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Simultaneous determination of 5-hydroxyindoleacetic acid and 5-hydroxytryptamine in urine samples from patients with acute appendicitis by liquid chromatography using poly(bromophenol blue) film modified electrode

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Abstract

The fabrication and application of a novel electrochemical detection (ED) system with a poly(bromophenol blue) (PBPB) film chemically modified electrode (CME) for high performance liquid chromatography (HPLC) were described. The electrochemical behaviors of 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) at this CME were investigated by cyclic voltammetry (CV) and differential pulse voltammetry (DPV). It was found that the PBPB CME efficiently exhibited electrocatalytic effect on the current responses of 5-HT and 5-HIAA with relatively high sensitivity, stability and long life of activity. In HPLC–ED, the two analytes had good and stable current responses at the CME and their linear ranges were over four orders of magnitude ($R \geq 0.9992$) with the detection limits being 0.25 nmol L^{-1} for 5-HT and 0.50 nmol L^{-1} for 5-HIAA. The application of this method for the determination of 5-HT and 5-HIAA in urine samples from patients with acute appendicitis (AA) was satisfactory.

Keywords: Poly(bromophenol blue) film; Acute appendicitis; 5-Hydroxytryptamine; 5-Hydroxyindoleacetic acid; HPLC–ED

1. Introduction

Appendicitis is the most common diagnosis for patients with a surgical condition of the abdomen [1]. Epidemiologic studies have shown that the lifetime prevalence of appendicitis is 7%, with the highest incidence occurring between 10 and 30 years of age [2,3]. A prompt and accurate diagnosis of acute appendicitis (AA) is crucial. False negative diagnosis may result in appendiceal rupture that is associated with a high rate of morbidity and mortality and, again, increased medical costs [4–6]. Classically, appendicitis has been primarily a clinical diagnosis. Radiographic studies [7–9], including plain abdominal roentgenograms, focused ultrasonography, and barium enema have been utilized in the evaluation of patients with suspected appendicitis. Continuous improvements in technology, technique and interpretation achieved over the past 10 years have substantially increased the accuracy of diagnosis. However, the rates of appendiceal perforation and negative appendectomy have remained unchanged. False negative appendectomy rate was noted to be up to 9–19% in recent years [10,11].

It has been shown that in the ongoing inflammatory process of the appendix, blood serotonin level increases. Upon release, 5-hydroxytryptamine (5-HT) is rapidly metabolized in the liver by the monoamine oxidase (MAO) system to 5-hydroxyindoleacetic acid (5-HIAA) and, thereafter, is secreted in the urine [12,13]. Measurement of the urine 5-HT and 5-HIAA may be a reliable marker of inflammation of the appendix. But up to now, less is known about the diagnostic values of 5-HT and 5-HIAA concentration in appendicitis. A method that can fastly separate and sensitively determine 5-HT and 5-HIAA is needed.

A variety of techniques have been utilized for the determination of 5-HT and 5-HIAA including spectrophotometer [14], fluorescence [15], chemical luminescence [16], polarography [17], voltammetry [18], and capillary electrophoresis [19]. Some of these methods have been applied in biological matrices and pharmaceuticals. However, the applications of fluorometric or other photometric methods for determination of monoamine neurotransmitters are limited because these analytes are absent

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from spectrum group and they are present with very low concentrations in biological matrix. However, the high performance liquid chromatography (HPLC) with electrochemical detection (ED) has gained great acceptance for its sensitivity, simple operation, low cost and no sample derivation [20–23]. Especially, as an important aspect of ED, it is convenient in enhancing sensitivity and selectivity by using chemically modified electrode (CME).

Recently, the use of conducting films as electrode modification agents for ED of some important organic and biological compounds has been demonstrated. Among the main conducting films, organic dye film has been attracting great scientific interest, because it can be easily synthesized from aqueous solution and is stable in the presence of air and water [24–26]. For the last decade, these compounds with low molecular weight have been extensively investigated due to potential optical, electrooptical, and sensor applications [27–29]. Many reports have demonstrated that the quinonimine dye such as neutral red, methylene blue, methylene green, and brilliant cresyl blue can be widely used for preparation of CME [30,31]. But based on our knowledge, few reports have appeared in the literature on the application of triphenylmethane dye, which was effective electron redox mediator due to their conjugated systems.

In this work, poly(bromophenol blue) (PBPB) film was synthesized electrochemically on glassy carbon (GC) electrode. The modifying process was simple and fast. It was found that the PBPB CME could effectively catalyze the oxidation of 5-HT and 5-HIAA. When the CME was used as an amperometric detector for HPLC, it showed a good linear relationship between the current responses and the concentrations of 5-HT and 5-HIAA with low detection limit. The method for simultaneous determination of 5-HT and 5-HIAA in urine samples was satisfactory. The difference of 5-HT and 5-HIAA contents in urine samples between controls and AA patients was also investigated in this experiment.

2. Experimental

2.1. Reagents

Bromophenol blue (BPB) was obtained from Shanghai Aisi Chemical Reagents (China). 5-Hydroxytryptamine and 5-hydroxyindoleacetic acid were of analytical grade and purchased from Sigma Chemicals (St. Louis, MO, USA). All reagents were of at least analytical-reagent grade and double-distilled deionized water was used for all solutions. Prior to use, all solutions were degassed with purified nitrogen for 20 min.

2.2. Apparatus

Electrochemical experiments were performed with a CHI-832 electrochemical system (CHI Co., USA). The three-electrode system consisted of a PBPB CME or a GC electrode as working electrode, a saturated calomel electrode (SCE) as reference electrode and a gold wire electrode as counter electrode.

HPLC experiments were conducted on a model 510 pump and a U6K injector (Waters Assoc., USA). The injection volume was 20 μL. The column was Luna 5 μm C_{18} (25 cm × 4.6 mm) (Phenomenex, USA) directly attached to a C_{18} precolumn (15 mm × 1.0 mm). The detector consisted of a laboratory-made thin-layer cell and a CHI-832 Electrochemical Analyzer. A PBPB CME or GC electrode was used as the working electrode. The mobile phase was 0.2 mol L^{-1} phosphate buffer solution (pH 5.0) containing 15% methanol, which was delivered at a constant flow rate of 1.0 mL min^{-1}. All the experiments were performed at room temperature and the pH value was calibrated with a pH meter (Horiba, Japan).

2.3. Preparation of PBPB modified electrode

Prior to preparation of the PBPB CME, the GC electrode surface was polished with 0.3 μm alumina on a polishing microcloth and rinsed with deionized water. Subsequently, it was ultrasonicated thoroughly with acetone, NaOH solution (50%, w/w), HNO_3 (1:1, v/v) and doubly distilled water.

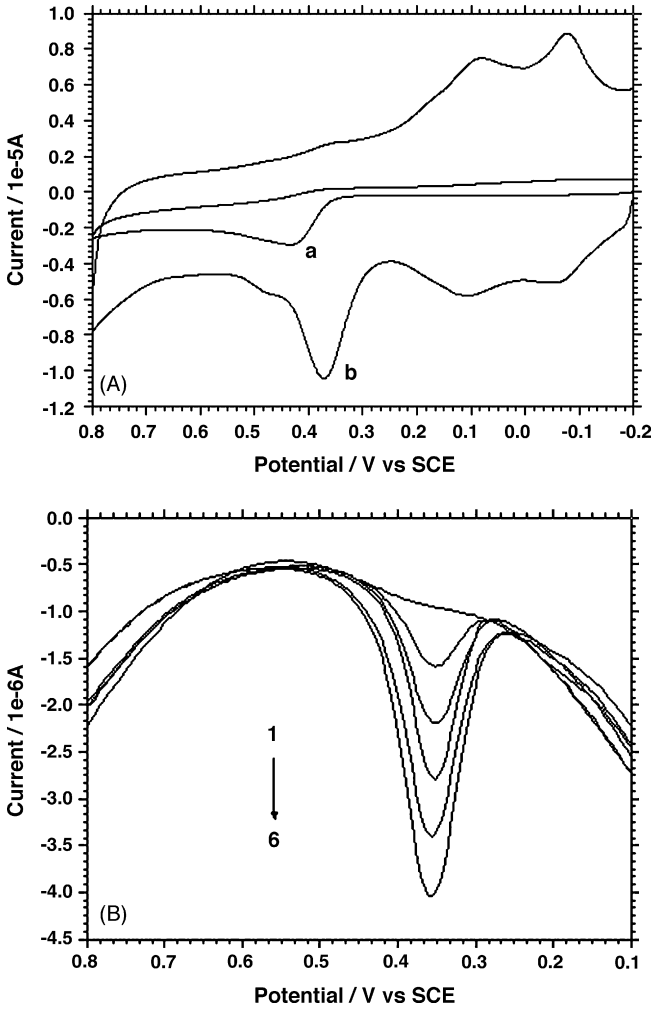


Fig. 1. (A) Cyclic voltammograms of 5-HT at GC electrode (a) and PBPB CME (b), containing 10.0 μmol L^{-1} 5-HT. (B) Differential pulse voltammograms of 5-HT at the PBPB CME: (1) 0 mol L^{-1} 5-HT; (2) 2.0 μmol L^{-1} 5-HT; (3) 4.0 μmol L^{-1} 5-HT; (4) 6.0 μmol L^{-1} 5-HT; (5) 8.0 μmol L^{-1} 5-HT; (6) 10.0 μmol L^{-1} 5-HT. Electrolyte: 0.2 mol L^{-1} phosphate buffer solution (pH 5.0).

After pretreating, the GC electrode was cycled in 0.5 mol L^{-1} H_2SO_4 with the potential range from −0.5 to +1.4 V at 0.1 V s^{-1} until the reproducible background was obtained. Then, the PBPB film was deposited in 0.1 mol L^{-1} phosphate buffer solution (pH 5.6) containing 0.1 mmol L^{-1} BPB with the cycling potential from −1.0 to +1.8 V at 0.1 V s^{-1} for 40 segments.

2.4. Collection and treatment of urine samples

Fifteen patients with the diagnosis of acute appendicitis and 15 control subjects without clinical diagnosis of appendicitis and absence of history of ingestion of the special foods that can raise urinary concentration of 5-HIAA in the local hospital participated in the study. The patients were treated with surgery. Each person gave written informed consent. Urine samples were obtained from the patients and the controls during the admission period. All the urine samples were acidified by 12 mol L^{-1} HCl and kept at −20 °C until analysis.

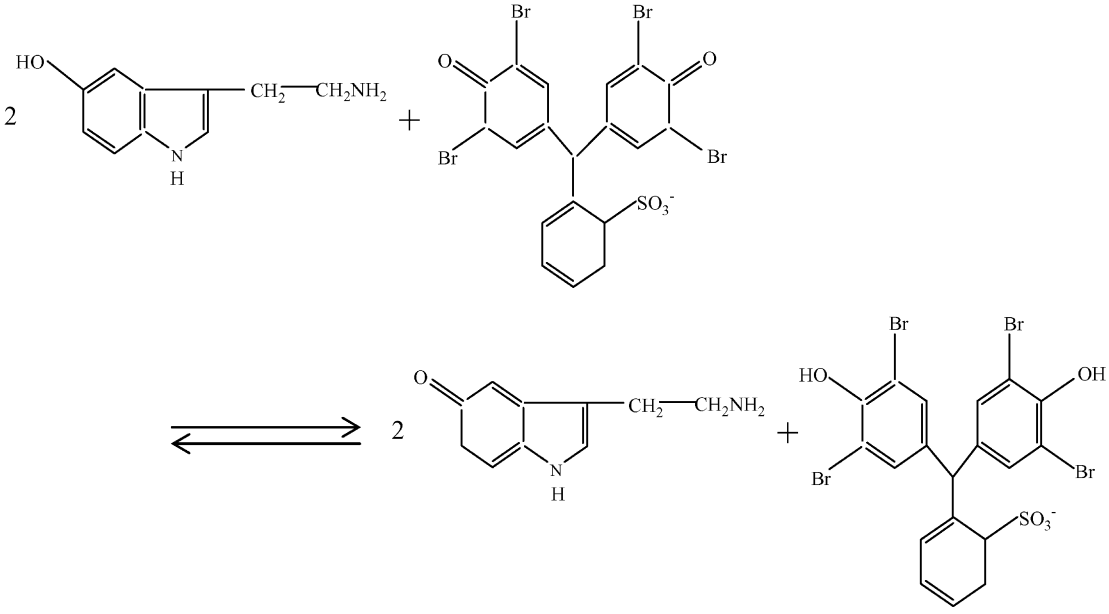
Before injected into HPLC system, the urine samples were centrifuged and filtered through a Millipore filter (0.22 μm pore size) and were diluted 10 fold with 0.2 mol L^{-1} phosphate buffer solution (pH 5.0).

3. Results and discussion

3.1. Electrocatalytic oxidation of 5-HT and 5-HIAA at PBPB CME

Fig. 1 shows the cyclic voltammograms and differential pulse voltammograms of 5-HT at the bare GC electrode and the PBPB CME in 0.2 mol L^{-1} phosphate buffer solution (pH 5.0). With the addition of 5-HT in the buffer solution, an oxidation peak of 5-HT was observed at both the electrodes in Fig. 1A. However, compared to the GC electrode, the peak current of 5-HT at the PBPB CME increased greatly and the peak positon of 5-HT moved to the negative direction. It means that the PBPB CME had a good catalytic effect on the oxidation of 5-HT. This is due to the fact that the structure of BPB is triphenylmethane with many conjugated systems, and therefore the PBPB film could act as promoter to increase the electron transfer rate. When PBPB film was synthesized electrochemically on GC electrode, it participated in the redox reaction of 5-HT. The reactions of electron transfer between 5-HT and PBPB film can efficiently increase the activity of 5-HT oxidation.

The catalytic mechanism can be presented as:



The differential pulse voltammetry (DPV) responses of 5-HT with different concentrations at the PBPB CME are shown in Fig. 1B, which indicated that the oxidation currents had a good linear correlation with 5-HT concentrations. A similar phenomenon was obtained at the PBPB CME when 5-HIAA was added to the buffer solution. All these results indicated that the PBPB CME had an effective catalysis function and could be used as the electrochemical detector for 5-HT and 5-HIAA in HPLC.

3.2. Hydrodynamic voltammetry (HDV)

HDV is a suitable method to select the appropriate potential applied on HPLC–ED. In this study, standard solutions of the mixed sample containing 1.0 μmol L^{-1} 5-HT and 5-HIAA were repetitively injected at the PBPB CME while the HPLC–ED operating potential increased from 0.1 to 0.6 V in 0.1 V increments. When the applied potential was larger than 0.1 V, the current response of 5-HIAA increased significantly and reached the higher value at 0.3 V. However, the current response of 5-HT reached the great value at 0.4 V. When the potential increased larger than 0.4 V, the current response of 5-HT had a little increase. But the baseline current also became high and other substances maybe responded at the electrode. In order to obtain

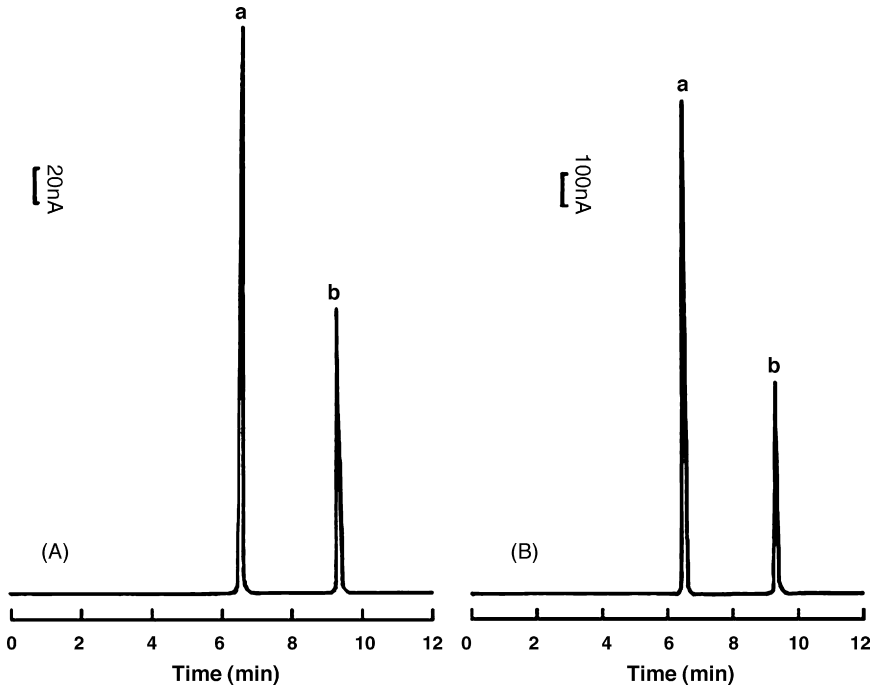


Fig. 2. Chromatograms of mixed standard solution of two analytes at the GC electrode (A), and at the PBPB CME (B). The mixed standard solution: 1.0 μmol L^{-1} 5-HT (a) and 5-HIAA (b). The working potential is set at +0.4 V vs. Ag/AgCl; column was Luna 5 μm C_{18} (25 cm × 4.6 mm) and directly attached to a C_{18} precolumn (15 mm × 1.0 mm); injection volume: 20 μL; mobile phase: 0.2 mol L^{-1} phosphate (pH 5.0) containing 15% methanol; and flow rate: 1.0 mL min^{-1}.

the best selectivity and signal/noise ratio, 0.4 V was chosen as the optimum detection potential for detecting the two analytes.

3.3. Effect of methanol content in mobile phase

The methanol content in mobile phase had great influences on the separation of 5-HT, 5-HIAA and many interfering compounds that coexisted in urine samples at high concentrations such as uric acid and ascorbic acid. With the increase of methanol content in mobile phase, the retention time of all the analytes was shortened. However, when the methanol content in mobile phase was higher than 16%, uric acid and 5-HT could not reach baseline separation. On the other hand, if the methanol content in mobile phase was lower than 14%, the separation process would be prolonged. In this experiment, 15% (v/v) was selected for the methanol content in mobile phase.

3.4. HPLC–ED of 5-HT and 5-HIAA

Fig. 2 shows the current responses of 5-HT and 5-HIAA at the GC electrode and at the PBPB CME in HPLC–ED, respectively. It can be found that both the current responses of the two analytes are much smaller at the GC electrode than at the PBPB CME. That is to say, the PBPB CME showed electrocatalytic oxidation of 5-HT and 5-HIAA. This is consistent with the results from cyclic voltammetry (CV) and DPV.

To determine the linearity of 5-HT and 5-HIAA at the PBPB CME in HPLC–ED, a series of mixed standard solutions of these analytes ranging from 0.10 to 100.0 μmol L^{-1} were tested. The ranges of the linear relationships observed between currents and concentrations were over four orders of magnitude, and the correlation coefficients were larger than 0.9992. The detection limits of these analytes at the CME were also investigated and the data were shown in Table 1.

The repeatability was estimated by making repetitive injection (eight times) of a standard solution containing 1.0 μmol L^{-1} mixture for the two analytes under the same conditions every 30 min. The relative standard deviations (R.S.D) of the peak currents were found to be 1.6% for 5-HT and 1.2% for 5-HIAA.

In addition, the long-term stability of the PBPB CME stored at 4 °C in PBS was examined by checking its relative activity periodically. No apparent change in the current responses of

Table 1
Analytical data of the two analytes by LC–ED at the PBPB CME[a]

Analytes	Regression equation[b]	Correlation coefficient (R^2)	Linear range (nmol L^{-1})	Detection limit (nmol L^{-1})[c]
5-HT	$Y = 1.434X + 0.010$	0.9995	0.50–10000	0.25
5-HIAA	$Y = 0.637X + 0.002$	0.9992	1.0–10000	0.50

[a] LC–ED conditions are as in Fig. 2.
[b] Where Y and X represent the peak current (μA) and the concentration of the analytes (μmol L^{-1}), respectively.
[c] The detection limits of the analytes were investigated using a signal-to-noise ratio of 3 (S/N = 3).

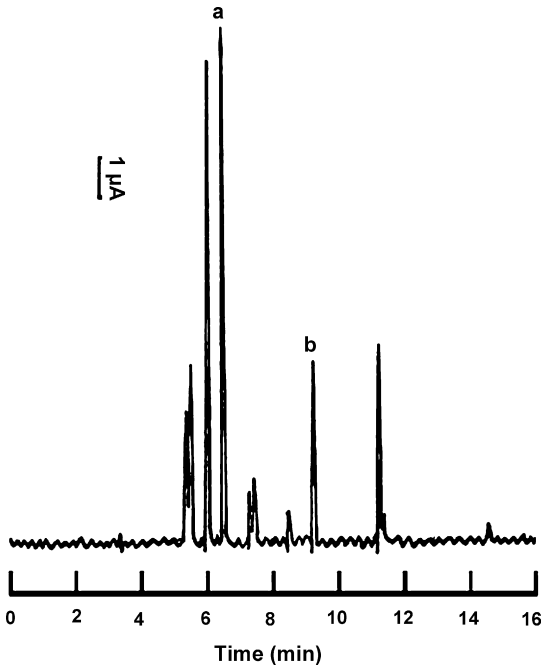


Fig. 3. Chromatograms of 5-HT (a) and 5-HIAA (b) in urine from one control patient by LC–ED. Other conditions are as in Fig. 2.

these analytes was observed over 3 weeks. The results indicated that the PBPB CME had good stability and reproducibility when it was used as the HPLC detector to determine 5-HT and 5-HIAA.

3.5. Determination of 5-HT and 5-HIAA in urine samples

To demonstrate the feasibility of the proposed PBPB CME as the ED for HPLC, the determination of 5-HT and 5-HIAA in urine samples was performed. Fig. 3 shows the chromatograms of 5-HT and 5-HIAA in urine sample from one control patient. The average concentrations of the analytes in urine samples from the patients and controls were given in Table 2.

The average of 5-HIAA in urine sample of control individuals was $6.73 \pm 1.15\ \mu mol\ L^{-1}$. The average of 5-HIAA in AA patients was $27.36 \pm 3.24\ \mu mol\ L^{-1}$, which was four times greater than normal individuals ($P < 0.01$). The best cutoff point for patients and controls of the 5-HIAA concentration was at $20\ \mu mol\ L^{-1}$ [13]. The mean value of 5-HIAA in AA patients showed significant difference from control groups. It has been reported that 5-HT present in the inflammatory sites excites sensory neurons, and pain may occur when 5-HT is released from platelets and mast cells during injury and inflammation of AA [32]. 5-HT is metabolized primarily by monoamine oxidase into 5-HIAA, then degrades; thus, the stimulation of MAO activity leads to the decrease of 5-HT, and the corresponding increase of 5-HIAA, contents. So the urinary level of 5-HIAA may be considered to be a new method for the diagnosis of AA. On the other hand, the levels of 5-HT in AA patients ($11.90 \pm 1.48\ \mu mol\ L^{-1}$) and in controls ($8.51 \pm 0.64\ \mu mol\ L^{-1}$) had no obvious difference ($P > 0.05$). One possibility is that abundant 5-HT release in plasma and platelet could reinforce the positive feedback of conglomeration and release reaction which was caused by endogenous platelet. Thus, the total amount of 5-HT in blood platelet did not decrease. These results demonstrated that the increase of 5-HIAA level in urine could be a warning sign of AA. Now further studies are proceeding in our laboratory.

Table 2
The contents of 5-HT and 5-HIAA in urine from controls and patients ($n = 15$)[a]

Analytes	Controls ($\mu mol\ L^{-1}$)	AA ($\mu mol\ L^{-1}$)
5-HT	8.51 ± 0.64	11.90 ± 1.48
5-HIAA	6.73 ± 1.15	27.36 ± 3.24

[a] The values shown are calculated from the calibration curves and are means of $n = 3$ in each case.

4. Conclusion

In this paper, the fabrication and application of the PBPB CME were studied. Both CV, DPV and liquid chromatographic experiments showed that the PBPB CME had excellent catalytic activity for the oxidation of 5-HT and 5-HIAA. In LC–ED, the sensitivity for determination of these analytes was improved greatly at the PBPB CME compared to those at the GC electrode. The method was successfully applied to the simultaneous determination of 5-HT and 5-HIAA in urine samples from patients with AA.

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