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Application of Disposable Screen-printed Electrode as an Epirubicin Sensor and Relation among Whole Blood and Tissue Concentrations of Epirubicin

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A disposable Multiwalled carbon nanotubes (MWNT) modified screen printed electrode (SPE) was developed for the clinical determination of Epirubicin（EPI）. EPI was determined by square wave voltammogram in HAc-NaAc buffer of pH 4.5 in the concentration range of 0.01 to 1.28μg/mL. The reproducibility of the analytical signal was characterized by a relative standard deviation smaller than 5%, and the calculated values of detection limits were 8.0 ng/mL. The applicability of the developed method was evaluated by determining EPI in a real sample. After administration of EPI, the concentration-time curve in rabbit blood was analyzed with the MWNT-SPE and the results compared well with those measured by high performance liquid chromatography (HPLC). The concentration-time curve study was also performed to investigate the whole blood- tissue correlation of EPI in mice. Whole blood, heart, liver, kidney and bone marrow samples were collected and analyzed using the validated MWNT-SPE. Good correlations between tissue and whole blood EPI levels were found. These correlations showed the feasibility and rationality of estimating tissue EPI concentration using whole blood, and would be helpful in the routine monitoring of EPI in clinical use.

Keywords: Epirubicin; Screen-printed electrode; Multiwalled carbon nanotube; Whole blood- tissue correlation

1. INTRODUCTION

Epirubicin (EPI), an anthracycline antibiotic and antitumour derivative of Doxorubicin, has been used to treat a wide range of cancers. However, the clinical application of EPI is limited because of the strong toxicity, as cumulative dose-related cardiotoxicity and myelosuppression[1, 2]. For this reason, monitoring the concentration of EPI in blood and tissues is beneficial to optimize drug dosing for achieving the maximal therapeutic effect with minimal toxicity reactions [3].

The determination of EPI has been achieved by high performance liquid chromatography (HPLC) [4-9]. However, HPLC analysis is costly, time-consuming, and requires specialized equipments and skilled technicians. The pretreatment of biological samples contains multiple steps (plasma/serum preparation, centrifugation, separation, etc.). An easy, precious method for clinical usage is called for urgently.

Electrochemical methods are generally employed due to their relatively rapid, high sensitivity and selectivity response[10]. Based on the electroactive behavior of anthracycline compounds, the electrochemical detecting methods for Epirubicin or Doxorubicin have been established by different electrodes, such as Ni ion-implanted Electrode[11], cobalt ion implantation modified electrode[12], glassy carbon electrode[13-15], carbon paste electrode [16], paraffin-impregnated graphite electrode[17] and indium-tin oxide electrode[18]. These previous studies are focused on the electrochemical behavior of EPI. However, the detection of EPI utilizing electrode in biological samples has rarely been investigated before, as far as we know. Furthermore, each electrode of these methods needs to be carefully refreshed after every measurement. The regeneration of electrode is time-consuming and requires highly trained personnel, which may not be suitable for on-site uses. Since disposable sensors offer significant advantages such as fast speed, high efficiency, low cost, and small sample size, we report here a screen-printed electrode strip (SPE) sensor for determination of EPI [19]. The proposed sensor strip is fabricated with working, counter and reference electrodes [20-22]. Electrodes modified with carbon nanotubes (CNTs) have been employed for the improved detection of either inorganic or biological molecules. CNTs represent a new kind of nanomaterials, composed of graphitic carbon with one or several concentric tubules. Recent studies have demonstrated the ability of CNTs to improve the electrochemical behavior, which may be attributed to their electronic structure, high electrical conductivity, and active sites [23-28].

This article reported the development of a new disposable MWNT modified screen-printed electrode (MWNT-SPE) for the analysis of EPI in biological samples. Importantly, the MWNT-SPE was applied to the analysis of whole blood and results correlated well with the clinical plasma analysis by HPLC method. In addition, we investigated the correlations between EPI concentrations in whole blood and tissues (heart, kidneys, liver, and bone marrow) in vivo. These results suggested that MWNT-SPE would be a promising monitoring analysis of EPI in clinic uses.

2. EXPERIMENTAL PART

2.1. Reagents

5mg/mL stock solution of EPI was prepared by dissolving EPI (Zhejiang Hisun Pharmaceutical Co., Zhejiang, China) into redistilled water and then stored at -20℃. The MWNT (purity > 90%) was obtained from Chengdu Organic Chemicals Co.(Sichuan, China). Dicetyl phosphate (DCP) was purchased from Sigma. Acetonitrile (ACN) and methanol of HPLC grade were obtained from Sinopharm Chemical Reagent Co. (Shanghai, China). Sodium dodecyl sulfate (SDS) was purchased

from Sigma-Aldrich Co. (St. Louis, MO, USA). All other chemicals were of analytical grade from a variety of suppliers.

2.2. Apparatus

The voltammetric measurements were performed with a CorrTest 2000 Electrochemical Workstation (CorrTest Instrument, Wuhan, China) connected to a computer. Scanning electron microscopic (SEM) image of the SPE electrode was obtained with a FE–SEM instrument (Quanta 200, FEI Corporation, Holland). The pH measurements were carried out using a Digital pH-meter (HI 98128, Beijing Hanna Instrument. Ltd., China). Digital Ultrasonic Cleaner (PS-10A, Jie Kang Technology Ltd., Hong Kong) was used for ultrasonication agitation.

HPLC system comprises P230 high-pressure constant-current pump and UV detector (Elite Analytical Instruments., Dalian, China). Reversed phase separation was performed on Hypersil ODS2 column (200×4.6 mm, C18, 5 μm, Elite Analytical Instruments., Dalian, China).

2.3. Preparation of real samples

Healthy New Zealand rabbits (2.0±0.5 Kg) and KM mice (20±2g) were obtained from Laboratory Animal Center of Wuhan University. The certification numbers were No.00002039 and No.00011801 (SCXK 2008-0004, China). The study protocol was in accordance with the guideline for animal research.

Blood samples were collected with anticoagulative tubes (EDTA-2Na). The whole blood samples were electrochemically detected by MWNT-SPE after diluting up to three-folded with HAc-NaAc (pH 4.5) buffer solution. The samples were subjected for HPLC in parallel. Plasma was separated from the remaining sample after vortex-mixing with 600μL Methanol and centrifugation at 15000 rpm for 10 min. As EPI is mainly distributed in plasma, indicating plasma and whole blood are equally acceptable for testing EPI.

Tissues (heart, liver, and kidneys) were collected after animals were sacrificed. The bone marrow was extracted from both femurs and pooled. All tissue samples were suspended in 0.2mL cold (4℃) 0.9% saline at a concentration of 25% (w/w) and homogenized on ice using a glass homogenizer. After homogenization, tissue samples were centrifuged at 3000 rpm for 10 minutes. Then supernatant was added into the HAc-NaAc solution and quantified. Drug concentration in each tissue was expressed as the percentage of the injected dose in the whole organ.

2.4. Preparation and modification of MWNT- SPE

According to the literature [28-30], a semi-automatic screen-printer was used to prepare disposable SPE (Fig.1.). Briefly, a SPE consisted of carbon-working electrode (a), Ag/AgCl reference electrode (b) and carbon-counter electrode (c). Conducting silver ink (BY2200, China) was initially printed on a flexible polypropylene film (90mm×130mm) to make the effective conductive nature of

the SPE. Then the carbon ink (Acheson, ED423SS, American) was printed upon the printed silver layer to fabricate the working electrode and the counter electrode with some part of the silver ink layer uncovered. After drying, the Ag/AgCl paste (Acheson , ED6038SS, American) was printed as reference electrode. At last, an insulating layer (JUJO, AC-3G, Japan) was printed over the SPE leaving the working area of 0.031 cm^2.

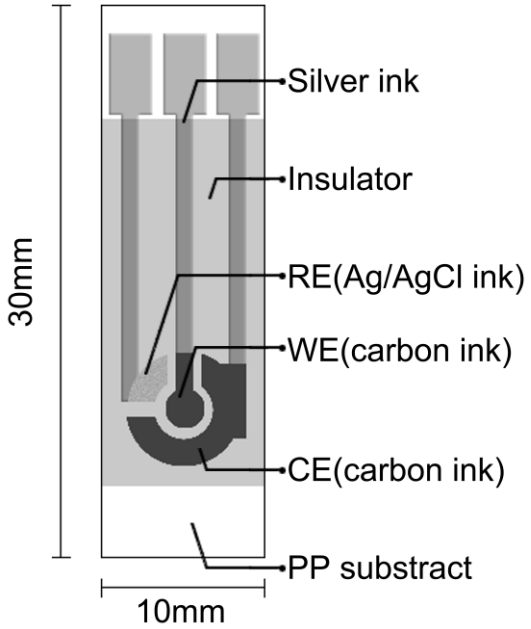

Figure 1. Image of the screen-printed electrode (SPE) consisting of a carbon working electrode, a carbon counter electrode, and Ag/AgCl reference electrode.

The MWNT was dispersed into water with the presence of dicetylphosphate (DCP), a kind of surfactant with two long carbon–hydrogen chains. The MWNT–DCP suspension was achieved via dispersing 5 mg MWNT and 5mg DCP in 5 mL of redistilled water by 30min ultrasonication agitation. Then MWNT-DCP solution was cast on the surface of working electrode, air-dried at room temperature.

2.5. Electrochemical procedure

In the model systems and real samples, EPI was measured in 0.2 mL of solution of different concentrations, to which 0.4 mL of HAc-NaAc (pH 4.5) buffer solution was added. The cyclic voltammetric curves (CV) were recorded over the potential range from –1.0 to 1.0 V versus the Ag/AgCl reference electrode. The scan rate in the CV was 100mV/s. The accumulation step was carried out under open-circuit for 10min. After that, square wave voltammogram (SWV) from -1.0 to -0.4 V were recorded after 10 s quiet time under open-circuit. The deaerated solutions (nitrogen stream,

2 min) were measured at ambient temperature without filtering. Each SPE was discarded after a single use.

2.6. Chromatography

According to the Chinese Pharmacopoeia[31], the mobile phase was a mixture of acetonitrile-Sodium dodecyl sulfate solution (SDS1.44g, $H_3PO_4$0.68mL, water500mL)-methanol at a ratio of 500:500:60(v/v). The separation was performed isocratically at a flow rate of 1.0mL/ min. The column temperature was held at 25 °C. EPI was detected at a working wavelength of 232 nm and a retention time of 8 min. The concentration of the investigated compound was determined from the area of the corresponding peak.

3. RESULTS AND DISCUSSION

3.1 SEM images of SPE surface

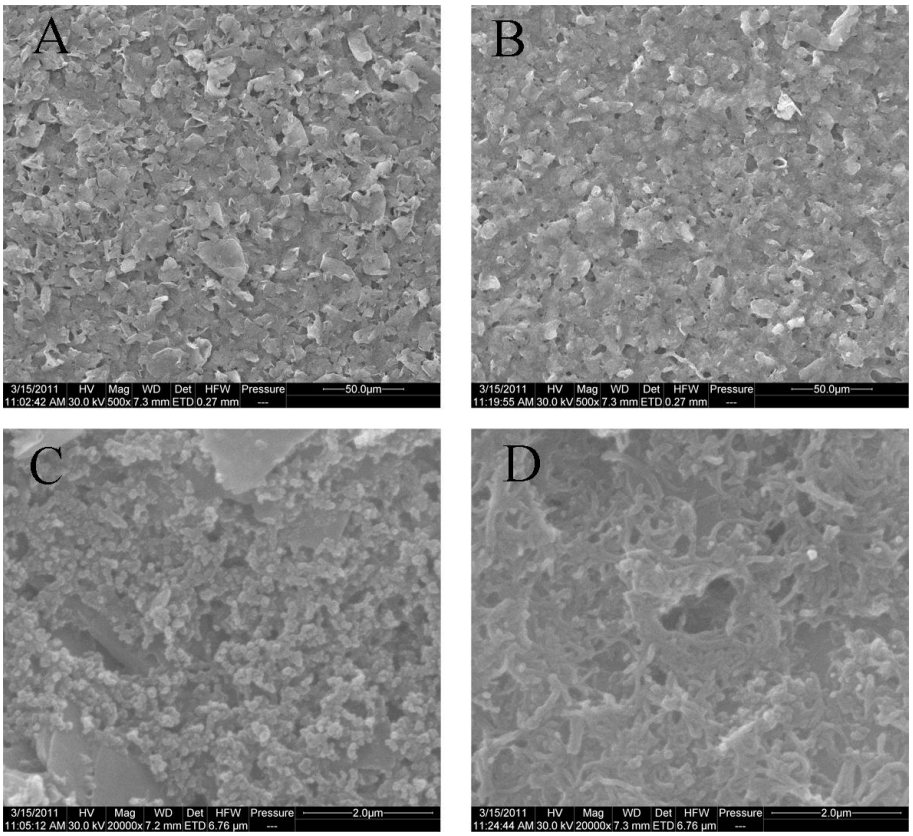

Figure 2. SEM images of bare-SPE (A,C) and MWNT-SPE (B,D) surface. Scale bar:50.0µm(A,B) and 2.0µm(C,D).

The SEM images of the working electrode from SPE and MWNT modified SPE (MWNT-SPE) are shown in Fig.2A-D. The surface of bare SPE is shown in Fig.2A. The original carbon surface is

constituted by flake-shaped particles. The high-resolution SEM image (Fig.2C) shows that the carbon surface is covered with a great content of graphite particles. However, the MWNT-SPE is characterized by a higher microporous structure of flake shape with nonuniform distribution, along with cavities (Fig.2B). And high-resolution SEM shows that the MWNT is highly dispersed and can be discernible (Fig.2D).The surface area of the MWCNT-SPE should be greater than the area of the unmodified SPE due to its roughly nanostructure[32],which could be used to accumulate EPI and avoid surface fouling. In addition, the remarkable catalytic effect of MWNT can promote the electron transfer reaction and enhance detection signal.

3.2. Electrochemical behaviors of EPI at the MWNT-SPE

The electrochemical behaviors of EPI at SPE were examined using cyclic voltammetry (CV) in HAc-NaAc with pH of 4.5 (Fig.3.). Upon addition of EPI, two pairs of redox peaks (R_1/O_1 and R_2/O_2) are observed. According to literature data[15], these correspond to the reduction of quinone (1.) and the oxidation of hydroquinone (2.) centers. The forward (oxidative) component corresponds to the oxidation of hydroquinone groups, while the backward (reductive) component of response corresponds to the reduction of quinone groups. The backward component of the first response is much bigger than one of the second response; consequently, it is used as the determining signal of EPI in this work.

At the bare SPE, EPI yields a very low peak current (Fig.3b), whereas the ratio of peak current to background current is much smaller than that at the MWNT-SPE (Fig. 3a.) at the same potential. This can be ascribed to the unique structure of MWNT (much larger specific surface area, subtle electronic properties and strong adsorptive ability). Thus it can be thought that the MWNT-SPE is suitable for the determination of EPI.

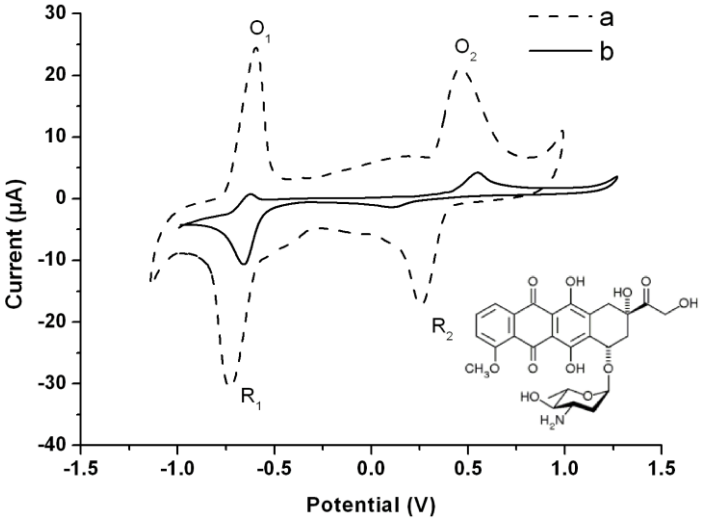

Figure 3. Cyclic voltamograms of 100μg/mL EPI in 0.1M, pH 4.5 HAc-NaAc at different electrodes. Curve (a):MWNT–SPE; curve (b): bare SPE. Scan rate: 100mV/s. The inset shows the structural of EPI.

3.3 Optimum experimental condition for EPI response

Various supporting electrolytes, such as HAc–NaAc, phosphate buffer, and borate buffered (each 0.1 M), were tested. HAc–NaAc was found to be the best, because the voltammogram of EPI was well defined and the sensitivity was reasonably high. To optimize the response of EPI, various pH values of HAc–NaAc solution were tested over the range of 3.0~8.0. The results show that the peak potential is pH dependent and the peak potential shifts towards a negative potential with increasing pH. The peak current increases from pH 3.0 to 4.5, and then decreases from pH 4.5 to 8.0. Thus, a solution of 0.1 M (pH4.5) HAc–NaAc was used in the following experiments.

3.4 Influences of the MWNT–DCP film thickness

The relationship between the volume of the MWNT-DCP suspension and the peak current was examined. An increase in the peak current was noticed when the MWNT suspension was increased from 1.0 to 7.0μL. However, the peak current decreased conversely when the volume exceeds 7.0μL. Because DCP is an insulator and it decreases the electrical conductivity of the film. Due to uncompensated resistive effect or lowering of the charge transfer rate, the peak current contrarily decreased when the MWNT-DCP film was too thick[33]. Therefore, 7μL was chosen as the optimized amount of the MWNT-DCP suspension.

3.5 Effects of the accumulation time

The accumulation was performed under the open-circuit condition. The peak current increased with the accumulation time in the range of 1~20 min. The peak current increased significantly in the first 10 min, and then almost remained constant. A further increase in the accumulation time did not increase the amount of EPI at the electrode surface, owing to surface saturation. To compromise between the sensitivity and running time, accumulation step in this study was performed under open-circuit for 10 min.

3.6 Calibration curve

SWV was further employed to increase the EPI signal. Prior to the practical assays, the SWV parameters were systemically optimized with the strip. The obtained optimized SWV parameters were as follows: potential window =-1.0 to -0.4V, step width =2mV; amplitude =25mV, frequency =10 Hz. The quantitative SWV determination of EPI at a MWNT-SPE in model systems (Fig. 4) was based on the linear relationship between the peak current intensity (|Ip|) and the EPI concentration (c). For the tested range of 0.01–1.28μg/mL, the following equation was obtained: $|Ip| = 0.301+14.683c$（$r = 0.9998$）. The relative standard deviation (RSD) at 0.1μg/mL EPI on different electrodes was about 4.1% (n =10), indicating excellent reproducibility. And the storage stability of this MWNT-SPE was also well maintained for over three months.

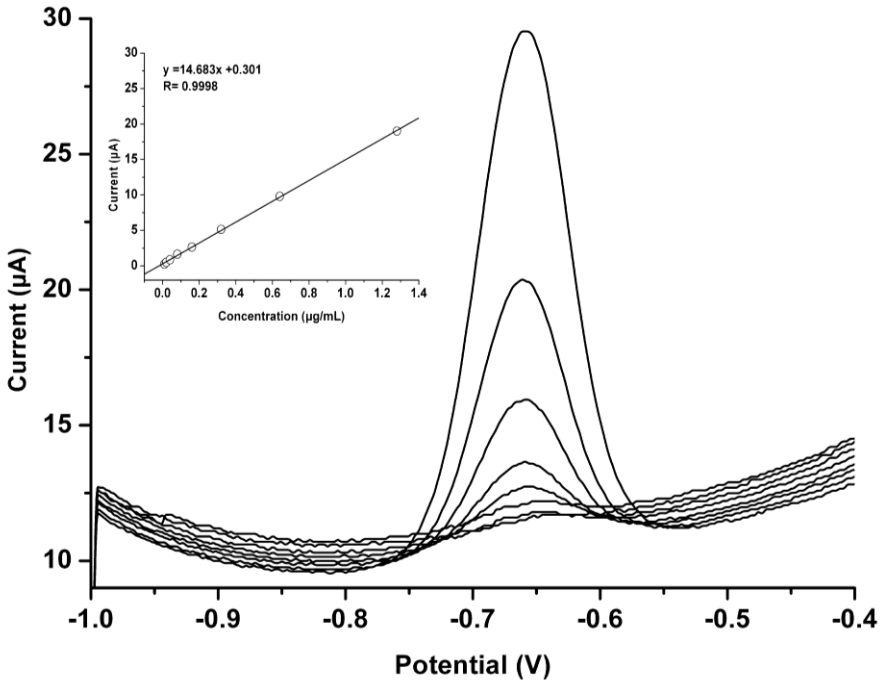

Figure 4. Square-wave voltammetry recorded at the MWNT-SPE for different concentrations of EPI (0.01, 0.02, 0.04, 0.08, 0.16, 0.32, 0.64, 1.28 μg/mL) in HAc-NaAc buffer solution. The inset shows the corresponding calibration plot.

3.7 Interferences

Some organic compounds that perhaps exist in biological sample were investigated on the reduction peak current of EPI. These common interferences, such as ascorbic acid, dopamine, uric acid, vitamin A, glucose, cholesterol, ascorbic acid (AA) vitamin A, vitamin E (concentration is 5×10^{-6} mol/L), did not interfere with the signal of 0.1μg/mL EPI (signal below 5%). These results indicated that the proposed method had good selectivity for the determination of EPI.

3.8. Comparison between MWNT-SPE analysis versus HPLC method in blood samples

3.8.1. Method validation.

In whole blood sample, the linear correlation was found in the range of 0.02–2.56μg/mL (r = 0.999), and the detection limit was 0.01μg/mL. Furthermore, the EPI concentrations determined by SWV agreed well with the results of the comparative HPLC analysis (Table 1). In whole blood, overall coefficients of variation were below 15 % for low and medium concentration and below 10 % for high concentrations for EPI as shown in table 1. The mean calculated recoveries of EPI for the low, medium and high concentrations were calculated as 108.65 %, 90.64 % and 102.05 %. Stability experiments were also carried out to investigate the stability of EPI in samples stored at -20℃. The results indicated that EPI was stable for at least three weeks in whole blood samples at -20℃.

Table 1. EPI measurements obtained by the MWNT-SPE and HPLC in blood samples (*n* =5).

C(μ g/mL)	Intra-day Precision (%)		Inter-day Precision (%)		Recovery (%)	
	MWNT-SPE	HPLC	MWNT-SPE	HPLC	MWNT-SPE	HPLC
0.02	10.52	4.81	14.82	10.89	108.65	113.47
0.16	10.27	3.13	10.06	5.64	90.64	103.86
2.56	8.66	5.68	9.75	3.17	102.05	91.55

3.8.2. Concentration-time curve study in rabbits.

EPI was injected via rabbit ear vein at a dose of 0.5 mg/kg (equivalent to human 60mg/m^2). Blood samples were collected from ear vein to anticoagulative tubes at 5, 10, 30 minutes and 1, 2, 4, 8, 12, 18, 24 hours post-dose.

The concentrations of EPI were found to vary in the range of 0.24-1.27μg/mL. A comparison of blood samples analyzed by HPLC and SPE yielded strong correlation: y = 0.1698 +0.8586x, r = 0.96. Results using the new method were compared with those from a standard HPLC method (Fig.5**.**).

Considering that the dose-limiting side effect was believed to depend on peak drug levels, and the antitumor effect was assumed to be relevant to the area under the concentration-time curve [3], MWNT-SPE had been shown to provide convenient and inexpensive determination of EPI in 'real world' samples.

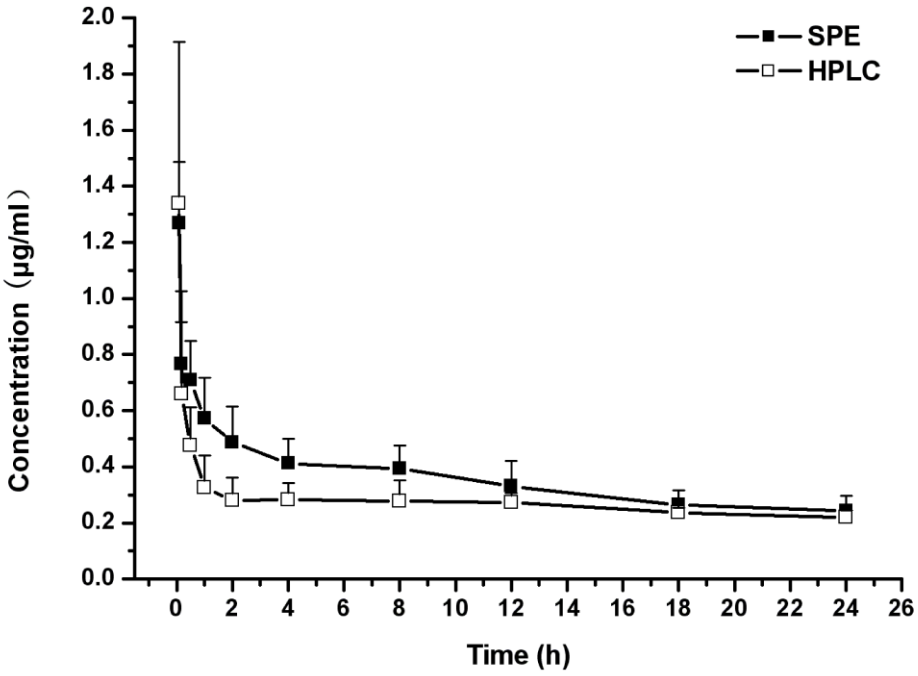

Figure 5. The concentration-time curve of EPI using MWNT-SPE and HPLC.

Conventionally the concentration of EPI is determined by chromatographic methods. These methods are cumbersome, complex and require apparatus for pretreatment of blood sample. Processing of blood specimens is a time consuming procedure and these approaches are thus not convenient for fast analytical assays. The direct analysis of EPI in whole blood is more feasible. So this disposable type MWNT-SPE holds promise in application area and might be valuable for on-site analysis.

On the other hand, various electroanalytical methods and sensors for the detection of anthracycline antibiotic, such as Doxorubicin, have been reported. However, few works concern the direct determination of EPI (not to mention the detection in biological samples). H. Zhang[15]have been reported electrochemical method for the detection of EPI injection samples. The calibration in the range of 5×10^{-8}~1×10^{-5} M with detection limit of 2×10^{-8} M were obtained by using single-walled carbon nanotube-modified glassy carbon electrode. And the relative standard deviation (RSD) at 5×10^{-7} M was about 6% (n=10). In this work, the MWNT-SPE was successfully applied for the determination of EPI in the concentration range 0.01~1.28μg/mL (1.84×10^{-8}~2.36×10^{-6} M). And the of detection limits was 8.0 ng/mL(1.47×10^{-8} M). The RSD at 0.1μg/mL EPI on different electrodes was about 4.1% (n =10). Concluded from the evaluation of the regarding aspects, such as sensitivity, limit of detection, linear range, stability and reproducibility, the MWNT-SPE was already successfully prepared for the determination of EPI concentration. More important, compared with traditional electrodes, the use of low-cost SPE has the advantage that the devices may be used once and then discarded. The use of disposable SPE eliminates the problems of fouling and surface regeneration of the electrochemical device. So the MWNT-SPE has appreciable workability for the EPI quantification assays.

3.9. Correlation of EPI in whole blood and tissues.

EPI was given into a caudal vein at a dose of 20 mg/kg (equivalent to human 60mg/m^2).After the i.v. injection, animals (four mice/group) were sacrificed at different intervals (5, 10 and 30 min, 4, 8 and 12 h). Various samples, including the heart, liver, kidneys, bone marrow and whole blood were collected.

The concentration-time curves of EPI in the whole blood, bone marrow, heart, liver, and kidney were presented in Fig.6. The EPI values in whole blood were found to vary in the range of 0.01-1.08 μg/mL with variations on the reported range of 0.04-1.72μg/mL[34], indicating possible disorders associated with interindividual differences. The concentrations of EPI were 0.04-0.78μg/mL in the bone marrow, 0.04-2.08μg/g in the liver, 0.22-1.77μg/g in the kidney, and 0.10-0.91μg/g in the heart. According to the principle of pharmacokinetics, the distribution of EPI in the body depended on the organ blood flow and tissue affinity. Therefore, a comparatively higher level of EPI was found in the liver and kidneys, and after 10 min, the drug concentration reached its highest level, and then declined rapidly. The correlation coefficients were 0.946 between the heart and whole blood, and 0.971 between the bone marrow and whole blood. After the maximum concentration, the correlation coefficients were 0.984 between the liver and whole blood, 0.977 between the kidney and whole blood. These correlation coefficients suggest that an excellent correlation existed between the blood and tissues. The

t test was also carried out to evaluate the correlation between blood and tissues. A high, significant correlation ($P<0.01$) was found between each tissue and blood. Thus, the EPI content in the blood could reflect the content in tissues.

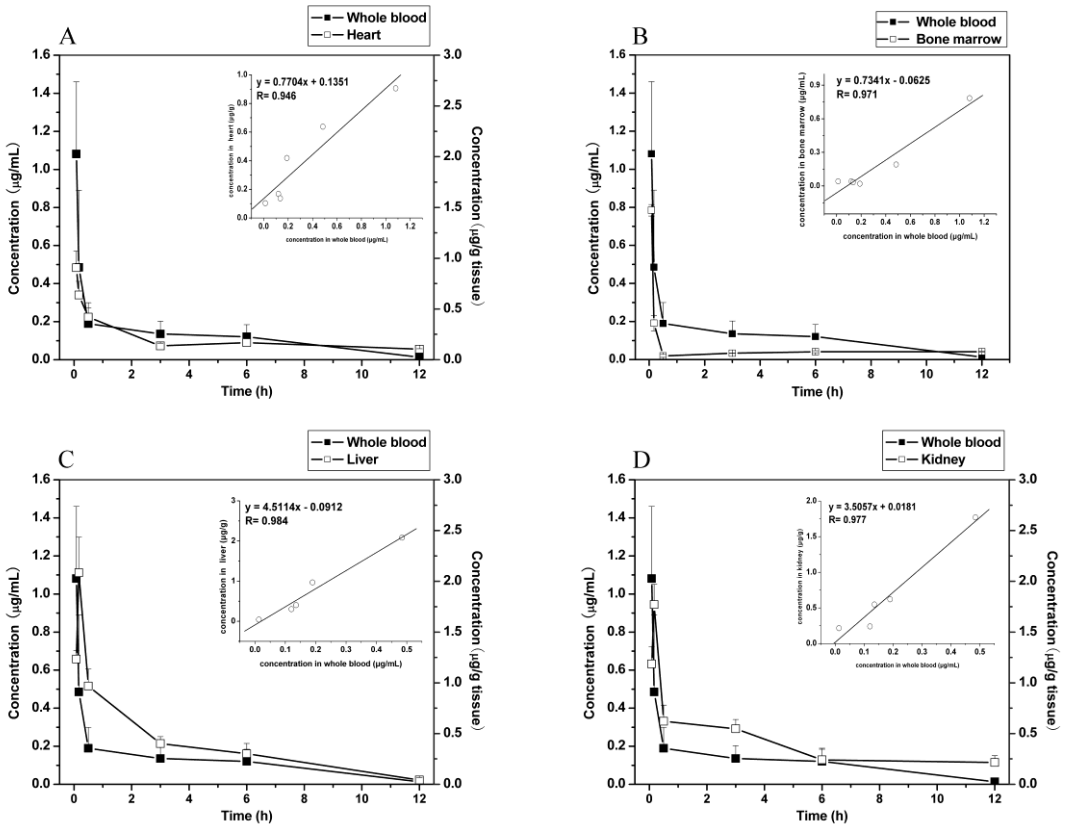

Figure 6. Correlation of EPI concentration-time curves in whole blood and heart (A),bone marrow (B),liver (C), and kidney (D). The inset shows the corresponding calibration plot. Data points are the means of four mice. Bars (SD), if not shown, are within the size of the symbols.

According to the report of the Medicines and Healthcare products Regulatory Agency (MHRA), the use of EPI has special warnings and precautions. During treatment, possible complications due to myelosuppression should be carefully monitored both before and during each cycle of therapy. The cardiac function also must be carefully monitored during treatment to minimize the risk of heart failure. Because EPI is mainly eliminated via the liver, the dosage should be reduced in patients with impaired liver function for avoiding an increase of overall toxicity. In addition, lower starting doses should be considered in patients with severe renal impairment. For these reasons, monitoring the concentration of EPI is beneficial to optimize drug dosing to achieve the maximal therapeutic effect with minimal adverse reactions. Equations shown in Fig.6 described the functional relationship between the EPI concentrations of whole blood and tissues. These equations would be useful for the estimation of the EPI concentration in tissues, in order to reduce cumulative dose-related cardiotoxicity, myelosuppression and avoid liver, kidney damage. Considering whole blood is the most

appropriate sample for drug monitoring, these correlations would be helpful in the routine monitoring of EPI in tissues.

EPI is used as a single agent. The recommended dosage in adults is 60-90 mg/m^2 body area. The dose should be repeated at 21 day intervals, depending upon the patient's haematological status and bone marrow function. Above a cumulative dose of 900-1000 mg/m^2, the risk of irreversible congestive cardiac failure increases greatly. Results above demonstrated the concentration-time curves of EPI in whole blood and tissues in12 hours post-dose. So our further experiments are being planned to determine the correlation existences between the blood EPI level and the tissues EPI level after long-term administration. Moreover, experiments will be performed to estimate the correlations between tissue EPI content and cumulative adverse reactions, such as cardiotoxicity and myelosuppression.

4. CONCLUSIONS

The MWNT-SPE showed a stable EPI signal by square-wave voltammetry. The test required minimal expertise, no sample pre-treatment, and provided the first result within 15 minutes. This test showed good correlation with HPLC and is well-suited for routine clinical use. In addition, excellent correlations were observed between the whole blood and tissues. Based on these correlations, the concentration in whole blood would be useful for the estimation of EPI in tissues, in order to reduce the risk of adverse reactions by adjusting the dose.

The use of MWNT-SPE bears the advantage of disposability, so after one use they are discarded avoiding the problems related with the memory of the previous measurements. And it could be stored at ambient temperature for more than three months. Furthermore, it is convenient to mass-production at lower cost and to assemble into portable chip based sensing devices suitable to unskilled users.

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