Rapid Determination of 7-hydroxycoumarin Using a Nanogold/poly-Thionine Modified Glass Carbon Electrode

Yanjie Zheng,*,**,**† Tianhua Zhong,**** Yichun Xu,* Li Chen,* Xinyang Yin,* Fei Lin,* Qiang Dai,* Shaohuang Weng,*,**† Xinhua Lin*,,**†

* Department of Pharmaceutical Analysis, School of Pharmacy, Fujian Medical University, No 1 Xueyuan Road, Minhou, Fuzhou 350122, China
** Nano Biomedical Technology Research Center, Fujian Medical University, No 1 Xueyuan Road, Minhou, Fuzhou 350122, China
*** Fujian Key Laboratory of Drug Target Discovery and Structural and Functional Research, Fujian Medical University, No 1 Xueyuan Road, Minhou, Fuzhou 350122, China
**** Key Laboratory of Marine Biogenetic Resources, Third Institute of Oceanography, Ministry of Natural Resources, No 178 University Road, Xiamen 361005, China

† To whom correspondence should be addressed.
E-mail: gillzheng@mail.fjmu.edu.cn (Y. Z.); wengshaohuang@163.com (S. W.); xhl1963@sina.com (X. L.)
Abstract

This paper presents a novel voltametric procedure for 7-hydroxycoumarin determination by nanogold/poly-Thionine modified electrode. The characterization of nanomaterials has been conducted by scanning electron microscopy (SEM) and electrochemical methods. The electrochemical sensing of 7-hydroxycoumarin was investigated by cyclic voltammetry (CV) and differential pulse voltammetry (DPV). Through a combination of the excellent electrocatalytic property of nanogold and polymer materials, this sensor shows an improved electrochemical response for 7-hydroxycoumarin detection with a good linear relationship in the range of \(5.0 \times 10^{-6} \text{~to~} 3.0 \times 10^{-5} \text{ mol·L}^{-1}\), and the detection limit was \(1.0 \times 10^{-6} \text{ mol·L}^{-1}\). This method solves the problem that 7-hydroxycoumarin cannot be accurately quantified on bare glassy carbon electrode and improves the detection sensitivity in the meantime. This is expected to play a huge potential in quantitative analysis of quality control, plasma concentration monitoring and mechanism research in vivo of this drug.
Introduction

Coumarin is a kind of compound with benzo-α-pyrene nucleus, which is a natural polyphenolic phytochemical widely distributed in the plant kingdom.\textsuperscript{1,2} Coumarin compounds have attracted great attention because of their biological activity with medicinal value, such as anti-inflammatory,\textsuperscript{3} anticoagulant,\textsuperscript{4} antibacterial,\textsuperscript{5} antiviral,\textsuperscript{6,7} and anti-cancer activities.\textsuperscript{8,9} 7-hydroxycoumarin (structural formula as shown in Fig. 1) is a derivative of coumarin, with the in-depth study of its biological activity, more and more potential applications have been found out. For instance, Narshimamurthy Anegundi \textit{et al.} used zebrafish embryo angiogenesis as a biological model and proved the anti-angiogenic effect of 7-hydroxycoumarin which was expected to play a role as a tumor angiogenesis inhibitor in early treatment of cancer.\textsuperscript{10} Li \textit{et al.} reported that 7-hydroxycoumarin was promising to be an antidote to MG preventing the progression of MG-related diseases for protecting HepG2 cells from methyl glyoxal(MG)-induced toxicity through the NRF2 activation pathway.\textsuperscript{11} Furthermore, Ramasamy \textit{et al.} demonstrated that 7-hydroxycoumarin could lower ultraviolet B-induced oxidation and NF-κB activation, regulate the expression of inflammatory/photoaging molecules in human dermal fibroblast cells. The photoprotective properties indicate its potential as a sunscreen ingredient to protect the skin from exogenous and endogenous harmful substances.\textsuperscript{12} In view of the many medicinal values of 7-hydroxycoumarin, the establishment of a rapid, sensitive and accurate detection method for its quantification is of certain significance to intensive study for the rational use of this drug and its pharmacokinetics effect in vivo.

The preferred position for Figure 1

Until now, the methods for 7-hydroxycoumarin detection mainly focus on high
performance liquid chromatography,\textsuperscript{13,14} capillary electrophoresis\textsuperscript{15,16} and thin-layer chromatographic-fluorescence.\textsuperscript{17} The coupling of these techniques may provide high selectivity of the assay, but brings also some disadvantages of operating complexity, time and reagent consuming and high cost. Therefore, it is essential to develop simple, rapid methods for their determination in routine analysis. Among various methods, electrochemical methods have been widely used for the detection of drugs and biological samples due to their advantages of easy operation, rapid response, low cost and less time consuming.\textsuperscript{18-21} On account of the weak electroactive and irreversible property of 7-hydroxycoumarin, it is difficult to determined accurately on bare electrode directly. Joseph Wang and colleagues have found that a linear function of 7-hydroxycoumarin concentration was in the range from 0.5 mM to 5 mM.\textsuperscript{22} Thus, how to improve the electrochemical response of 7-hydroxycoumarin becomes the key problem to be solved in design of electrochemical method. Conductive polymers have been generally used as ideal chemically modified materials for electrode due to their good stability, excellent electrical conductivity, and strong electrocatalytic activity.\textsuperscript{23-26} Consequently, how to improve the detection sensitivity so as to achieve the detection of 7-hydroxycoumarin by electrochemical means could be a worth thinking problem. Thionine, as a redox dye,\textsuperscript{27,28} could be fixed on the electrode surface by polymerization while this conductive film could be a redox mediator with good redox reversibility, stability and fast charge transfer capacity.\textsuperscript{29-31} Moreover, as one of the inert metal nanoparticles, nanogold is widely used for the electrode interface modification due to their high surface-to-volume ratio and surface activity, excellent electrocatalytic property and desirable biocompatibility.\textsuperscript{32-34} On account of good conductivity of nanogold, it can improve electron transfer and amplify electrochemical signals of electroactive molecules.\textsuperscript{35} When polymer film combining with nanogold materials were
modified on the electrode surface successively, the improved properties of the composites would be beneficial for molecular determination with low electrochemical activity.36

In this paper, nanogold/poly-Thionine composite modified electrodes were prepared by electropolymerization and electrodeposition, respectively. The surface morphology and electrochemical properties of the modified electrodes were studied using scanning electron microscopy(SEM) and electrochemical methods, and the electrochemical behavior of 7-hydroxycoumarin on the modified electrode was investigated. Owing to the remarkable electrocatalytic effect of nanogold/poly-Thionine composite on 7-hydroxycoumarin, the modified electrode was used as a working electrode to determine the content of 7-hydroxycoumarin successfully while the mechanism of 7-hydroxycoumarin determination by this method is shown in Fig. 2. It provides a new idea for the quality control of the drug and a good electrochemical sensing platform for drug monitoring in complex biosamples which is simple, rapid, stable and green.

The preferred position for Figure 2

Experimental

Reagents and chemicals

Thionine was obtained from Sigma-Aldrich (St. Louis, MO, USA). Chloroauric acid was purchased from Sinopharm Chemical Reagent Co., Ltd. (China). 7-hydroxycoumarin was from Beijing Bailingwei Technology Co., Ltd. (China). All other chemicals were of analytical grade and used without any further purification. Phosphate buffer solutions (PBS) were prepared by mixing the stock solutions of 10 mM NaCl and 10 mM NaH2PO4-Na2HPO4, and then adjusting pH with 50 mM H3PO4
or 50 mM NaOH. \([\text{Fe(CN)}_6]^{3-/4-}\) solution acted as the electrolyte solution in electrochemical impedance spectrum (EIS) analysis containing 10 mM K_3[Fe(CN)_6]-K_4[Fe(CN)_6], 0.1 M KCl. Acetate buffer solution (ABS) was prepared by 0.2 M HAc and NaAc, and pH was adjusted to 4.5 with 0.2 M HAc. All solutions were prepared with double-distilled water.

**Apparatus**

The morphology of nanogold/poly-Thionine was analyzed by SU8010 Ultra-high Resolution Scanning Electron Microscope (Hitachi, Japan). Electrochemical Impedance Spectroscopy were performed by Autolab electrochemical workstation (PGSTAT 302F, Netherlands). All electrochemical measurements were carried out on a CHI 660D electrochemical workstation (Shanghai CH Instrument, China). A traditional three-electrode system was used for all electrochemical experiments, the working electrode was a bare or modified glassy carbon electrode, a silver-silver chloride electrode (Ag/AgCl) and a platinum electrode were used as the reference and the auxiliary electrode, respectively.

**Preparation of poly-Thionine modified electrode**

A bare glassy carbon electrode (GCE, Φ=3 mm) was polished successively with 0.3, 0.05 μm of Al_2O_3 slurry. Then, it was rinsed with double distilled water and sonicated in HNO_3 solution (HNO_3:H_2O=1:1), ethanol, and double distilled water in turn for 10 min. After being cleaned, electrochemical modification of GCE was performed using cyclic voltammetry (CV) in HAc-NaAc buffer (pH=4.5) containing 1.0 mmol·L⁻¹ Thionine solution in the potential range of -0.4 ~ 1.5 V for 25 cycles at a sweep rate of 100 mV·s⁻¹. After the electropolymerization, the electrode was rinsed thoroughly with distilled water for further use.

**Preparation of nanogold/poly-Thionine modified electrode**
Electrodeposition was used to prepare nanogold/poly-Thionine modified electrode, the polymer electrode was placed in a 0.1 mmol·L⁻¹ sulfuric acid solution containing 5.0 mmol·L⁻¹ of chloroauric acid, electrodeposition was enforced under CV sweeping from -1.5~0 V at 100 mV·s⁻¹ for 5 cycles. After deposition, the modified electrode was rinsed thoroughly with distilled water.

**Results and Discussion**

**SEM characterization of poly-Thionine and nanogold/poly-Thionine modified electrodes**

SEM image provides detailed information about the surface morphology of modified electrode. As shown in Fig. 3A, a slight loose, fog-like, heterogeneous film appeared on the electrode surface which indicated Thionine successfully polymerized on the surface of GCE. On the basis of poly-Thionine modified electrode, when electrodeposition was conducted by chloroauric acid (Fig. 3B), the interface of GCE was covered with a relatively uniform nanogold with a certain thickness in low magnification field of view (10.0 k×), and when observing in 100 k× (Fig. 3C), three-dimensional structure could be seen clearly different from that on the bare glass carbon substrate electrode, further indicating nanogold film indeed partially covered on the electrode. Furthermore, the elemental analysis was investigated with EDX, as shown in Fig. 3D. EDS analysis obtained from the surface of nanogold/poly(thionine)-modified electrode confirmed the stable and main ingredient of Au and C elements along with a small amount of S (Tab1) confirming the chemical state of the nanogold/poly(thionine) combined with SEM image.
The preferred position for Figure 3

Electrochemical characterization of nanogold/poly-Thionine modified electrode

Electrochemical impedance spectroscopy (EIS) can give information on the impedance changes of the electrode surface in the modification process. So EIS was also used to evaluate electron transfer efficiency at different stages of interfacial electrode shown in Fig 4. With the increase of the polymerization cycle, electron transfer resistance (Ret) increased significantly with the increase of polymerization cycles (Fig. 4A, curve b-1, c-2, d-3; Fig 4B, curve a-5, b-25 cycles, respectively) when aggregated for 25 cycles (Fig. 4B, curve b), the Ret value was nearly 210000 Ω. It was evident that a poly-Thionine film on bare GCE surface was already formed after one cyclic of electropolymerization. Subsequently, when deposited by chloroauric acid on the poly-Thionine modified electrode (Fig. 4C, curve b), Ret decreased sharply and nearly close to the response of bare GCE interface (Fig. 4C, curve a), indicating that the deposition behavior of gold nanoparticles on the film layer was conducive to the electron exchange for $[\text{Fe(CN)}_6]^{3-}/^{4+}$ on the electrode surface. Combined the diagrams of SEM with and EIS, it can be determined that nanogold film has been formed on poly-Thionine modified electrode successfully which play a vital role in accelerating electron transfer. Furthermore, the electrochemical characterization of nanogold/poly-Thionine modified GCE electrode was performed in 0.5 mol·L$^{-1}$ H$_2$SO$_4$ at a scan rate of 100 mV·L$^{-1}$. For comparison, voltammetric behavior of a bare glass carbon electrode and a bare gold electrode with the same geometric surface area were jointly investigated by using cyclic voltammetry. As shown in Fig. 4D, there was no obvious response on the bare glassy carbon electrode in sulfuric acid solution (Fig. 4D, curve a) while the current signal was significantly enhanced on the nanogold/poly-Thionine
modified electrode (Fig. 4D, curve c) than that of the bare gold electrode (Fig. 4D, curve b). By assuming the reduction of monolayer of gold oxide requires $386 \, \mu \text{C}\cdot\text{cm}^{-2}$, the real surface of the nanogold/poly-Thionine) modified electrode was approximately 9.7 times larger than that of bare gold electrode with the same geometric area. Combining the graphs of Fig.3 and Fig.4, obviously, nanogold had successfully been deposited on the surface of the pretreated electrode by thionine, resulting in the electrode having a larger electroactive surface and higher conductivity.

The preferred position for Figure 4

Electrochemical behavior of 7-hydroxycoumarin

Fig. 5 showed CV curves of bare and nanogold/poly-Thionine modified GCE in PBS solution with and without 7-hydroxycoumarin. In PBS solution, there was no obvious current response signal on either bare GCE (Fig. 5, curve a) or nanogold/poly-Thionine modified electrode (Fig. 5, curve b), when adding 5.0 μmol·L$^{-1}$ 7-hydroxycoumarin, no obvious peak current response could be observed on GCE, basically coincided with the response of PBS solution. Even though the concentration went up to 15.0 μmol·L$^{-1}$, a weak and broadened oxidation peak appeared at 0.712 V (Fig. 5, curve c) which could not be accurately quantified. However, a well-shaped oxidation peak appears at 0.764 V after adding 5.0 μmol·L$^{-1}$ 7-hydroxycoumarin on the nanogold/poly-Thionine modified electrode (Fig. 5, curve d). The oxidation peak potential was positively shifted by 52 mV with significantly improved peak shape and increased peak current in contrast to that at bare GCE. The remarkable peak current enhancement can undoubtedly be attributed to the unique structure and properties of nanogold/poly-Thionine (such as large specific area, strong adsorptive ability, and subtle electronic properties). In short, this new approach could be suitable to the direct determination of
7-hydroxy coumarin and improve the detection sensitivity concurrently. The possible electrochemical reaction of 7-hydroxy coumarin on the modified electrode is illustrated in Fig. 6.

The preferred position for Figure 5

The preferred position for Figure 6

Furthermore, in order to make sure the different electrochemical response of 7-hydroxy coumarin on each modified electrode interface, electrocatalytic properties of different modified electrodes (such as poly-Thionine, nanogold and nanogold/poly-Thionine modified electrode) were used as the working electrodes for 7-hydroxy coumarin detection and the electrocatalytic properties were compared. As shown in Fig. 7, a weak oxidation peak with unsatisfactory peak shape and peak height appeared on the bare (curve a), poly-Thionine (curve b) and nanogold modified GCE (curve c) at the same concentration at 0.712, 0.796 and 0.801 V, respectively. However, the peak current response at nanogold/poly-Thionine modified electrode increased significantly (curve d) with distinctly improved shape of peak. It shows that the electrode modified by nanogold/poly-Thionine composite material has a better and more significant electrocatalytic effect on 7-hydroxy coumarin than other monolayer film ones, which makes quantitative determination of 7-hydroxy coumarin possible.

The preferred position for Figure 7

Optimization of experimental conditions
The number of deposition cycles directly determines the thickness of the polymer film, and a proper increase in the thickness of the nanogold also influences electrostatic
interaction between the modified electrode and 7-hydroxycoumarin. Thus, the influence of different deposition behavior on oxidation peak was investigated. As shown in Fig. S1, when the number of electrodeposition cycles was 5, the peak appeared at 0.764 V (Fig. S1, curve b) with the peak current reached at 3.039 μA. As cycle number was went up to 10, the peak potential moved to 0.788 V (curve c) with the peak current decreasing to 1.882 μA. Combined with the CV curves in Fig. 5, the increasing deposition amount of nanogold has increased the coverage on the surface of poly-Thionine film leading to the reduction capacity of electron transfer between the active component and the 3D electrode interface to a certain extent, and then, the current signal decreases. Therefore, it is advisable to choose 5 cycles for the preparation of nanogold film.

The preferred position for Figure S1

The electrochemical oxidation behavior of 7-hydroxycoumarin in various media, such as PBS, Britton-Robinson buffer, HAc-NaAc buffer, Tris-HCl buffer and NH$_3$·H$_2$O-NH$_4$Cl buffer, were compared. The best oxidation response was obtained in pH 7.4 PBS since the peak current is the highest and the peak shape was well-defined. In addition, the influence of pH value on the oxidation peak current of 7-hydroxycoumarin was also examined in PBS solution (Fig. S2). The $E_{pa}$ versus pH graph clearly indicates that the catalytic peak shifts to a more negative potential with the increase of pH. The oxidation peak potential has a good linear relationship with pH value which the linear equation was $E_{pa}(V) = 1.1574-0.04914 \text{pH}$ in the range of pH 4.0–10.0, with a correlation coefficient of 0.9952. The peak current increases as the pH increases and reaches the largest at pH 7.4, subsequently, the peak current singal decreases as pH continues to go up. Therefore, PBS of pH 7.4 was chosen as the
determining medium.

**The preferred position for Figure S2**

Furthermore, the effect of scan rate on the anodic peak current of 7-hydroxycoumarin was studied in this paper. As the scan rate increased, $I_{pa}$ was directly proportional to the scan rate $v$ over the range of 40–160 mV·s$^{-1}$ (as shown in Fig. S3). The linear regression equation was $I_{pa}=0.0063v+0.8161$ with a correlation coefficient of 0.9958, suggesting a surface-controlled process on the modified electrode surface. The excessive sweep speed will lead to the increase of charging current and the decrease of peak current, so 100 mV·s$^{-1}$ was chosen as the optimized condition.

**The preferred position for Figure S3**

**Electrochemical detection of 7-hydroxycoumarin**

Since DPV has a much higher current sensitivity and better resolution than CV, it was used in the determination of 7-hydroxycoumarin concentration at the nanogold/poly-Thionine modified electrode and the estimation of the lower limit of detection. As shown in Fig. 8, the dependence of the peak current on the concentration of 7-hydroxycoumarin is in a linear relationship in the range of 5.0 to 30.0 μmol·L$^{-1}$. The linear equation is $I_{pa}=0.0217C+0.2739$ with a correlation coefficient of 0.9951, and the detection limit is $1.0 \times 10^{-6}$ mol·L$^{-1}$ (S/N=3). The relative standard deviation of the same electrode in 9 successive scans is 2.1 % for 15.0 μmol·L$^{-1}$ 7-hydroxycoumarin and 3.2 % for interelectrodes, indicating that this modified electrode has an excellent reproducibility. This proposed method has effectively improved the detection sensitivity of this drug which is difficult to accurately quantified on bare GCE. The analytical
performance of several strategies containing HPLC and CE along with DPV approaches was summarized and compared in Tab. 2. The LOD of this proposed assay is even lower than those of previously reported approaches.\textsuperscript{13-16,22} Although this method could not achieve a higher sensitivity than some reported one,\textsuperscript{17} this method makes 7-hydroxycoumarin detection by electrochemical method more sensitively than the previously literature which has never been dug deep into after Joseph Wang reported.\textsuperscript{22} Meanwhile, it is worth noting that this detection could meet the requirement of rapid and accurate determination for 7-hydroxycoumarin with simple and facile operation, indicating its potential for the possible analysis of some drugs with weak electrochemical activity.

The preferred position for Figure 8

Interferences

To evaluate the interferences of foreign species on the determination of 7-hydroxycoumarin, the effect of the presence of possible co-existence interference was investigated by DPV. In the study, no obvious interference was found for the detection of 5.0 \( \mu \text{mol}\cdot\text{L}^{-1} \) 7-hydroxycoumarin when adding 100 M of \( \text{K}^{+}, \text{Zn}^{2+}, \text{Ca}^{2+}, \text{NO}_3^{-}, \text{Cl}^{-}, \text{SO}_4^{2-} \), sodium citrate, lactose in the mixture solution. The results indicate that modified electrode possesses excellent anti-interference capability for the determination of 7-hydroxycoumarin in complex sample.

Analytical application to human urine

In order to evaluate the feasibility of the method in practical applications, the detection of 7-hydroxycoumarin in human urine was carried out. Three treated urine samples were respectively spiked with 5.0 and 10.0 \( \mu \text{mol/L} \) of 7-hydroxycoumarin. Tab.3
showed that the measured recovery of 7-hydroxycoumarin in urine was between 96.6 % and 103.7 % with the RSD less than 5.2 %. Thus, the measured recovery demonstrated the reliability and accuracy of the proposed method for the detection of 7-hydroxycoumarin in human urine samples, which can be an attractive candidate for drug monitoring.

Conclusions

In this study, a nanogold/poly-Thionine modified electrode was successfully prepared. The SEM measurement indicates that this composite film could be assembled layer-by-layer on the surface of electrode. This composite film has good electrocatalytic activity toward the oxidation of 7-hydroxycoumarin and significantly improves the detection sensitivity which could probably solve some components analysis with weak electrochemical activity. Furthermore, this method has the advantages of simple preparation, low cost and good stability, which can provide a new idea for the quality control of 7-hydroxycoumarin and the monitoring of its clinical urine or blood concentration.

Acknowledgements

The authors gratefully acknowledge the financial support of the National Science Foundation of China (21705021 and 21775023), Open project(HY201703) and COMRA program (DY135-B2-08) funded by Key laboratory of biological genetic resources from Ministry of Natural Resources, Medical Innovation Project of Fujian Province of China (2016-CX-44), Joint Funds for the innovation of science and Technology, Fujian province (2017Y9121 and 2019Y9011).
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Fig. Captions

Fig. 1  Structure of 7-hydroxycoumarin.

Fig. 2  Schematic diagram of determination of 7-hydroxycoumarin

Fig. 3  SEM images of poly-Thionine (A) and nanogold/poly-Thionine modified electrode (B, C) with different magnification (A, B, ×10.0 k; C, ×100 k) and EDS (D) results of the surface of poly-Thionine modified electrode

Fig. 4  Electrochemical impedance spectrum (EIS) of different electrodes. (A) bare electrode (a); poly-Thionine modified electrode with 1 cycle (b); 2 cycles (c); 3 cycles (d); (B) 5 cycles (a); 25 cycles (b); (C) bare electrode (a); nanogold/poly-Thionine modified electrode (b). (D) CV curves of bare GCE (a), bare gold electrode (b) and nanogold/poly-Thionine modified electrode (c) in 0.5 M H₂SO₄ solution at a scan rate of 100 mV/s

Fig. 5  CV curves of 15.0 μmol·L⁻¹ 7-hydroxycoumarin at bare electrode and 5.0
μmol·L⁻¹ 7-hydroxycoumarin at nanogold/poly-Thionine modified electrode in PBS of pH 7.4 (scan rate:100mV.s⁻¹).

Fig. 6  The electrochemical behavior of 7-hydroxycoumarin on nanogold/poly-Thionine modified electrode

Fig. 7 CV curves of 5.0 μmol·L⁻¹ 7-hydroxycoumarin at bare electrode (a), poly-Thionine modified electrode (b), nanogold modified electrode (c) and nanogold/poly-Thionine electrode (d) in PBS (pH 7.4) (scan rate:100mV.s⁻¹).

Fig. 8 DPV curves with varying amounts of 7-hydroxycoumarin on nanogold/poly-Thionine modified electrode in PBS (pH 7.4 ) (from bottom to top: 5.0, 7.0, 9.0, 11.0, 13.0, 15.0, 18.0, 20.0, 22.0, 25.0, 30.0 μM). The inset shows linear fitting curve between \( I_{pa} \) and the concentrations of 7-hydroxycoumarin.

Tab.1  EDAX ZAF Quantification

Tab.2  Comparison of the reported methods for 7-hydroxycoumarin detection

Tab. 3  The recovery of the proposed method in 50% human serum
Graphical Index

Fig. 1

![Graphical Image 1]

Fig. 2

![Graphical Image 2]
Fig. 3
Fig. 4
Fig. 5
Fig. 6

\[
\text{OH} \quad \text{+ H}_2\text{O} \quad \xrightarrow{-2\text{H}} \quad \text{+ 2H}^+ 
\]
Fig. 7
Fig. 8
| Element | Weight (%) | Atom (%) |
|---------|------------|----------|
| C K     | 23.24      | 82.11    |
| S K     | 1.22       | 1.62     |
| AuL     | 75.54      | 16.28    |
| Total   | 100.00     | 100.00   |
| Detection strategies                          | Linear range                  | Detection limit     | Ref  |
|----------------------------------------------|-------------------------------|---------------------|------|
| High-performance liquid chromatography       | 3.08--616 μM (0.5-100 μg/ml)  | 3.08 μM (0.5 μg/ml) | [13] |
| High-performance liquid chromatography       | 30.8--616 μM (5-100 μg/ml)    | 30.8 nM (50 ng/ml)  | [14] |
| Capillary electrophoresis                    | 0-308 μM (0-50 μg/ml)         | 6.17 μM (1 μg/ml)   | [15] |
| Capillary zone electrophoresis               | 0-150 μM (0-24.3 μg/ml)       | 6.17 μM (1 μg/ml)   | [16] |
| Thin-layer chromatographic-fluorescence      | 0.0308-61.6 μM (5-10 000 ng/ml) | 6.17 nM (1 ng/ml) | [17] |
| Differential pulse voltammetry at bare GCE   | 0.5-5 mM (81-810 μg/ml)       | 10 μM (1.62 μg/ml)  | [22] |
| Differential pulse voltammetry at nanogold/poly-Thionine modified GCE | 5-30 μM (0.81-4.86 μg/ml) | 1 μM (0.162 μg/ml) | This work |
**Tab. 3** The recovery of the proposed method in 50% human serum

| Sample number | \( C_{\text{added}} \) (μM) | \( C_{\text{tested}} \) (μM) | Recovery (%) | RSD(%) |
|---------------|-------------------------------|-------------------------------|--------------|--------|
| 1             | 5.0                           | 5.07                          | 101.4        | 4.5    |
| 2             | 5.0                           | 4.83                          | 96.6         | 5.2    |
| 3             | 4.89                          | 97.8                          | 2.7          |
| 4             | 9.88                          | 98.8                          | 2.9          |
| 5             | 10.0                          | 103.7                         | 3.7          |
| 6             | 9.75                          | 97.5                          | 3.1          |