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Journal of Pharmaceutical and Biomedical Analysis
32 (2003) 505–512
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Study of the interaction of 6-mercaptopurine with protein by microdialysis coupled with LC and electrochemical detection based on functionalized multi-wall carbon nanotubes modified electrode

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Received 9 September 2002; received in revised form 4 February 2003; accepted 25 February 2003

Abstract

Microdialysis sampling coupled with liquid chromatography and electrochemical detection (LC–ECD) was developed and applied to study the interaction of 6-Mercaptopurine (6-MP) with bovine serum albumin (BSA). In the LC–ECD, the multi-wall carbon nanotubes fuctionalized with carboxylic groups modified electrode (MWNT-COOH CME) was used as the working electrode for the determination of 6-MP. The results indicated that this chemically modified electrode (CME) exhibited efficiently electrocatalytic oxidation for 6-MP with relatively high sensitivity, stability and long-life. The peak currents of 6-MP were linear to its concentrations ranging from 4.0×10^{-7} to 1.0×10^{-4} mol l^{-1} with the calculated detection limit (S/N = 3) of 2.0×10^{-7} mol l^{-1}. The method had been successfully applied to assess the association constant (K) and the number of the binding sites (n) on a BSA molecular, which calculated by Scatchard equation, were 3.97×10^{3} mol^{-1} l and 1.51, respectively. This method provided a fast, sensible and simple technique for the study of drug–protein interactions.

Keywords: Microdialysis; Drug–protein interaction; Multi-wall carbon nanotubes; Carboxylic groups; Chemically modified electrode

1. Introduction

6-Mercaptopurine (6-MP) was widely used as a clinical agent in therapy of human leukemia and as an immunosuppressive drug. However, the drug was harmful to the kidney and liver of human beings [1]. When the drug entered into plasma of body, it was bound more or less to plasma proteins such as albumin and α_1-acid glucoprotein in the blood [2]. Only the unbound drugs could diffuse from the blood, reached the action site and exhibited pharmacological activity and/or side

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0731-7085/03/$ - see front matter
doi:10.1016/S0731-7085(03)00158-4

effects. In this respect, plasma protein binding was an important factor in establishing pharmacokinetic and pharmacodynamic properties of a drug, as only the free fraction of the drug was pharmacologically active. Unfortunately, studies about the interaction of 6-MP and proteins were very few.

Nowadays, there were various methods for the drug–protein phenomena. Equilibrium dialysis and ultrafiltration [3,4] had been commonly used to determine unbound drug concentrations in both in vivo and in vitro samples. However, these methods had potential problems. For example, the equilibrium between bound and unbound drug might shift during the experiment due to a volume shift. It seemed that the advent of high-performance frontal analysis (HPFA) overcomed these problems [5], but HPFA was not suitable for the analysis of weak protein binding. Although using a micro HPFA injection column or the CE/FA mode could solve the problem, more expensive instruments were needed [6].

In recent years, microdialysis was an excellent in vivo sampling technique. It had been extensively used for monitoring the unbound drug in vivo. Its pharmacokinetic applications had been well covered in recent reviews [7,8]. Microdialysis sampling with a semi-permselective probe membrane could prevent the large molecules from diffusing into the perfusate, and selectively monitor the concentration of macromolecule-free substance. Hence, the microdialysis had the characteristic of in situ sampling cleaned-up and monitoring. Compared with other methods reported for the study of the drug–protein interaction, It had many advantages such as a simple operation, time saving, less required quantity of drug, not shifting the equilibrium between bound and unbound drug, and easy connection to analytical instruments. Therefore, its utility also showed a powerful potential in the studies of the drug–protein interaction [9–11]. Before the wide use of microdialysis, there was a question need to be solved. Since the microdialysis sample available was small and the concentration of interesting drug in sample was very low, an analytical technique coupled with the microdialysis must had an effective separation and sensitive detection ability.

Liquid chromatography and electrochemical detection (LC–ECD) coupled with the microdialysis sampling [12–14] had been much of attentions for in vitro and in vivo determination because of its small detection volume, high sensitivity, effective separation, and small injection volume. However, electrochemical detection of 6-MP was often hampered by the slow electron-transfer kinetics at the electrode surface, so that the sensitivity for determining 6-MP was very low. The CMEs could solve the problem. At present, the Nafion/indium hexacyanoferrate CME had already been reported for determining the 6-MP in the urine sample with LC–ECD [15]. But the limit of detection was only 1×10^{-6} mol l^{-1}.

Since carbon nanotubes was discovered in 1991 [16], they had attracted much attention because of remarkable nanostructures combining high surface area, high electrical conductivity, good chemical stability and significant mechanical strength. The subtle electronic properties suggested that carbon nanotubes had the ability to promote electron-transfer reactions when used as an electrode in chemical reactions. In fact, carbon nanotubes had been used to fabricate carbon nanotube electrodes. Their performance had been found to be superior to other carbon electrodes in terms of reaction rates and reversibility [17,18]. Recently, the carbon nanotubes fuctionalized with carboxylic groups modified electrode (MWNT-COOH CME) was developed. It was first found that it showed excellent catalytic activity for the oxidation of 6-MP. Therefore, higher sensitivity and lower limit of detection could also be achieved.

There were also various methodologies based on high performance liquid chromatography for detection 6-MP in plasma [19–21]. However, they had involved more complicated extraction procedures. And most important was that those methods could not be used to detect the unbound 6-MP in plasma. The HPLC method presented in this paper was simple method. Coupled with microdialysis, it allowed the direct quantitation of the unbound 6-MP, and also gave the opportunity to investigate the interaction of 6-MP–BSA in vitro.

2. Experimental

2.1. Reagents

Muti-wall carbon nanotubes (MWNTs) with the diameter of 10–30 nm and the length of 1–10 μm were obtained from Sun nanotech Co. Ltd., China. The TEM image of MWNTs was shown in Fig. 1. 6-MP was purchased from Fluka Chemie AG (Switzerland). Bovine serum albumin (BSA) and disodium ethylene diamine tetraacetate (Na_2EDTA) were obtained from Shanghai Chemical Reagents (China). All of reagents were at least analytical-reagent grade. Double-distilled deionized water was used for all solutions.

2.2. Apparatus

Electrochemical experiments were performed on a CHI-830 Electrochemical Analyzer (CH Instruments, USA) with a three-electrode system. A glassy carbon (GC) disc electrode (diameter 2 mm, BAS Co., Japan) or MWNT-COOH CME was served as working electrode. A Saturated Calomel electrode (Model 232C, Jiangsu Electroanalytical Instruments Factory, China) was used as reference electrode and a platinum electrode (Model 213, Jiangsu Eletroanalytical Instruments Factory, Jiangsu, China) as counter electrode.

Liquid chromatographic experiments were conducted on a HP1090 liquid chromatography (Hewlett–Packard Company, USA). A homemade thin-layer radial flow cell with a CHI-830 Electrochemical Analyzer was used as the detector. The working electrode was the GC disc electrode (diameter 5 mm, BAS Co., Japan) or MWNT-COOH CME. The stainless steel was served as counter electrode and the potentials were referenced to an Ag/AgCl (saturated KCl) electrode. The HPLC system was equipped with a HP ODS Hypersil column (4.6 mm i.d. × 200 mm, 5 μm particle, HP, USA). The injection volume was 25 μl.

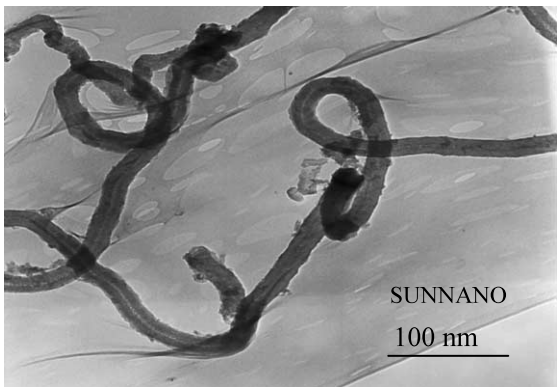

Fig. 1. TEM image of MWNTs.

Microdialysis was carried out on a CMA/101 microdialysis pump (CMA Microdialysis AB, Stockholm, Sweden) and a PES 12 microdialysis probe with a membrane diameter of 0.5 mm and a length of 2.0 mm (BAS Co, Japan). The probe was perfused with Ringer's solution at a rate of 1.0 μl min^{-1}, which consisted of 140 mmol l^{-1} NaCl, 1.0 mmol l^{-1} $MgCl_2$, 1.2 mmol l^{-1} $CaCl_2$ and 5.0 mmol l^{-1} $NaHCO_3$, pH 7.4.

Fourier transform (FT) IR spectra were recorded on a NEXUS 670FT IR spectrometer (Nicolet Co., USA).

2.3. Preparation of the MWNT-COOH modified electrode

MWNTs functionalized with carboxylic acid groups were prepared by refluxing with HNO_3 for 4–5 h. Then the MWNTs functionalized with carboxylic acid groups were washed with double-distilled deionized water until the pH was nearly 7.0. On the FTIR spectra of the MWNT-COOHs, the appearance of the peaks at 1716 and 1575 cm^{-1} corresponded to $\nu_{(C=O,\ -COOH)}$ and $\nu_{(C=O,\ -COO^-)}$, respectively, which is in accordance with the literature [22,23], indicated that –COOH and $-COO^-$ were present on the surface of the MWNTs. The MWNT-COOH suspension was prepared by dispersing one milligram MWNT-COOH in 10 ml of *N*,*N*-dimethylformamide (DMF) with the aid of ultrasonic agitation.

Prior to preparation of the MWNT-COOH CME, a GC electrode was polished with 300 nm alumina, and sonicated sequentially in acetone, NaOH (1 mol l^{-1}), HNO_3 (1:1, v/v) and doubly distilled water. The MWNT-COOH CME was prepared by dropping the above suspensions of MWNT-COOHs (2 μl) on the GC electrode sur-

face and then evaporating the solvent under an infrared lamp.

2.4. Procedure for studying the interction of 6-MP with BSA

The interaction of 6-MP–BSA was determined with the microdialysis sampling coupled with LC–ECD based on MWNT-COOH CME. The standard stock solution of 6-MP (1.0×10^{-3} mol l^{-1}) and BSA (1.0×10^{-4}mol l^{-1}) were prepared in Ringer's solution and mixed at different molar ratios (6-MP:BSA μmol l^{-1} = 20:50, 30:50, 40:50, 50:50, 60:50, 70:50). The unbound 6-MP was sampled by means of a microdialysis and determined with LC–ECD. During the whole process of microdialysis sampling, the mixed 6-MP–BSA solutions were thermostated at 37 °C. The binding parameters were estimated according to Scatchard equation [24], as shown below:

$$\frac{\gamma}{C_f} = nK - K\gamma$$

where γ was the ratio of the bound drug to the protein in molar concentration, C_f was the concentration of the unbound drug, K was the association constant, and n was the number of binding sites on a protein molecule.

3. Results and discussion

3.1. Characterization of MWNT-COOH CME and its electrocatalytic activity for oxidation of 6-MP

Curve b of Fig. 2B showed the cyclic voltammogram of the MWNT-COOH CME in 0.1 mol l^{-1} phosphate buffer solution (pH 7.0). In the potential range from −0.4 to 0.9 V versus SCE, a pair of stable redox waves appeared with the cathodic peak potential of −0.08 V versus SCE and the anodic peak potential of −0.04 V versus SCE, which was related to the redox of the carboxylic groups [23].

Fig. 2 also showed the cyclic voltammograms of 6-MP on bare GC electrode and MWNT-COOH CME. With the addition of 6-MP in the phosphate

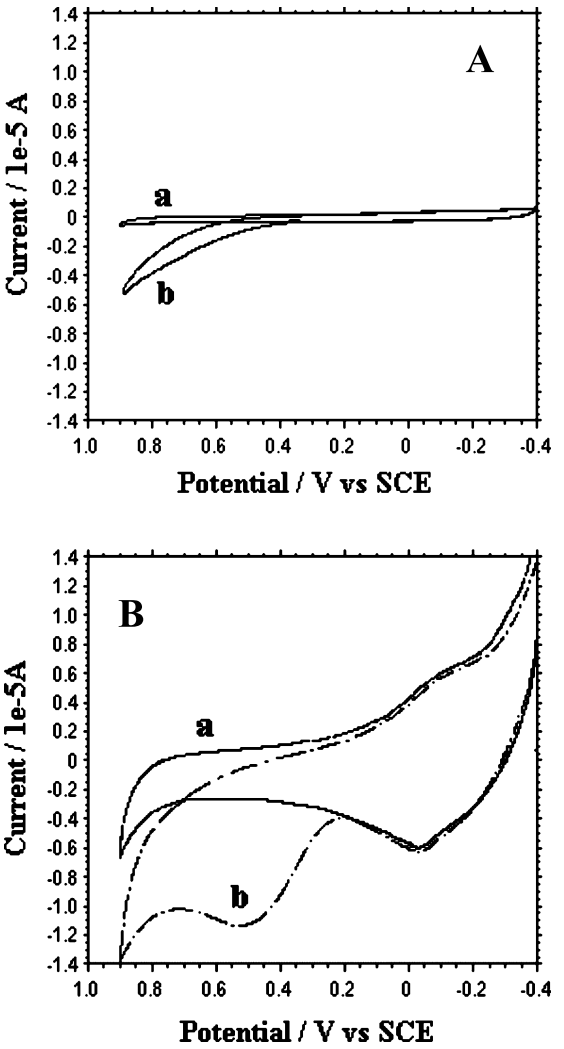

Fig. 2. Cyclic voltammograms of 6-MP on GC electrode (A) and MWNT-COOH CME (B); (a) in 0.1 mol l^{-1} PBS solution (pH 7.0); (b) in 0.1 mol l^{-1} PBS solution (pH 7.0) containing 1×10^{-3} mol l^{-1} 6-MP. Scan rate: 0.1 V s^{-1}, Initial potential: −0.4 V vs. SCE.

buffer solution, the irreversible oxidation currents were observed at both electrodes. However, the CV response of 6-MP on MWNT-COOH CME exhibited a large well-defined peak at +0.53 V versus SCE while the response on bare GC electrode exhibited a small oxidation current. It meant that the MWNT-COOH CME had an efficient catalytic activity for the oxidation of 6-MP. This could be explained by the facts: like

single-wall carbon nanotube CME [25], MWNT-COOHs could act as promoter to enhance the electrochemical reaction, increasing the rate of the heterogeneous electron transfer. Also they could increase the effective area of the electrode, so the oxidation currents increased significantly. In addition, the behaviors of the 6-MP on the electrode modified with MWNTs, which was not functionalized with carboxylic acid group, were also investigated. It was found that MWNTs CME did not show catalytic activity for it. So the carboxylic acid groups of the MWNT-COOH might also be involved in the process of catalytic oxidation for 6-MP.

3.2. Liquid chromatography condition for determining 6-MP

The pH value of the mobile phase was one of the important factors for the determination of 6-MP in the LC–ECD. With the pH value of the mobile phase increasing, the current responses of 6-MP on the MWNT-COOH CME increased. When the pH reached 7.0, the CME gave relatively better response for the determining 6-MP. So pH 7.0 could be selected as the optimum pH for the mobile phase.

Hydrodynamic voltammetry (HDV) was employed to select the optimum applied potential for the determination of 6-MP in LC–ECD. In this study, a standard solution of 1.0×10^{-4} mol l^{-1} 6-MP was repetitively injected to the LC–ECD and each peak current was recorded, at the same time the applied potential was increased from +0.3 to +1.2 V versus Ag/AgCl by 0.1 V increments. Fig. 3 showed the HDVs of 6-MP on MWNT-COOH CME and the bare GC electrode. On MWNT-COOH CME (Fig. 3A), when the applied potential was lower than +0.4 V versus Ag/AgCl, there was essentially no evidence for the oxidation for 6-MP. When the potential was higher than +0.4 V versus Ag/AgCl, the peak currents for the oxidation of 6-MP increased quickly. In the potential of +0.8 V versus Ag/AgCl, the current response of 6-MP reached the most. With the increase of potential higher than +0.8 V versus Ag/AgCl, the current response would decrease quickly and noise level also became very high. So +0.8 V versus Ag/AgCl was chosen as the optimum applied potential for the determination of 6-MP in LC–ECD, where the signal-to-noise (S/N) ratio was the highest. On the GC electrode the response of 6-MP was quite different. The current response did not reached the most until the potential was applied at +0.9 V versus Ag/AgCl (Curve B in the Fig. 3). In addition, the response at the same potential was less sensitive than that on MWNT-COOH CME. All of these also proved the MWNT-CME exhibited an excellent catalytic activity to 6-MP.

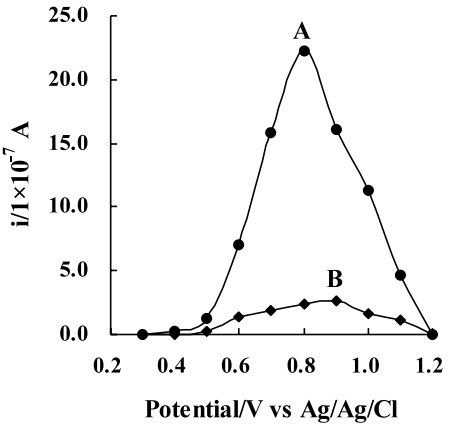

Fig. 3. Hydrodynamic voltammograms of 1×10^{-4} mol l^{-1} 6-MP (A) on the MWNT-COOH CME; (B) on the bare GC electrode. Mobile phase: methanol–PBS solution (0.1 mol l^{-1} and containing 1×10^{-4} mol l^{-1} Na_2EDTA, pH 7.0,) (5:95, v/v); flow rate: 1 ml min^{-1}.

3.3. Linearity, detection limits, reproducibility and recovery

To determine the linear calibration curve, correlation coefficient and detection limit of 6-MP on the MWNT-COOH CME in LC–ECD, a series of 6-MP solutions were tested with the concentrations ranging from 5.0×10^{-8} to 2.0×10^{-3} mol l^{-1}. The results showed that the peak currents of 6-MP were linear to its concentrations ranging from 4.0×10^{-7} to 1.0×10^{-4} mol l^{-1} ($i_P = 0.0222\ C_{6\text{-MP}} + 5.0 \times 10^{-10}$, nine data points) with the detection limit of 2.0×10^{-7} mol l^{-1} and the correlation coefficient of 0.9991. The detection limit was calculated according to S/

N = 3, and the average of the noise values were determined from the blank injection (n = 11). The detection limits of 6-MP on the GC electrode was also investigated. It was found that the detection limit of 6-MP on GC electrode were about 1.3×10^{-6} mol l^{-1} (S/N = 3). It meant that the MWNT-COOH CME gave better detection limit.

The repeatability was estimated by repetitive injection (n = 8) of 1.0×10^{-6} mol l^{-1} 6-MP under the same conditions. The relative standard deviation (R.S.D.) of the peak currents was found to be 2.0%. In addition, the sensitivity of the MWNT-COOH CME showed no observable change after 1 month of storage in PBS solution at 4 °C or the successive potential applied at +0.8 V versus Ag/AgCl, indicating that the CME was very stable and long self-life. Hence, the currents on the MWNT-COOH CME were stable and reproducibility.

The recoveries of 6-MP were determined by the standard addition; and the recoveries were in the range of 98–102% (n = 3).

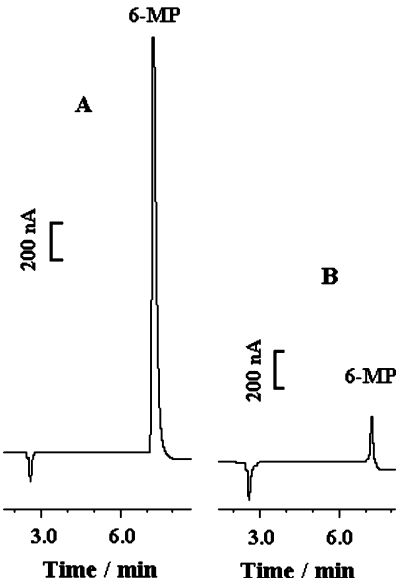

Fig. 4. Chromatograms of 1×10^{-4} mol l^{-1} 6-MP on (A) MWNT-COOH CME and (B) the bare GC electrode. The working potential was set at +0.8 V vs. Ag/AgCl; other conditions were as in Fig. 3.

3.4. Comparison of response of 6-MP on GC electrode and the MWNT-COOH CME in the LC–ECD

The typical response of 6-MP on the GC electrode and the MWNT-COOH CME was shown in Fig. 4. Compared with GC electrode, the current response of 6-MP on the MWNT-COOH CME was improved greatly. This also proved that the MWNT-COOH CME showed catalytic activity to the oxidation of 6-MP. Therefore, the MWNT-COOH CME could be used for determining 6-MP with relatively high sensitivity in LC–ECD.

3.5. Microdialysis sampling experiment

The drug–protein interaction was studied by a microdialysis sampling system and an analytical system. The principle of microdialysis was based on the passive diffusion of analytes across the semi-permeable membrane of a microdialysis probe. In the mixed drug–protein solution, the unbound 6-MP could diffuse into the probe by the concentration gradient without the alternation of the analyzed system. Meanwhile, the 6-MP, which were bound to BSA, would be cut-off by the membrane. Then the microdialysate with unbound 6-MP was constantly renewed and sampled for further analysis.

Before the study of drug–protein interaction, the relative recovery of microdialysis for 6-MP was studied and determined. It was well known that the relative recovery of microdialysis probe for the analyte was equal to the ratio of its concentration in the microdialysate, i.e. the outlet from the probe (C_{out}), to its concentration in the medium surrounding the probe (C_{in}). Therefore, the recovery was C_{out}/C_{in}. The relative recovery of a microdialysis probe was affected by the microdialysis rate. The lower microdialysis rates were, the higher relative recoveries were. In this paper, the relative recoveries of 6-MP at the rate of 5.0, 4.0, 3.0, 2.0, 1.0 μl min^{-1} were investigated, according to 1.0×10^{-5} mol l^{-1} 6-MP. It was found that the relative recovery of 6-MP was too low to be detected by the MWNT-COOH CME in LC–ECD when the rate was over 5.0 μl min^{-1}. So in order to detect 6-

Table 1
The unbound 6-MP concentrations and the binding fractions with BSA solution[a]

Ratio 6-MP–BSA (μmol l^{-1}:μmol l^{-1})	Unbound concentration (μmol l^{-1})	bound concentration (μmol l^{-1})	Bound fraction (%)	R.S.D. (%)
20:50	15.6	4.4	22.0	2.3
30:50	23.5	6.5	21.7	2.0
40:50	31.6	8.4	21.0	1.9
50:50	39.7	10.3	20.6	1.6
60:50	47.9	12.1	20.2	1.7
70:50	56.2	13.8	19.7	1.6

[a] LC–ECD conditions as in Fig. 4. The values shown were calculated from the calibration curves and were mean of n = 5 in each case.

MP rapidly and accurately, the optimum microdialysis rate of 1.0 μl min^{-1} was selected. The microdialysis relative recovery was determined to be the average (n = 5) of 34.5% for 6-MP.

3.6. Interaction between 6-MP and BSA

The interaction between 6-MP and BSA was studied in the mixed solutions with different concentration ratio of 6-MP to BSA. The unbound concentration and the binding fractions of 6-MP in BSA solution could be determined by the microdialysis sampling and the LC–ECD, which were listed on Table 1. The parameters of the 6-MP–BSA interaction such as binding constant (K), the number of the binding sites on a BSA molecule (n) were calculated according to the Scatchard analysis. The results showed that the Scatchard plot was linear with good correlation coefficient of 0.997, the K and n were 3.97×10^3 mol^{-1} l and 1.51, respectively (95% confidence level) (Indicated in Fig. 5). Besides, compared with high affinity drugs (nK was about 10^6 mol^{-1} l), the nK value for 6-MP was very small, indicating that the studied drug was a lightly binding drug.

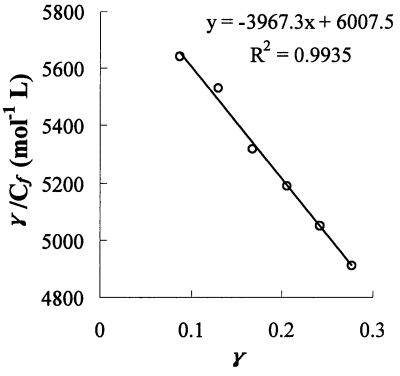

Fig. 5. Scatchard plot of 6-MP–BSA interactions obtained using microdialysis sampling coupled with LC–ECD.

4. Conclusions

In this paper, an MWNT-COOH CME was developed. It was first found that the CME showed excellent catalytic activity for the oxidation of 6-MP with relatively high sensitivity, stability and long-life. Thus, the sensitivity for determination of 6-MP was improved greatly; and lower detection limit was obtained. When it combined with LC–ECD and microdialysis, it was successfully applied to study the interaction of 6-MP with BSA. In addition, from the results, it was also indicated that microdialysis was a powerful potential in the studies of the drug–protein interaction. Compared with other methods reported for the study of the drug–protein interaction, microdialysis sampling had many advantages such as a simple operation, time saving, less required quantity of drug, not shifting the equilibrium between bound and unbound drug, and capability of easy connection to analytical instruments.

Acknowledgements

This work was supported by the Natural Science Foundation of China (No. 20175006) and BAS Company of Japan.

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