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Determination of thiols by capillary electrophoresis with electrochemical detection using a palladium field-decoupler and chemically modified electrodes

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Abstract

Off-column electrochemical detection has been used for the determination of cysteine and related compounds after separation by capillary electrophoresis. For the decoupling of the high-voltage field, an improved palladium decoupler was developed with a dead volume of less than 10 nl. Micro-electrodes, bulk-modified with cobalt phthalocyanine, were used in a nanoliter-volume cell for detection of the thiol compounds. With a detection potential of +0.6 V vs. Ag/AgCl a high selectivity and sensitivity was obtained.

Baseline separation of cysteine, homocysteine and glutathione could be realized in 25 min, using a formate buffer with pH 2.7 as background electrolyte. Detection limits of 0.5 μmol l^{-1} for homocysteine, 1 μmol l^{-1} for cysteine and 5 μmol l^{-1} for glutathione were obtained. The concentrations of free cysteine and total cysteine (after treatment with dithiothreitol) in urine samples could be measured.

1. Introduction

Capillary electrophoresis (CE) has been shown to be a powerful analytical tool with its high separation efficiency [1]. While new applications are still being explored and fundamental research is carried out with great interest in many laboratories, CE has already become an alternative or complemental analytical technique to high-performance liquid chromatography (HPLC) for routine analysis [2]. UV-absorption detection is by far the most commonly used detection technique, but it has a major inherent drawback: the dependence of the sensitivity on the light-path length, which is small in CE. This drawback does not exist in electrochemical detection (ED). ED is based on the reaction of analytes on an electrode surface and only molecules in a thin diffusion layer contribute to the signal intensity. For compounds which can not be detected favourably by UV absorption, ED can be an alternative.

ED in capillary electrophoresis has received considerable attention from several research groups [3–16]. A prerequisite to perform ED in CE is to isolate the electrophoretic current generated by the high-voltage (HV) used for separation, from the electrochemical detection current. To achieve this, there are basically two approaches: the end-column mode [3–7] and the off-column mode [8–16]. End-column detection, where the detection electrode is positioned close to the capillary exit in the grounded end-vial, is restricted to very narrow capillaries (<25 μm

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I.D.) with a low electrophoretic current to minimize the interference of the high voltage on the detection current. Normal-sized capillaries (25–100 μm I.D.) can only be used in off-column detection, provided that the influence of electrophoretic current is eliminated from the electrochemical detection signal. To achieve this, the separation capillary is connected to the detector through a field-decoupler made of porous material [8–11], an ion-exchange tube [12,13] or palladium metal [14–16]. This decoupler acts as the grounded end of the CE system. In this way, the electrophoretic current is separated from the electrochemical current.

Thiols play an important role in several biological processes. It is of clinical and pharmaceutical importance to determine these compounds in biological fluids and tissues [17]. The separation of thiols using HPLC has been reviewed [18]. Because of the absence of strong chromophores and fluorophores, pre- or post-column derivatization procedures are often used for sensitive and selective detection. Derivatization is not required in ED [19,20], but thiols are not easily detected with a normal carbon electrode, since their oxidation requires a high overpotential [21]. It has been shown that modification of the electrode with cobalt phthalocyanine (CoPC) as electrocatalyst reduces the required overpotential, so that thiols can be detected at a lower, more selective potential [22,23].

Recently, the separation of thiols by CE with UV [24–27] or fluorescence detection [28] has been described. O'Shea and Lunte [29,30] developed an off-column ED method for the determination of various thiol compounds in CE. They used a Nafion joint to decouple the electric field and amalgated gold [29] or CoPC-modified carbon-paste electrodes [30] for detection.

In previous work we have shown that conductive carbon cement (CCC) is a suitable matrix material for the preparation of chemically-modified electrodes to be used in flowing solutions [16,23,31], giving a higher electrode stability than carbon paste. With CoPC-modified CCC electrodes thiols could be reproducibly determined in urine samples after separation by liquid chromatography [23]. Following up on the work of O'Shea and Lunte, in the study described in this paper CoPC-CCC microelectrodes were tested for the determination of underivatized cysteine compounds separated by CE. First, however, we report on an improvement of the palladium union we use to decouple the electric field. Since cysteine compounds are to be separated as positive ions, migrating in the same direction as the electroosmotic flow, the demands on the dead volume of a decoupler are very strict [14]. The dead volumes of two new types of the decoupler were determined by comparing the variances in off-column (UV) detection with that in on-column detection. The improved Pd-decoupler was used for off-column ED of reduced thiols using CoPC-modified CCC electrodes. The experimental conditions to separate cysteine, homocysteine and glutathione were studied. The determination of free and total cysteine in urine samples is shown.

2. Experimental

2.1. Apparatus

The field-decouplers were made from 6-mm diameter palladium rod. Fused-silica capillaries with an outer diameter of 375 μm and different internal diameters were obtained from Polymicro Technologies (Phoenix, AZ, USA). For off-column amperometric detection, the experimental set-up was as described before [16]. A Prince programmable injector for capillary electrophoresis, including a 30-kV high-voltage supplier was obtained from Lauer Labs (Emmen, Netherlands). The 75 μm I.D. separation capillary was 85 cm long. New capillary was etched with 1 *M* HCl for one hour before use unless stated otherwise. A 7-cm piece of poly ether ether keton (PEEK) tubing (Upchurch, Oak Harbor, WA, USA) with 63.5 μm I.D. and 0.5 mm O.D. was used as the coupling capillary in ED. A piece of PEEK tube (I.D. 0.5 mm, O.D. 1/16″) was used to construct CoPC-modified carbon working electrodes in the fashion described earlier for cuprous oxide modified microelectrodes

[16]. An Amor (Spark, Emmen, Netherlands) potentiostat/amplifier was used. The signals were registered with a HP 3394A integrator and a Kipp and Zonen strip-chart recorder. Samples were introduced hydrodynamically. The volume of sample loaded was calculated from the equation:

$$V_{inj} = \frac{\pi P_{inj} d_1^4 d_2^4 t}{128\eta(d_1^4 L_2 + d_2^4 L_1)} \quad (1)$$

where P_{inj} is the injection pressure, t the injection time, η the viscosity, and L_1, L_2 and d_1, d_2 are the lengths and internal diameters of the separation and the coupling capillaries, respectively.

For UV absorbance detection, a Spectra 100 variable-wavelength UV detector (Spectra-Physics) was used, and a 2-mm detection window was made by burning off the coating of the capillary.

2.2. Chemicals and solutions

All chemicals were used as received. Conductive carbon cement (CCC) was supplied by Gerhard Neubauer (Münster, Germany). Cobalt phthalocyanine (CoPC) and *dl*-homocysteine (HCYS) were obtained from Aldrich (Milwaukee, WI, USA), MES hydrate from Aldrich (Steinheim, Germany). 1-Naphthol (NOH) was purchased from BDH, 1-cysteine (CYS), 1-naphthalenemethylamine (NMA^+), 6-hydroxynaphthalenesulfonic acid ($HNSA^-$) from Merck, and 2-naphthol-3,6-disulfonic acid ($NDSA^{2-}$) from Fluka. Glutathione (reduced, GSH) and dithiothreitol (DTT) were obtained from Janssen Chimica. All other chemicals were analytical reagent grade. Concentrated stock solutions of NOH, NMA^+, $HNSA^-$ and $NDSA^{2-}$ in water or methanol were kept at 4°C, and diluted to 10^{-4} mol l^{-1} with buffer for injection. Stock solutions of thiols were prepared daily in buffer solutions and stored at 4°C and diluted to the desired concentrations before use. Both MES buffer and formate buffer contained 1 m*M* EDTA and 0.1 m*M* NaCl. Subboiled demineralized water was used to prepare solutions. Buffers were filtered through a Millipore membrane filter (0.45-μm pore size) and degassed with helium before used. Before each CE run, the inlet buffer vial was replaced with a vial filled with freshly degassed buffer, and the capillaries were washed with the buffer for 5 min.

2.3. Sample preparation

Fresh urine samples were diluted with the running buffer, filtered, and immediately subjected to injection. DTT-treated urine samples were prepared as follows. To 2 ml of urine sample, 1.8 ml of 0.1 *M* Na_2HPO_4 (pH 8.0) and 0.2 ml of 0.1 *M* DTT were added and mixed. After 15 min the mixture was centrifuged and 2 ml of supernatant was mixed with 2 ml of formate buffer and subjected to injection.

3. Results and discussion

3.1. Evaluation of Pd decouplers

The decoupler used in previous studies suffered from a relatively large dead volume (ca. 15 nl). Two new types of palladium decouplers were developed (Fig. 1). Type A has the same configuration as the reported one [15] but with a narrower connecting channel (100 μm). With

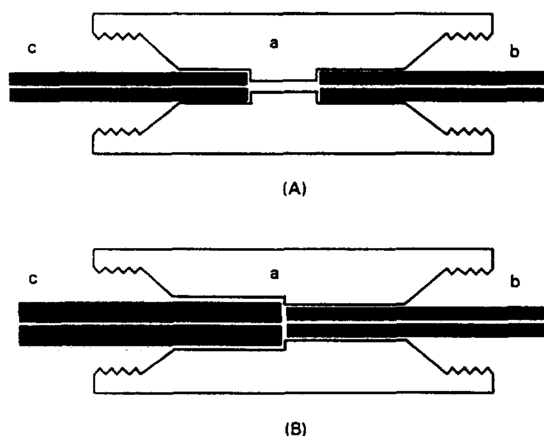

Fig. 1. Cross-section of the palladium decouplers type A and B. (a) Pd union; (b) separation capillary; (c) coupling capillary (not on scale).

type B the separation and coupling capillaries are positioned directly against each other, and electrical contact with the solution is made through the thin gap between them. These two decouplers were tested as described before [15]. The ability of the decouplers to dissipate the generated hydrogen was tested by measuring current–voltage relations. For both types of Pd decouplers, the electrophoretic current increased linearly with the applied voltage up to ca. 50 μA. At higher voltages the linearity was lost but currents up to 150 μA could be passed.

To determine the zone-broadening contribution of the decouplers, the zone widths of four differently charged naphthalene derivatives, obtained with on-column and off-column UV detection, were compared. With on-column detection a 85 cm × 75 μm I.D. capillary with the detection window at 60 cm was used. For off-column detection, the separation capillary was 60 cm × 75 μm I.D.. A 15 cm × 50 μm I.D. coupling capillary was used with a detection window at 10 cm from the decoupler. A compensating pressure of 100 mbar was used to eliminate the laminar backflow in the separation capillary. The results are given in Table 1. The difference between the calculated value of the zone variance and the experimental value obtained with off-column detection may be attributed to the contribution of the dead volume in the palladium decouplers. Typical dead volumes of 9 nl for type A decoupler and 7 nl for type B decoupler were found. The electroosmotic and electrophoretic mobilities from off-column detection match perfectly with those from on-column detection, which indicates that the calculation method [16] is reliable. In further experiments a decoupler of type B was used.

The influence of the applied high voltage on the noise level of off-column amperometric detection was investigated. As shown in Fig. 2A, a higher noise was obtained at higher voltages. It is not clear whether this is caused by an incomplete decoupling of the electrophoretic current or by the appearance of small hydrogen bubbles at high voltages. Interestingly, the noise on the electrophoretic current increases in a similar way with the applied voltage (Fig. 2B).

Table 1
Comparison of on-column detection and off-column detection[a]

Parameter	Mode	Decoupler type	Compound			
			NMA^+	NOH	$HNSA^-$	$NDSA^{2-}$
Elution time	On-column		185	248	376	560
(s)	Off-column	Type A	209	274	404	575
		Type B	208	272	402	590
Mobility	On-column		2.75	8.07[c]	−2.72	−4.47
(10^{-4} cm^2 V^{-1} s^{-1})	Off-column[b]	Type A	2.86	8.02[c]	−2.78	−4.53
		Type B	2.87	8.07[c]	−2.80	−4.57
Zone variance	On-column exp		0.41	0.76	1.37	5.06
σ^2 (s^2)	Off-column calc[d]		1.06	1.31[e]	2.04	5.87
	Off-column exp	Type A	1.82	3.1	3.50	6.60
		Type B	1.59	1.8	2.34	6.30
$\Delta\sigma$ (nl)		Type A	9.3	14.3[e]	7.2	9.1
		Type B	7.8	7.5[e]	5.8	7.0

[a] For experimental details, see text.
[b] Calculated from the equations of Ref. [16].
[c] Electroosmosis.
[d] The calculated variance contributions of the coupling capillary were added to the results of on-column detection.
[e] Diffusion coefficient estimated [14].

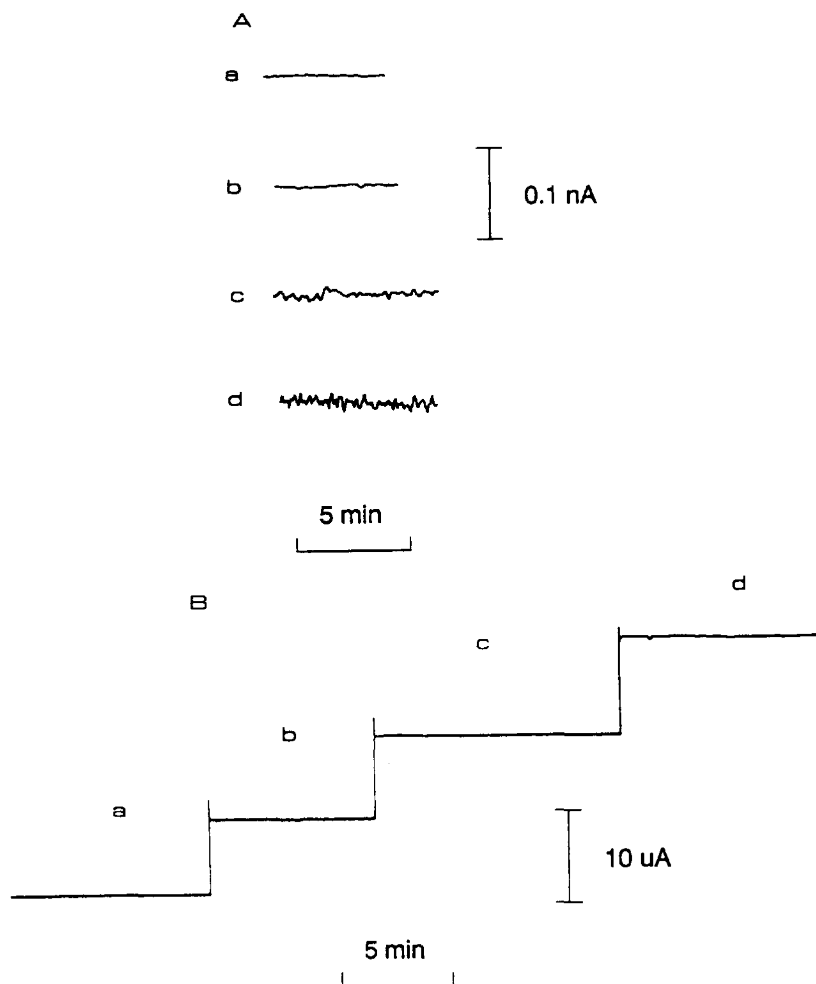

Fig. 2. (A) Influence of the applied voltage on the electrochemical detector baseline noise; (B) electrophoretic current trace at different applied voltages. (a) 0 kV; (b) 10 kV; (c) 20 kV; (d) 30 kV.

3.2. Detection and separation of thiols

In previous work CCC electrodes bulk-modified with CoPC have been used for detection of thiols in HPLC [23]. A miniaturized CoPC-CCC electrode, with an active surface of 0.2 mm^2, was constructed for ED in CE. Cyclic voltammetry was used to evaluate the response of the microelectrodes to CYS. As illustrated in Fig. 3, CYS can be oxidized at anodic potentials higher than +0.4 V with two anodic peaks. The mediated electrochemical process is identical to that observed with large-area (7 mm^2) electrodes. For detection in CE, a potential of +0.6 V vs. Ag/AgCl was chosen.

Although in the literature the separation of thiols using a MES buffer has been described [29,30], we have found no separation between CYS, HCYS and DTT with this buffer. In fact, at the pH of a MES buffer (pH 5.5–6.7), CYS and HCYS are close to their isoelectric point [32] and DTT is also neutral [33]. A baseline separation between the four model compounds was achieved using a formate buffer with pH 2.7, where the compounds are positively charged (see Fig. 4).

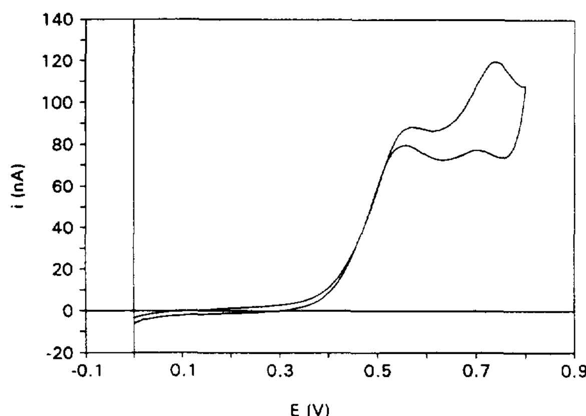

Fig. 3. Cyclic voltammogram of 1 mM cysteine in 5 mM formate buffer (pH 2.7) using a CoPC-modified micro-electrode. Scan rate: 20 mV s^{-1}.

At pH 2.7 an osmotic mobility of $(1.4 \pm 0.1) \cdot 10^{-8}$ m^2 V^{-1} s^{-1} was found using UV detection and phenol as neutral marker. It can be calculated [16] that with this osmotic mobility a compensating pressure of 3.5 mbar is required to

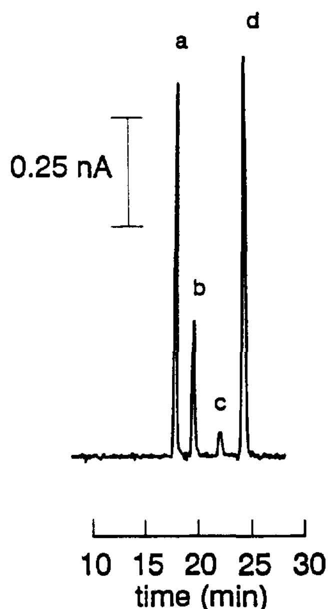

Fig. 4. Electropherogram of the separation of (a) homocysteine; (b) cysteine; (c) glutathione; (d) DTT. Concentrations: 10 μM for each compound. Buffer: 5 mM formate + 1 mM EDTA + 0.1 mM NaCl (pH 2.7); injection volume: 20 nl; applied voltage: 30 kV; P_{cmp}: 5 mbar; CoPC-CCC electrode operated at +0.6 V.

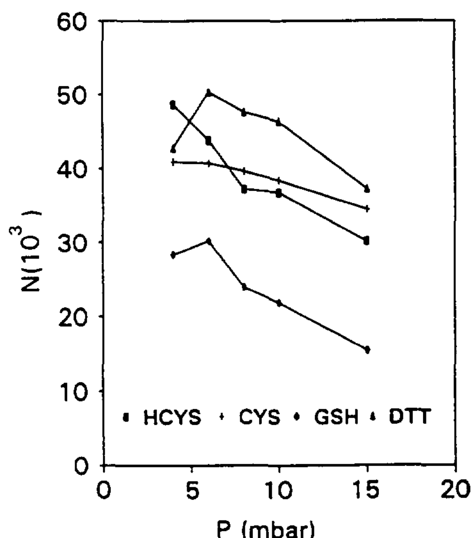

Fig. 5. Influence of the applied pressure on the observed plate numbers in formate buffer.

preserve the flat flow profile in the separation capillary. In Fig. 5 the influence of the applied compensation pressure on the observed plate numbers is shown. As has been pointed out before [14], the influence of P_{cmp} on the separation efficiency is not very profound for zones migrating in the same direction as the osmotic flow. Still, optimum values between 3 and 6 mbar were observed, with maximum plate numbers of approximately 50 000 for HCYS, CYS and DTT and 30 000 for GSH. Typical detection limits of 0.5 μM for HCYS, 1 μM for CYS and 5 μM for GSH were found. Calibration plots from 10 to 200 μM were linear ($r = 0.9967$). Although appreciable differences in sensitivity between individual electrodes were found, the within-day reproducibility using the same electrode was within 8%.

An example of the application of the method for the determination of free and total (after treatment with DTT) CYS in urine is shown in Fig. 6. In this particular urine sample a free concentration of 35 μM CYS was found, which is well within the normal range. The total concentration, after reduction of cystine and other cysteine-containing compounds [34,35], was found to be 201 μM (Fig. 6B).

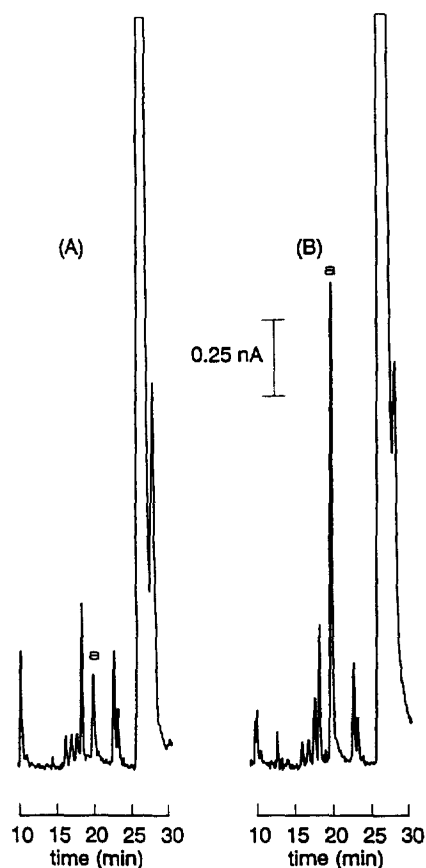

Fig. 6. Electropherograms of a urine sample (A) diluted with formate buffer and (B) treated with DTT. Total dilution factor 4. Peak (a): cysteine. Conditions as in Fig. 4.

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