyclic voltammetric scan and selection of detection potential

Derivatization with acetaldehyde aids CE–ECL detection of amino acids (34). Primary amines can react with acetaldehyde to form secondary amine derivatives, which can then react further to produce tertiary amine derivatives, and these can be oxidized by electrogenerated $Ru(bpy)_3^{3+}$ to produce stronger light emission than the initial primary amines (35–37). Although a tertiary amine group exists in the metformin molecule, electron-withdrawing substituents attached to the α-carbon atom will further destabilize positive or electron-deficient radical ions, and hence tend to reduce the ECL activity of the compound (36). A nitrogen atom that is attached to a carbon atom is more electronegative than carbon, and hence is electron withdrawing by an inductive effect. This group results in a low ECL intensity of MH. Derivatization of the primary amine and secondary amine of MH molecule to tertiary amines with acetaldehyde enhances ECL intensity, due to an increase in the number and length of alkyl chains attached to the nitrogen atom, and the formed symmetrical structure that stabilizes radical ion (Fig. 1).

The ECL intensity is dependent on the rate of the light-emitting chemical reaction, which is in turn dependent on the potential applied to the electrode. ECL intensities were recorded in the ECL cell of the different components of the buffer solution, both with and without derivatization and with and without MH (Fig. 2); no CE was applied. The ECL intensities with acetaldehyde only (curve b), MH only (curve c) and acetaldehyde + MH without derivatization (curve d) were very weak. These peak signals were undetectable in the ECL intensity vs. potential scans due to ECL noise from $[Ru(bpy)_3]^{2+}$. In comparison, the buffer solution containing acetaldehyde-derivatized MH (curve e) had obviously

enhanced ECL intensity. This suggests that derivatization of MH increased the reduction rate of $[Ru(bpy)_3]^{3+}$.

The relationship of ECL intensity to the applied potential (1.05–1.40 V) was investigated. The ECL intensity was enhanced when the potential increased from 1.05 to 1.25 V, and then the intensity decreased after 1.25 V. Therefore, 1.25 V was selected as the applied potential for the following experiments.

Effect of pH and concentration of buffer on derivatization reaction

The optimal pH for the buffer solution was investigated in the range 5.5–8.5 (Fig. 3A). The maximum intensity was obtained at pH 7.5. The pH dependence of ECL intensity was attributed to formation of an amino alcohol intermediate. Low pH was unfavourable to initial nucleophilic attack on the aldehyde by the lone-pair electrons of the amine group (34). At higher pH, the dehydration rate of the amino alcohol intermediate increased with increasing pH. Thus, the ECL intensity decreased.

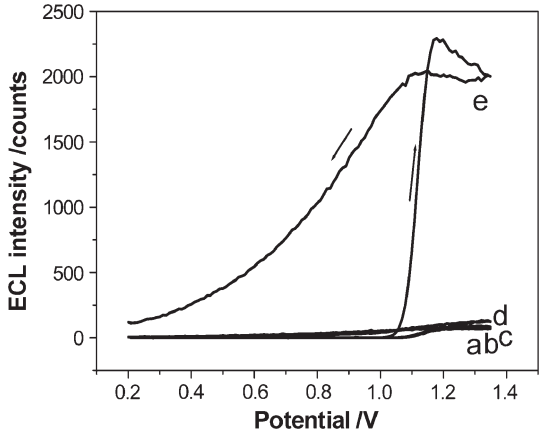

Figure 2. Profile of electrogenerated chemiluminescence. Analysis conditions: scan rate, 100 mV/s; a, 2.5 mmol/L $[Ru(bpy)_3]^{2+}$ + 50 mmol/L phosphate buffer, pH 7.5; b, a + 1.4 mmol/L acetaldehyde; c, a + 6.16 μg/mL MH; d, b + 6.16 μg/mL MH with no derivatization; and e, d with derivatization.

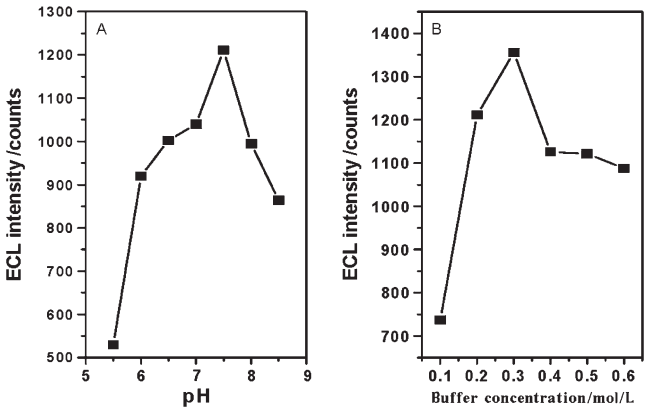

Figure 3. Effect of the phosphate buffer pH (A) and concentration (B) on the derivatization reaction. Analysis conditions for (A): detection potential, 1.25 V; sample, 7.7 μg/mL MH; derivatization reagent, 0.68 mmol/L acetaldehyde; derivatization reaction time, 60 min; derivatization reaction buffer, 0.2 mol/L phosphate; running buffer, 7.5 mmol/L phosphate + 7.5 mmol/L borate + 2 mmol/L β-CD + 6 mmol/L Na_2SO_4, pH 10.0; ECL cell, 50 mmol/L PBS, pH 7.0, +5.0 mmol/L $[Ru(bpy)_3]^{2+}$; injection, 10 kV for 10 s; and separation voltage, 12 kV. Detection conditions for (B): derivatization reaction buffer, pH 7.5 phosphate; and other conditions as in (A).

The concentration of the derivatization buffer solution also affected the ECL intensity (Fig. 3B). With an increase in buffer solution concentration, ECL intensity increased until 0.3 mol/L, after which it decreased. In subsequent experiments, 0.3 mol/L PBS was used as the derivatization buffer solution.

Effect of acetaldehyde concentration and derivatization reaction time

Effect of acetaldehyde concentration (0.16–3.4 mmol/L) on the reaction was examined (Fig. 4A). With a constant reaction time, the enhancement of the ECL signal increased with increasing acetaldehyde concentration. The ECL intensity rapidly reached a maximum value when the acetaldehyde concentration was >1.4 mmol/L, and this was selected as the optimal concentration for precolumn derivatization of MH.

The time that MH was reacted with acetaldehyde also affected the ECL intensity. After addition of acetaldehyde to MH, the ECL signal increased rapidly in the initial 30 min (Fig. 4B). Maximum ECL intensity was reached at 120 min. After 120 min, the ECL signal decreased with increasing reaction time because of the dehydration of the amino alcohol intermediate. Thus, a precolumn derivatization time of 120 min was chosen for detection of MH.

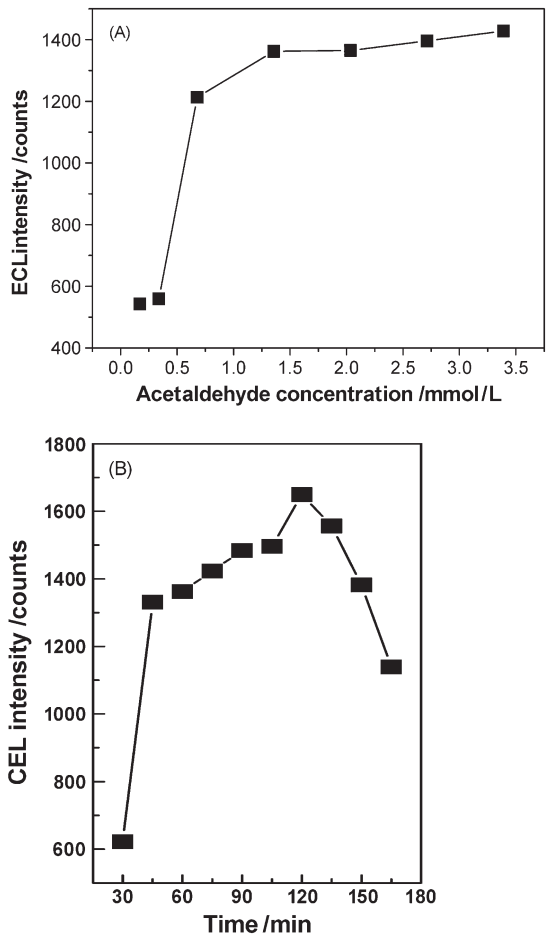

Figure 4. Effect of the acetaldehyde concentration (A) and the derivatization reaction time (B). Analysis conditions for (A): derivatization reaction buffer, 0.3 mol/L phosphate; and other conditions as in Fig. 3B. Detection conditions for (B): acetaldehyde concentration, 1.4 mmol/L, and other conditions as in (A).

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Optimization of buffer pH in ECL cell and pH of separation buffer

In order to obtain the optimal sensitivity of MH in the CE–ECL system, the effect of buffer pH in the detection cell was studied over a wide pH range (6.0–9.0; curve a in Fig. 5). The maximum ECL intensity was reached at pH 7.5, and this pH was used for all the following experiments.

The pH of the separation buffer was also examined in the range 7.0–11.5 (curve b in Fig. 5). The ECL intensity of MH increased with increasing pH to a maximum at pH 10.5, after which it decreased due to the wider ECL peak. Consequently, pH 10.5 was selected as the optimal separation buffer pH. It should be noted that the small amount of separation buffer used in the ECL cell did not alter the pH of the detection buffer (22).

A preliminary study indicated that borate buffer could reduce the background signal and shorten the retention time (35), and PBS could give a higher ECL intensity. Therefore, a mixture of 7.5 mmol/L PBS and 7.5 mmol/L borate solution was used as the separation buffer in this study. In addition, after optimizing a series of concentrations of Na_2SO_4 and β-cyclodextrin, 6 mmol/L Na_2SO_4 and 2 mmol/L β-cyclodextrin were included in the separation buffer to inhibit adsorption of samples and improve the separation (34,38–41).

Optimization of separation voltage, injection voltage and injection time

The influence of separation voltage (4–14 kV) on the ECL intensity was investigated. ECL intensity increased as the separation voltage increased up to 8 kV, and then it dropped as the voltage was increased further. Thus, 8 kV was chosen as the separation voltage.

As a key factor in CE, the effects of injection time and injection voltage were studied in detail. The injection voltage influence on ECL intensity was investigated in the range 4–14 kV. At a higher injection voltage, more analyte can enter into the diffusion layer and a higher ECL signal may be produced. However, the analyte diffuses into the solution, which results in peak retardation and broadening. Therefore, as a compromise between higher ECL intensity and improved column efficiency, an injection voltage of 8 kV was selected.

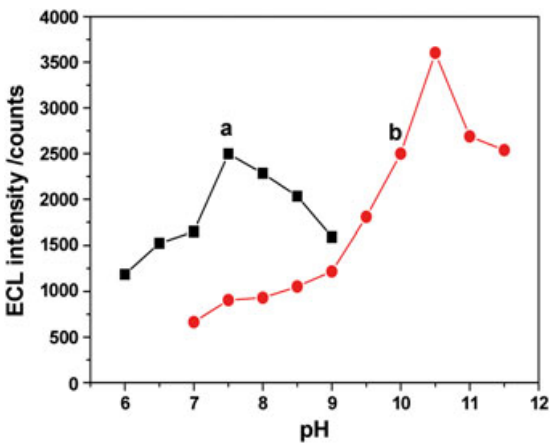

Figure 5. Effect of the phosphate buffer pH in the ECL cell (a) and the separation buffer pH (b) on ECL intensity. Analysis conditions for curve a: derivatization reaction time, 120 min, and other conditions as in Fig. 4B. Analysis conditions for curve b: ECL cell phosphate buffer, pH 7.5; and other conditions as for curve a.

The injection time (4–16 s) also affected ECL intensity. When injection time increased from 4 to 8 s, the ECL signal increased proportionally. Beyond 10 s, the ECL intensity increased slowly. Furthermore, the longer injection time resulted in a broader peak shape, due to an excessively large injection volume. Therefore, 8 s was selected as the optimum injection time.

Comparison of the ECL intensity of MH

Precolumn derivatization with acetaldehyde greatly increased the ECL intensity of MH in the presence of the electrogenerated $[Ru(bpy)_3]^{3+}$ (Fig. 6), and no obvious ECL emission from acetaldehyde was observed. The peak height of MH without acetaldehyde derivatization was 29 (Fig. 6A), while with the same MH concentration, the peak height was 3656 when acetaldehyde was used as a derivatization agent (Fig. 6B). Derivatization of MH with acetaldehyde resulted in an ECL intensity increase of more than 120 times that without derivatization.

Linearity, detection limit and precision of MH

Under the optimized experimental conditions (see above), the linear range and the detection limit were studied. The calibration curve for MH was linear over the concentration range 0.001000–15.00 μg/mL with a regression equation of $y = 471x + 29.2$ (where y is peak height, x is μg/mL, $r = 0.9992$). The detection limit was 0.31 ng/mL with a signal:noise ratio of 3. The quantitative limit for the method was 1.0 ng/mL. The relative standard deviation (RSD) of the ECL intensity (peak height) and the migration time for six consecutive injections of the same preparation (7.7 μg/mL MH) were 1.8% and 1.2%, respectively. The calibration was repeated every day for 5 days. The RSD of the slope and intercept for five calibrations were 1.3% and 1.7%, respectively. The detection limit of MH and linear range of the present method are better than those reported for other methods, which were 3–500 ng/mL and less than three orders of magnitude, respectively (2–12). The

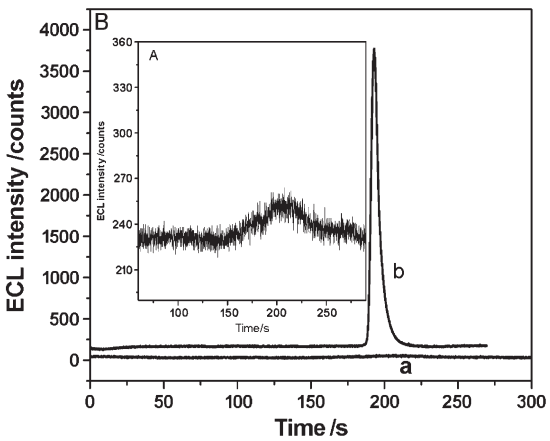

Figure 6. ECL of MH (A) and MH precolumn-derivatized with acetaldehyde (B). Analysis conditions for Fig. 6A: detection potential, 1.25 V; sample, 7.7 μg/mL MH; derivatization reaction time, 120 min; derivatization reaction buffer, 0.3 mol/L phosphate, pH 7.5; running buffer, 7.5 mmol/L phosphate + 7.5 mmol/L borate + 2 mmol/L β-CD + 6 mmol/L Na_2SO_4, pH 10.5; ECL cell, 50 mmol/L PBS, pH 7.5, + 5.0 mmol/L $[Ru(bpy)_3]^{2+}$; injection, 10 kV for 8 s; and separation voltage, 8 kV. Analysis conditions for (B) were as for (A) + derivatization reagent of 1.4 mmol/L acetaldehyde.

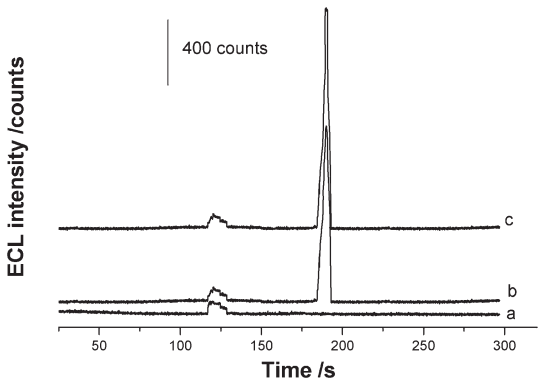

Figure 7. CE–ECL electropherograms of urine samples: a, blank human urine sample; b, human urine sample collected after MH ingestion; and c, sample b spiked with 0.7 μg/mL MH. Analysis conditions are as described in Fig. 6B.

comparison of detection limit between this method and previous reports is shown in Table S1 (see Supporting information).

Application

The proposed CE–ECL method was applied to determination of MH in human urine samples. Peaks were identified by spiking a standard into the sample solutions. ECL electropherograms were obtained from the blank urine sample, the urine sample collected after MH ingestion, and urine spiked with 0.7 μg/mL MH (Fig. 7). The spiked sample (curve c) had a higher peak at 190 s than the unspiked urine sample (curve b). At about 120 s the peak heights for all samples (curves a, b and c) were similar. Therefore, the peak at 190 s was considered to be MH. The volunteer took MH (250 mg) with 100 mL water and urine samples were then collected after 3 h. The content of MH in human urine was determined to be 2.31 μg/mL on average ($n = 5$). To determine the recovery of the method, MH spiked urine samples were prepared in the range 1.00–5.00 μg/mL. All the spiked samples were analysed as described above. The recoveries of MH in urine samples at the different concentrations were in the range 98.50–99.72%. The RSD of ECL peak height was <2%.

Conclusions

A new analytical procedure based on CE–ECL has been developed for determination of MH, using acetaldehyde as a derivatization reagent. The derivatization of MH with acetaldehyde increased the ECL intensity more than 120 times. The RSD of ECL intensity for six continuous injections of 7.7 μg/mL MH ($n = 6$) was <2%. The recovery was >98%. The linear range of the calibration curve with this method was larger than three orders of magnitude. The detection limit was 0.31 ng/mL with a signal:noise ratio of 3. The proposed method is simple, economical and sensitive for the determination of MH in urine.

Supporting information on the internet

Supporting information may be found in the online version of this article.

Acknowledgements

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