High Performance Liquid Chromatography with an Electrochemical Detector in the Cathodic Mode as a Tool for the Determination of *p*-Nitrophenol and Assay of Acid Phosphatase in Urine Samples

Yuji YAMAUCHI, Megumi IDO, Merime OHTA, and Hatsuo MAEDA*

Graduate School of Pharmaceutical Sciences, Osaka University; 1–6 Yamada-Oka, Suita, Osaka 565–0871, Japan.
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Utilizing a commercially available helium-purging device and PEEK tubes for all tubing, especially for connection between the mobile phase and pump, high performance liquid chromatography with an electrochemical detector (ECD/HPLC) at the cathodic mode is a simple and precise method for the determination of *p*-nitrophenol (NP). Studies with cyclic and hydrodynamic voltammetry indicated that 25% aqueous MeOH containing 0.1% (v/v) CF_3CO_2H and −0.8 V *vs.* Ag/AgCl are the best mobile phase and detection potential for cathodic ECD/HPLC. With the present system, the limits of detection and determination were 0.2 and 0.25 μM, respectively, and up to 50 μM, a linear calibration curve was afforded. Within-day precisions for the analysis of 5 and 50 μM NP were 0.8 and 0.7% (n=6), respectively, and between-day precisions (n=6) for these samples were 3.5 and 2.2%, respectively. Compared with the commonly used Bessey–Lowry–Brock method, cathodic ECD/HPLC was useful for the assay of acid phosphatase in urine samples with *p*-nitrophenyl phosphate disodium salt as a substrate.

Key words *p*-nitrophenol; cathodic detection; HPLC; electrochemical detector; phosphatase; *p*-nitrophenyl phosphate

Aromatic nitro compounds including nitrazepam, chloramphenicol, and *p*-nitrophenyl phosphate are often found in pharmaceuticals and as common reagents[1] for the assay of enzymes such as phosphatase and peptidase. The nitration of tyrosine residues in proteins has been recognized as an important process for regulating pathological conditions of various diseases induced by oxidative stress.[2] Thus, general and sensitive methods for the determination of aromatic nitro compounds in biological fluids are needed.

Spectrophotometric methods are currently used for the analysis of aromatic nitro compounds. As an alternative, cathodic detection can be considered, since aromatic nitro groups are known to undergo electrochemical reduction at a relatively positive potential.[3—5] Yet, few studies on the reductive detection of aromatic nitro compounds are to be found in the literature.[6—8] This is mainly because deoxygenation of the medium must be carried out for electrochemical detection in a cathodic mode.[3,9—12] Otherwise, dissolved oxygen, whose potential for reduction is quite low at common electrodes such as carbons, exhibits a cathodic response, preventing the sensitive and reproducible determination of analytes. Notably in high performance liquid chromatography with an electrochemical detector (ECD/HPLC) in the cathodic mode, a rather tedious procedure must be applied continuously for a mobile phase. However, a cathodic ECD/HPLC system has the following advantages over other HPLC systems: (1) in general, downsizing of an electrochemical HPLC system is more feasible than that of a spectrophotometric one; (2) cathodically active compounds are rare in biological fluids, so there are fewer problems from interference or separation in comparison to anodic ECD/HPLC. To develop a useful bioanalytical method based on these advantages, especially the second, we tested a cathodic ECD/HPLC system as a sensitive and precise tool for the determination of *p*-nitrophenol (NP). Recently, ECD/HPLC systems with a dual mode have been developed for the determination of a nitrophenol analogue, nitrotyrosine.[13—15] The detection of nitrotyrosine using such systems comprises two electrochemical processes for transformation and detection as follows: cathodic reduction to the corresponding aminophenol at an upstream electrode and oxidation of the product at a downstream (detection) electrode. Although dual ECD/HPLC was demonstrated to provide a highly sensitive tool for the analysis of nitrotyrosine, a sophisticated electrochemical device is required and anodic oxidation-based detection will encounter problems with separation since biological fluids contain a large number of anodically active compounds. The purpose of this study is to take full advantage of the potential utility of cathodic detection, to develop a simple and general ECD/HPLC system for NP in biological samples. Herein, we report that utilizing not only a commercially available helium-purging device but also PEEK tubes for all tubing makes the ECD/HPLC system a useful tool for the determination of NP and assay of acid phosphatase with *p*-nitrophenyl phosphate disodium salt as a substrate.

Experimental

Materials and Apparatus Milli Q water and MeOH of HPLC grade were used throughout this study. All other chemicals were of reagent grade and used without further purification. Glassy carbon (GC) rods (GC-20, 3 mmϕ×100 mm) were obtained from Tokai Carbon. A GC disk electrode was fabricated as reported previously,[16] and polished with a Maruto ML-150P polishing system with polishing paper (#1500) and alumina powder (0.05 μm) on a polishing cloth before being sonicated in water and MeOH prior to use. Cyclic voltammograms were obtained with an ALS model 600 electrochemical analyzer. A three-electrode configuration was employed: a GC electrode as a working electrode, a saturated calomel electrode (SCE) as a reference electrode, and a platinum wire electrode as a counter electrode. Test solutions were deoxygenated by bubbling nitrogen gas for several minutes and subjected to voltammetric measurements at room temperature and 100 mV/s over a potential range between 0.0 and −1.0 V *vs.* SCE under a nitrogen atmosphere. The HPLC system consisted of a Shimadzu LC-10AD$_{vp}$ pump, a Tosoh AS-8020 auto-injector with a 10 μl sample loop, a Shimadzu CTO-10AC$_{vp}$ column oven, a Shimadzu L-ECD-6A electrochemical flow cell loaded with a GC electrode (GC-20, 15×30×3 mm), a Huso HECS318 potentiostat, and a Shimadzu Chromatopac C-R6A recorder. The GC working electrode was polished as mentioned above, sonicated in water, MeOH,

* To whom correspondence should be addressed. e-mail: h-maeda@phs.osaka-u.ac.jp

and $CHCl_3$, and dried with a nitrogen stream prior to loading in the electrochemical cell. A Mightysil RP-18 GP column (3 μm, 100 mm×2.0 mm i.d., Kanto Chemical Co.) was placed in the column oven at 30 °C. PEEK tubes with a 2 mm i.d. were used for connection between the mobile phase and pump, and 0.13 mm i.d. for tubing in all other components. The mobile phase was filtered with a membrane filter (0.2 μm), continuously deoxygenated with helium gas using a GL-Sciences CR670 carrier reservoir, and pumped at 0.2 ml/min. Spectrophotometric measurements were made with a Hitachi U-3210 spectrophotometer.

Assay of Acid Phosphatase in Urine Samples Urine was taken from healthy volunteers, kept in a refrigerator at 4 °C, and centrifuged at 4 °C for 15 min at 1000 ***g*** prior to measurements. Citrate buffer (0.5 ml, pH 4.8) and 4.0 mg/ml of *p*-nitrophenyl phosphate disodium salt solution in water (0.5 ml) were added to the urine samples (0.1 ml). The resulting mixture was incubated at 37 °C for 30 min. The incubated mixture (0.4 ml) was diluted with the mobile phase (20 ml), and subjected to analysis using the present ECD/HPLC. Another 0.4 ml of the mixture was diluted with a 20 mM aqueous NaOH solution (20 ml) and subjected to spectrophotometric measurements at 405.2 nm according to the Bessey–Lowry–Brock method.[17]

Results and Discussion

On cyclic voltammetry in 25% aqueous MeOH containing 0.1 mM EDTA·2Na and 0.1 M KCl, NP exhibited cathodic and anodic waves at −0.86 and −0.76 V *vs.* SCE, respectively (Fig. 1a). As shown in Fig. 1b, the quasi-reversibility was lost by addition of CF_3CO_2H (TFA) at 0.1% (v/v), leading to a peak-potential shift in the positive direction and a marked increase in peak-current. The cathodic response in the presence of TFA was reproducible in successive voltammetric measurements, indicating that no electrode fouling was brought about through cathodic reduction of NP under the conditions. The observed voltammetric phenomenon was well in line with the fact that nitro compounds are more feasibly reduced at a cathode in the presence than absence of acids.[4,5] Effects of other acids on the voltammetric responses of NP were also evaluated. The results are summarized in Table 1. The organic and inorganic acids examined showed similar effects to TFA, the extent of which seemed to depend on their acidities. Among the acids, TFA allowed NP to exhibit the largest cathodic response at the most positive potential. Although adding TFA at a larger amount might induce a more favorable effect on the cathodic response of NP, the effect was not tested since common reversed phase columns cannot tolerate a highly acidic mobile phase. It was demonstrated that NP could be determined based on anodic oxidation of its phenolic moiety as well.[18—20] However, anodic detection of NP results in electrode fouling due to the formation of polymeric products on the electrode surface[20] as with other phenols.[21—24] Accordingly, in terms of the maintenance of electrode surfaces, cathodic reduction is regarded as a much more useful tool for ECD/HPLC analysis of NP than anodic oxidation.

Comparing chromatograms obtained at 0.8 and −0.8 V *vs.* Ag/AgCl for urine samples also demonstrated that cathodic detection for the ECD/HPLC of NP has an advantage over anodic detection. For the cathodic system, the mobile phase was continuously deoxygenated with a helium-purging device. Figure 2a indicates that urine samples contain a large number of compounds detected in the anodic mode under the conditions. In contrast, the chromatogram afforded by cathodic ECD/HPLC was quite simple (Fig. 2b). The observation suggests that with cathodic HPLC systems, the problem of separating target from non-target compounds is reduced, namely, additional peaks broadening over chromatograms

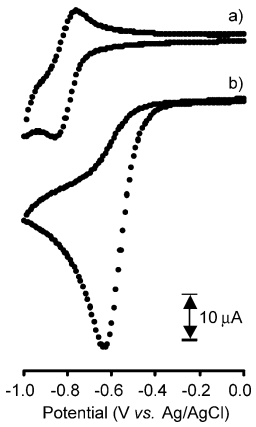

Fig. 1. Cyclic Voltammograms of 1 mM *p*-Nitrophenol in 25% Aqueous MeOH Containing 0.1 M KCl and 0.1 mM EDTA·2Na (a) without and (b) with 0.1% (v/v) CF_3CO_2H

Table 1. Effect of Acids Added at 0.1% (v/v) on Potential (E_{cp}) and Current (I_{cp}) of a Cathodic Wave of 1 mM *p*-Nitrophenol in Cyclic Voltammetry at a Glassy Carbon Electrode[a)]

Acid	E_{cp} (V *vs.* SCE)	I_{cp} (μA)
—	−0.86	23
CF_3CO_2H	−0.64	57
HCl	−0.64	52
$HClO_4$	−0.73	51
$ClCH_2CO_2H$	−0.75	51
H_3PO_4	−0.75	47
CH_3CO_2H	−0.77	49

a) Obtained in 25% aqueous MeOH containing 0.1 mM EDTA·2Na and 0.1 M KCl at room temperature and 0.1 V/s.

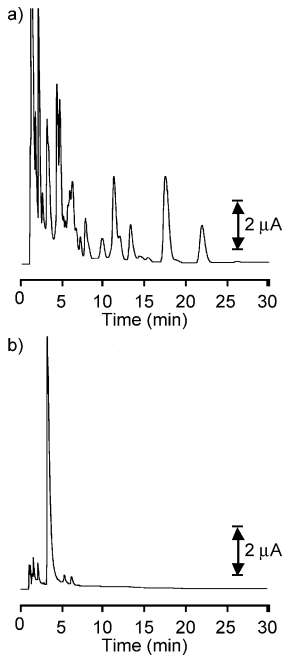

Fig. 2. Typical Chromatograms of Urine Samples Obtained with an ECD/HPLC System at a Detection Potential of (a) 0.80 and (b) −0.80 V *vs.* Ag/AgCl

Conditions: mobile phase, 15% aqueous MeOH containing 0.1 M KCl, 0.1 mM EDTA·2Na, and 0.1% (v/v) CF_3CO_2H; flow rate, 0.2 ml/min; column temperature, 30 °C; injection volume, 10 μl.

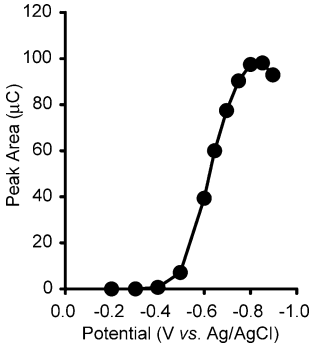

Fig. 3. A Hydrodynamic Voltammogram for 50 μM *p*-Nitrophenol

Conditions: mobile phase, 25% aqueous MeOH containing 0.1 M KCl, 0.1 mM EDTA·2Na, and 0.1% (v/v) CF_3CO_2H; flow rate, 0.2 ml/min; column temperature, 30 °C; injection volume, 10 μl.

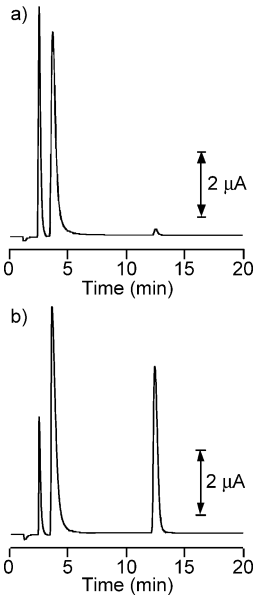

Fig. 4. Typical Chromatograms of Unine Samples (a) before and (b) after Incubation with *p*-Nitrophenyl Phosphate Disodium Salt at 37 °C for 30 min with the Proposed ECD/HPLC System

Conditions are the same as those in Fig. 3 except that the detection potential was −0.8 V *vs.* Ag/AgCl.

will be effectively eliminated. It should be mentioned here that the key to obtaining simple and reproducible chromatograms was the use of PEEK tubes, especially between the mobile phase and pump. When a Teflon tube was used instead, the background response for cathodic ECD/HPLC was high and unstable (data not shown), rendering the system useless as an analytical tool.

A hydrodynamic voltammogram for NP (50 μM) was obtained with a cathodic ECD/HPLC system utilizing PEEK tubes for all tubing as well as a helium-purging device (Fig. 3). As a mobile phase, 25% aqueous MeOH containing 0.1 M KCl, 0.1 mM EDTA·2Na, and 0.1% (v/v) TFA was used. As the detection potential was more negative than −0.4 V *vs.* Ag/AgCl, a larger peak due to reduction of the nitro compound was observed, and the peak area reached a maximum value at −0.8 V. Based on this result, the possibility of using ECD/HPLC with a detection potential at −0.8 V to measure NP was examined. Detection and determination limits were estimated as the lowest concentrations affording chromatographic responses with relative standard deviations (RSDs) less than 30 and 10%, respectively. The detection limit was 0.20 μM (2.0 pmol injected). The determination limit was 0.25 μM (2.5 pmol injected), and up to 50 μM (500 pmol injected), a linear calibration curve for NP was obtained, the slope and correlation coefficient (r) being 1.93 C/M and 0.999. The reproducibility of the present system was also examined by evaluating within-day and between-day precisions. When each standard solution of 5 and 50 μM NP was subjected to the HPLC analysis six times, RSDs were 0.8 and 0.7%, respectively. Based on the results for the same experiments repeated independently for 6 successive days, mean values of the peak areas obtained for six successive injections of these standard solutions on each day deviated by only 3.5 and 2.2% for 5 and 50 μM NP, respectively. Thus, it is demonstrated that the present cathodic ECD/HPLC is a sensitive and precise method for the determination of NP.

The assay of acid phosphatase is a clinical enzyme test, since acid phosphatase activity is a useful marker for various diseases.[25] Determination of the enzyme activity is achieved by spectrophotometric detection of NP enzymatically produced from *p*-nitrophenyl phosphate disodium salt.[25] One of the most common methods used is the Bessey–Lowry–Brock (BLB) assay.[17] However, the assay has the drawback that the spectrophotometric measurement must be performed for each sample twice: first for a test solution obtained by addition of excess aqueous NaOH after incubation and second for a control solution prepared by addition of conc. HCl to the test solution. This is not only because the enzyme substrate exhibits a spectrophotometric response as well, but also because in the spectrophotometric measurements, background responses for biological samples are different from each other and not negligible, and hence subtracting the spectrum of a control solution from that of a test sample is necessary. Thus, a simple and precise assay of phosphatases as an alternative to the BLB method is needed. Therefore, the bioanalytical utility of our method was examined by applying it to the assay of acid phosphatase in urine samples.

Figure 4 shows typical chromatograms obtained using the present ECD/HPLC system for urine samples before and after incubation with *p*-nitrophenyl phosphate disodium salt at 37 °C for 30 min. Only peaks due to reduction of the substrate, dissolved oxygen in injected samples, and NP were observed, demonstrating that cathodic ECD/HPLC is useful for the analysis of urine samples as mentioned above. Note that sample solutions were not deoxygenated, leading to the appearance of a peak. Seven urine samples were subjected to the assay of acid phosphatase by both the present and BLB methods. As shown in Fig. 5, a linear relationship with a slope and r of 1.06 and 0.998, respectively, was observed between the results afforded by these methods. The result clearly indicates that the cathodic ECD/HPLC is a useful and precise method for assaying acid phosphatase without need for tedious procedures such as measuring blank samples.

In summary, the present study demonstrates that by using PEEK tubes for all tubing as well as a helium-purging device, cathodic ECD/HPLC makes full advantage of its potential strengths, and has bioanalytical utility. To render the present method a more practical tool from a standpoint of clinical analysis, the measurement-time for one sample must be

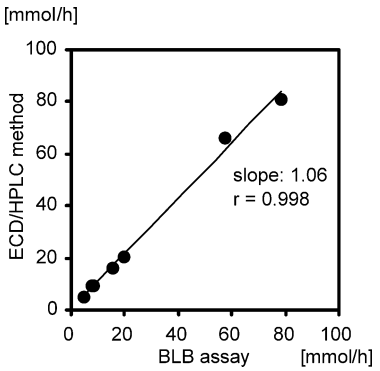

Fig. 5. Correlation of the Results Obtained by the Present and Bessey–Lowry–Brock Methods

shortened. Further study along this line is under way in our laboratory.

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