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High performance liquid chromatography equipped with a cathodic detector and column-switching device as a high-throughput method for a phosphatase assay with *p*-nitrophenyl phosphate

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Abstract

LC coupled to an electrochemical detector (LC-EC) operating in cathodic mode with a column-switching system realizes a high-throughput detection of *p*-nitrophenol (NP). The measurement-time for each NP sample was shortened to 20 s, and the successive analyses of 39 samples was completed within 13 min. In the present system, the limits of detection and quantification were 0.15 and 0.20 μM, respectively, and further, up to 25 μM, a linear calibration curve was afforded. Relative standard deviations for standard solutions of 0.20, 1.0, and 25 μM NP were 4.3, 2.0, and 1.1% ($n = 5$), respectively. Between-run precisions of the analysis of 5.0 and 25 μM NP over 6 days were 4.8 and 1.3%, respectively. A comparison with the commonly used Bessey–Lowry–Brock method indicates that the present LC-EC is useful for the high-throughput assay of acid and alkaline phosphatases in urine and blood samples with a *p*-nitrophenyl phosphate substrate.

Keywords: LC-EC; *p*-Nitrophenol; Bessey–Lowry–Brock assay; Acid phosphatase; Alkaline phosphatase; *p*-Nitrophenyl phosphate

1. Introduction

A number of aromatic nitro compounds are found in pharmaceuticals (including nitrazepam and chloramphenicol) and used as common reagents [1] for enzyme assays, such as phosphatase and peptidase. Spectrophotometric methods have typically been used for the analysis of the nitro compounds. Cathodic detection can be considered as an alternative method, since aromatic nitro groups are well known to undergo electrochemical reductions [2–4]. However, only scattered studies on reductive detection of aromatic nitro compounds have been found in literature [5–7]. This is primarily because the media must be deoxygenated for electrochemical detection in the cathodic mode [2,8–11]. This is because dissolved oxygen (DO) exhibits a cathodic response, which prevents the sensitive and reproducible determination of analytes. In high performance liquid chromatography coupled to an electrochemical detector (LC-EC) in the cathodic mode, the rather tedious deoxygenation procedure for the mobile phase must be continuously applied. This procedure is essential to obtain a stable baseline and reproducible responses of analytes on LC-EC. In addition, deoxygenation of the prepared sample solutions must be conducted as effectively as that of the mobile phase. When the deoxygenation of the sample solutions is insufficient, DO in the sample solutions exhibits a relatively large peak on a chromatogram.

Despite the apparent drawback, cathodic EC for LC systems is thought to be advantageous over other detectors, including anodic EC. One such advantage is that cathodically active compounds are extremely rare in biological fluids, thus minimizing problems with interference and separation. In fact, a urine sample was determined to afford a chromatogram with a large number of peaks on an LC system equipped with an anodic EC at 0.8 V versus Ag/AgCl, than a system with a cathodic EC at −0.8 V, which resulted in a simple chromatogram [12]. Fewer additional peaks on chro-

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matograms will enable analysts not only to be freed from the time-consuming optimization of the separation conditions, but also enable them to measure one sample by LC in a dramatically reduced time. Recently, it was demonstrated that a cathodic LC-EC system with PEEK tubing throughout (especially for the connection from the mobile phase reservoir to the pump) coupled to a commercially available helium-purging device realizes a simple and precise method of analysis for *p*-nitrophenol (NP) and for an acid phosphatase assay with *p*-nitrophenyl phosphate disodium salt as the substrate [12]. In this paper, we attempted to expand the utility of our cathodic LC-EC system into a high-throughput method for the identification of NP. Herein, an LC-EC system utilizing a column-switching device with a six-port valve serves as a sensitive and precise high-throughput method for analyzing NP and for the assay of acid and alkaline phosphatases in urine and blood samples, respectively.

2. Experimental

2.1. Chemicals and materials

Water was purified using a Millipore Milli-Q Gradient-A10 system coupled with an EYELA (Japan) SA-2100E automatic water distillation apparatus. HPLC grade MeOH was purchased from Nacalai Tesque (Japan). The mobile phase was filtered through an Advantec (Japan) PTFE membrane (0.2 μm) prior to use and was deoxygenated using a GL Sciences (Japan) CR670 carrier reservoir with helium gas at 0.1 kgf/cm^2. Inertsil and Mightysil columns were purchased from GL Sciences and Kanto Chemical Co. (Japan), respectively. *p*-Nitrophenol (NP) and *p*-nitrophenyl phosphate disodium salt were purchased from Wako Pure Chemicals (Japan) and Nacalai Tesque, respectively. All other chemicals were reagent grade and used without further purification. A glassy carbon working electrode (GC-20, 15 mm × 30 mm × 3 mm) was obtained from Shimadzu (Japan), and polished using a Maruto (Japan) ML 150-P polishing system equipped with polishing paper (#1500) and alumina powder (0.05 μm) on a polishing cloth, and followed by sonication in water, MeOH, and $CHCl_3$, and then dried with a nitrogen stream prior to loading into the electrochemical cell for LC-EC.

2.2. Preparation of reagents

The standard solutions of NP were prepared in aqueous 0.1% (v/v) CF_3CO_2H (TFA). An aqueous solution of 4.0 mg/mL *p*-nitrophenyl phosphate disodium salt was utilized as the enzyme substrate solution. The citrate buffer solution consisted of 20.7 g citric acid monohydrate, 180 mL aqueous 1 M NaOH, and 100 mL aqueous 0.1 M HCl in 1 L water. Glycine buffer was prepared by dissolving 7.50 g glycine, 0.20 g $MgCl_2{\cdot}(H_2O)_6$, and 85 mL aqueous 1 M NaOH in 1 L water. The substrate and buffer solutions were warmed at 37 °C prior to use for the assay.

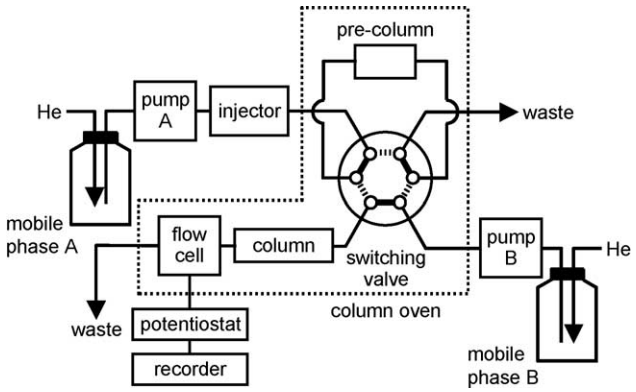

Fig. 1. A schematic diagram of the present column-switching cathodic LC-EC system.

2.3. Instruments

The LC-EC system equipped with a column-switching device was constructed as depicted in Fig. 1: pump A and B, Shimadzu LC-10AD$_{VP}$; column-switching valve, Shimadzu FCV-12AH; column-switching controller, Shimadzu SCL-10A coupled to a Shimadzu Sub-controller-vp; injector, either Rheodyne 7725i manual injector with a 10 μL PEEK sample loop or a Tosoh (Japan) AS-8020 auto-injector; column oven, Shimadzu CTO-10AC$_{VP}$; detector, Shimadzu L-ECD-6A electrochemical flow cell coupled to a Huso (Japan) HECS318 potentiostat; helium-purging device, GL Sciences CR670; recorder, Shimadzu Chromatopac C-R6A. A PEEK tube (2 mm i.d.) was used to connect the mobile phase reservoir to the pump. PEEK tubing (0.13 mm i.d.) was used for all other connections throughout the LC system. Spectrophotometric measurements were performed using a Hitachi (Japan) U-3210 spectrophotometer.

2.4. Assay for acid phosphatase in urine samples

Urine samples were collected from healthy volunteers, stored at 4 °C, and centrifuged at 4 °C for 15 min at 1000 × *g* prior to analysis. The supernatant (0.1 mL) was added to a mixture of citrate buffer (0.5 mL, pH 4.8) and the substrate solution (0.5 mL). The resulting mixture was incubated at 37 °C for 30 min, and cooled in an ice-water bath. The incubated mixture (0.4 mL) was diluted with mobile phase (20 mL), and analyzed by LC-EC in order to estimate the amount of enzymatically generated NP. Another 0.4 mL of the incubated mixture was diluted with aqueous 0.02 M NaOH (20 mL) and measured spectrophotometrically at 405.2 nm according to the procedure reported for the Bessey–Lowry–Brock method [13].

2.5. Assay for alkaline phosphatase in plasma samples

Heparinized whole blood collected from healthy volunteers was centrifuged at 4 °C and 1000 × *g* for 10 min. The obtained plasma sample (0.2 mL) was added to a mixture of glycine buffer (pH 10.5, 1.0 mL) and the substrate solution (0.5 mL). The resulting mixture was incubated at 37 °C for 30 min, and cooled in an ice-water bath. The incubated mixture (1.0 mL) was treated with aqueous 0.6 M $HClO_4$ (0.5 mL) and centrifuged at 4 °C and 10,000 × *g* for 10 min. The obtained supernatant (1.2 mL) was diluted with aqueous 0.1% (v/v) TFA solution (5 mL) and LC-EC was used to estimate the amount of enzymatically generated NP. Another 1.0 mL of the incubated mixture was diluted with aqueous 0.02 M NaOH (5 mL), and measured spectrophotometrically at 405.2 nm according to the procedure reported for the Bessey–Lowry–Brock method [13].

3. Results and discussion

3.1. Column-switching system as a tool for on-line deoxygenation

Our previous study on a cathodic LC-EC demonstrated that DO in a mobile phase can be effectively removed by utilizing PEEK tubing throughout the system coupled to a helium-purging device. The successful deoxygenation enabled the LC-EC system to produce a stable baseline with a low background response, which resulted in sensitive and reproducible results for the analysis of NP. However, one shortcoming of this system was a magnified response for DO in the sample solution due to the fact that the injected sample solutions were not deoxygenated in an effort to simplify the procedure. Fig. 2a shows a typical chromatogram for 50 μM NP obtained for the reported LC-EC system. A much larger peak than that of NP was observed, due to the DO in the injected sample. As the MeOH content of the mobile phase increased, NP eluted increasingly quicker, and as the MeOH content of the mobile phase decreased, NP was retained on the column longer. Contrastingly, the retention time of the DO remained constant regardless of the polarity of the mobile phase (data not shown). These observations indicate it difficult to markedly reduce the analysis time for NP on the cathodic LC-EC system by shortening the retention time by altering the content of the mobile phase or changing the analytical column unless the DO responses in sample solutions can be eliminated or at least significantly reduced.

A comparison of the chromatographic behavior of NP and DO, however, provided a clue to solving the DO setback without employing a tedious and impractical procedure, such as bubbling nitrogen gas through sample solutions. A column-switching device has been well documented for on-line sample clean-up on LC analysis [14,15]. Thus, the cathodic LC-EC was coupled to this device in which the line was switched between a pre-column and an analytical column via a six-port valve (Fig. 1). The injected NP solution was introduced to a pre-column with mobile phase A, which was expected to retain NP and allow DO to pass through the pre-column. With the switching device, the pre-column was connected to an analytical column and eluted with mobile phase B, resulting in the elution of NP. The chromatogram shown in Fig. 2b was obtained when the switching-time was set at 1.0 min after the injection of 50 μM NP (10 μL). As expected, the DO response was significantly reduced; 89% of the DO in the injected sample solution was removed by utilizing the column-switching device.

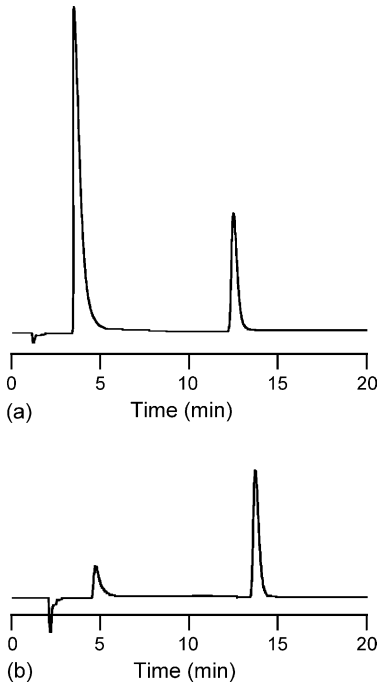

Fig. 2. Typical chromatograms for 50 μM NP obtained by the cathodic LC-EC system at −0.8 V vs. Ag/AgCl (a) without and (b) with the column-switching device. Running conditions for (a): column, Mightysil RP-18 GP (3 μm, 2 mm × 100 mm); column temperature, 30 °C; mobile phase, aqueous 25% MeOH, containing 0.1 M KCl, 0.1 mM EDTA·2Na, and 0.1% (v/v) CF_3CO_2H (TFA); flow rate, 0.2 mL/min; injection volume, 10 μL. Running conditions for (b): pre-column, Inertsil WP300 C_{18} (5 μm, 1.5 mm × 10 mm); analytical column, Mightysil RP-18 GP (3 μm, 2 mm × 100 mm); column temperature, 30 °C; mobile phase A, 0.1 M KCl, 0.1 mM EDTA·2Na, and 0.1% (v/v) TFA; mobile phase B, aqueous 25% MeOH, containing 0.1 M KCl, 0.1 mM EDTA·2Na, and 0.1% (v/v) TFA; flow rate for mobile phases A and B, 0.2 mL/min; injection volume, 10 μL.

The effects of the switching-time on the response of NP and DO observed for 50 μM NP injected in the present LC-EC were examined. In addition, the capability of the pre-column (5 μm, 1.5 mm × 10 mm) to retain NP and discard DO was determined to depend on the packing material of the column. Three ODS pre-columns with different surface densities and silica gel particle pore sizes were evaluated; Inertsil WP300 C_{18} (monomeric, 300 Å), ODS-3 (monomeric, 100 Å), and ODS-P (polymeric, 100 Å). Fig. 3 shows that DO in the sample solution was effectively cleaned up when the switching-time was set at more than 1.0 min for all of the

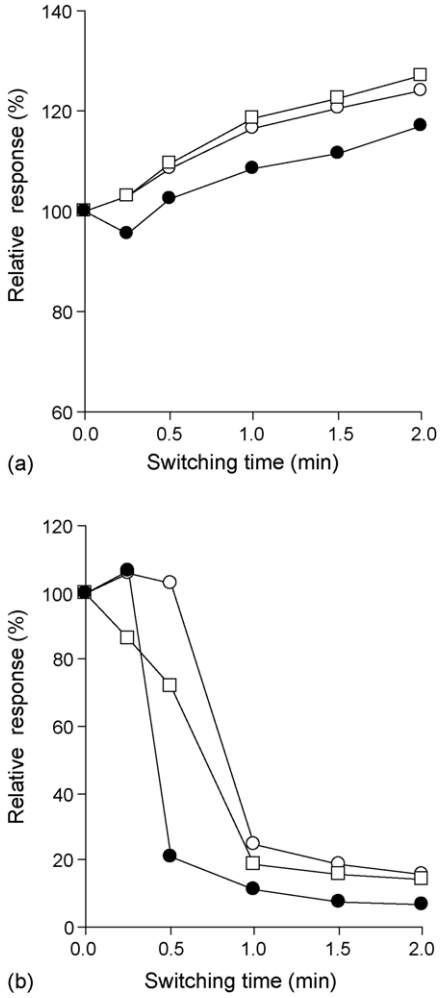

Fig. 3. Effects of the switching-time on (a) NP and (b) DO responses when 50 μM NP was subjected to the present LC-EC system with Inertsil WP300 C_{18} (closed circles), ODS-3 (open circles), or ODS-P (open squares) columns as the pre-column (5 μm, 1.5 mm × 10 mm). Responses relative to those obtained using the LC-EC without the column-switching device are plotted against the switching-time. The other conditions are the same as in Fig. 2b.

pre-columns evaluated: utilizing Inertsil WP300 C_{18}, ODS-3, or ODS-P pre-columns at a 1.0 min switching-time reduced the DO response by 89, 75, and 81%, respectively. Some response-enhancement effects for detecting NP were observed for the column-switching LC-EC system. The Inertsil WP300 C_{18} pre-column was selected and used in later studies due to its superior ability to discard DO. It is important to note that the NP peak was not observed to broaden in the present LC-EC system, even with a 2 min switching-time. Thus, the column-switching system was demonstrated to be a sufficient method for the on-line deoxygenation of sample solutions.

3.2. Optimization as a high-throughput method

In order to maximize the capability of the present LC-EC system as a novel tool for the high-throughput analysis of NP, a reduced analysis time was desired. This was achieved by optimizing the analytical column, flow rates of mobile phases A and B and the switching-time. An Inertsil WP300 C_8 column (5 μm, 1.5 mm × 10 mm, pore size; 300 Å) enabled the LC-EC system to analyze NP in about 2 min when the switching-time and flow rate for mobile phases A and B were set at 1.0 min and 0.2 mL/min, respectively. This analysis time was significantly shorter than those realized using other reversed phase columns (data not shown). The effects of the flow rate and the switching-time for the system using this analytical column are summarized in Table 1. The same flow rate was used for mobile phases A and B. Flow rates higher than 2.0 mL/min were not examined due to the relatively high column pressure produced from high flow rates. At flow rates higher than 0.2 mL/min, a shorter switching-time was sufficient for the on-line deoxygenation system in order to successfully eliminate the DO: the DO peak was reduced in about 90% of the all cases, compared with those for the cathodic LC-EC system without a column-switching device. As shown in Table 1, the higher flow rate allowed analysis of NP to be accomplished in a shorter time. The efficiency of cathodic reduction of NP on the electrode, however, was markedly depressed, leading to a marked decrease in the overall peak area. Fortunately, as the flow rate increased, the peak sharpened, resulting in an increase in peak height. Furthermore, the relative standard deviations (RSDs) of the peak heights were better than those for the peak areas. In order to evaluate the present system for efficiency as a high-throughput method for NP analysis, the peak heights were used instead of the peak areas. Thus, 2.0 mL/min and 6 s, respectively were considered the flow rate and switching-time of choice for constructing a high-throughput system. Under these optimized conditions for the LC-EC system, the

Table 1
Effects of the Flow rate and switching-time on the chromatographic responses of NP when 50 μM NP was subjected to the present LC-EC system[a]

Running conditions		Chromatographic responses of NP[b]		
Flow rate (mL/min)	Switching-time (sec)	Retention time (sec)	Peak area (μC)	Peak height (μA)
0.2	60	132	89.9 (1.5%)	4.6 (0.4%)
0.4	30	66	55.4 (0.4%)	5.3 (0.2%)
1.0	12	28	31.6 (1.6%)	6.5 (1.3%)
2.0	6	14	19.3 (2.7%)	6.3 (1.0%)

[a] Running conditions: pre-column, Inertsil WP300 C_{18} (5 μm, 1.5 mm × 10 mm); analytical column, Inertsil WP300 C_8 (5 μm, 1.5 mm × 10 mm); the other conditions are the same as in Fig. 2b.
[b] The numbers in parentheses are RSDs ($n = 6$).

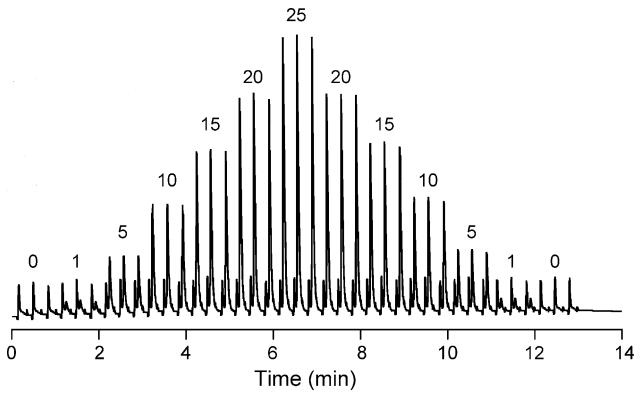

Fig. 4. Successive analysis of NP standard solutions (0–25 μM) using the present LC-EC system. Each of NP standard solutions were injected three times. Conditions: pre-column, Inertsil WP300 C_{18} (5 μm, 1.5 mm × 10 mm); analytical column, Inertsil WP300 C_8 (5 μm, 1.5 mm × 10 mm); flow rate for mobile phases A and B, 2.0 mL/min; switching-time, 6 s; the other conditions are the same as in Fig. 2b.

analysis of NP was completed in 20 s and 39 samples were successively analyzed within 13 min (Fig. 4).

3.3. Performance as a high-throughput method

Under these conditions for the cathodic LC-EC, detection and quantification limits were estimated as the lowest concentrations affording chromatographic responses with RSDs less than 30 and 10%, respectively [16]. The detection limit was 0.15 μM (1.5 pmol injected). The determination limit was 0.20 μM (2.0 pmol injected), and up to 25 μM (250 pmol injected), a linear calibration curve for NP was obtained. The slope and correlation coefficient (r) of the curve were 155.8 mA/M and 1.000, respectively. The reproducibility (within-run precision) of the present system was quite satisfactory: RSDs (n = 5) for standard solutions of 0.20, 1.0, and 25 μM NP were 4.3, 2.0, and 1.1%, respectively. Between-run precision was also evaluated as follows: 5.0 and 25 μM NP was subjected to the LC-EC successively six times, and the experiment were repeated for 6 days. The mean value of the peak heights obtained on each day deviated by 4.8 and 1.3% for 5.0 and 25 μM NP, respectively. Thus, the present cathodic LC-EC system was demonstrated to be a precise high-throughput method for the determination of NP.

3.4. High-throughput assay of phosphatase

An assay of alkaline or acid phosphatase is one of the typical clinical enzyme tests, since phosphatase activity is a useful marker for a number of diseases [17]. Determination of the enzyme activity was achieved by spectrophotometric detection of NP enzymatically produced from *p*-nitrophenyl phosphate disodium salt [17]. One of the most common methods is the Bessey–Lowry–Brock (BLB) assay [13]. Thus, to examine the bioanalytical utility of our present LC-EC system, its performance was compared with that of the BLB method as acid phosphatase assays for urine samples and alkaline phosphatase assays for blood samples with a *p*-nitrophenyl phosphate substrate.

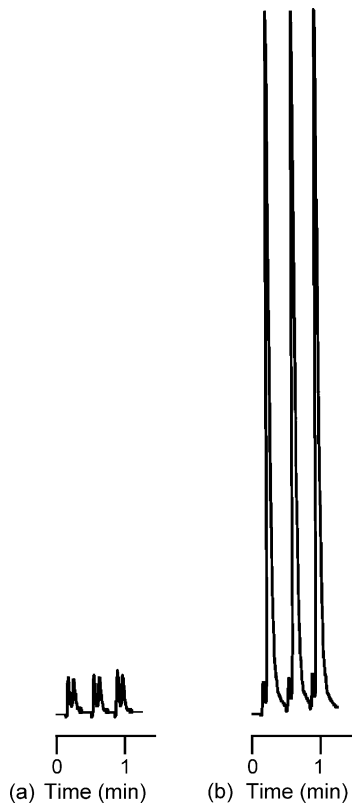

Fig. 5. Typical chromatograms for urine samples (a) before and (b) after incubation with *p*-nitrophenyl phosphate disodium salt using the present LC-EC system. Chromatographic conditions are the same as in Fig. 4.

Fig. 5 shows typical chromatograms obtained for urine samples before and after incubation with *p*-nitrophenyl phosphate disodium salt at 37 °C for 30 min under acidic conditions. Only two peaks due to DO in injected samples and NP were observed. The enzyme substrate was detected using the previously reported cathodic LC-EC system [12]. However, the column-switching system was useful for totally eliminating a polar compound, *p*-nitrophenyl phosphate, as well as for reducing the DO peak, leading to the disappearance of the peak corresponding to the substrate remaining after the enzymatic reaction. The present system gained additional advantage by using a column-switching device instead of purging procedure for eliminating DO in sample solutions. Seven urine samples were subjected to acid phosphatase assays and analyzed according to both the present method and the BLB method. Although the present method led to slightly higher activities than BLB method, a good linear relationship was observed between the results obtained in these methods; the slope, intercept, and r were 1.08, 1.04 mmol/h, and 0.998, respectively (Fig. 6). The present high-throughput method was also applicable to assay of alkaline phosphatase in blood samples, without any other peaks in the chromatograms. The activities of three plasma samples were determined to be 1.12, 1.42, and 1.00 mmol/h with RSDs (n = 3) less than 2.7%. The

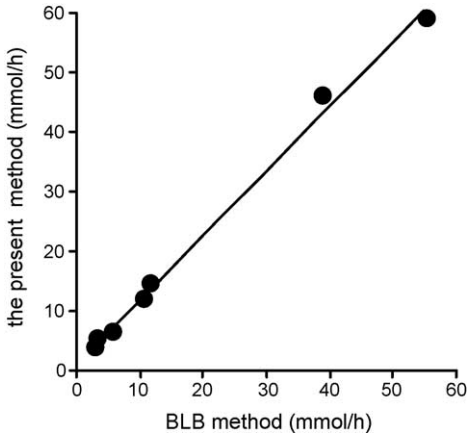

Fig. 6. Correlation of the activities for acid phosphatase in urine samples collected from seven volunteers determined using the present LC-EC system and the Bessey–Lowry–Brock method.

measured values for the same samples using the BLB method were 1.11, 1.37, and 0.98 mmol/h, respectively, RSDs ($n = 3$) being within 3.1%.

4. Conclusions

In this study, the combination of a cathodic LC-EC system and a column-switching device was shown to be advantageous: the former provides simple chromatograms for the analysis of biological samples, without additional peaks, and the latter serves as a tool for the on-line elimination of compounds that may otherwise co-elute with the analyte of interest, such as DO and the substrate for phosphatase in sample solutions discussed herein. As a result, a novel high-throughput method for the determination of NP and for acid phosphatase assays in urine samples and alkaline phosphatase assays in plasma samples has been developed. This method may be applied as a high-throughput method for various enzyme assays of commercially available substrates that are enzymatically transformed into NP and nitroaniline. In addition, the high-throughput method is applicable for the determination of endogenous aromatic nitro compounds, such as nitrotyrosine [18] and nitro(deoxy)guanosine [19] as markers for various diseases induced by oxidative stress. Further studies along these lines are currently underway in our laboratory.

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