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High-performance liquid chromatography of thiols with differential pulse polarographic detection of the catalytic hydrogen evolution current

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ABSTRACT

SH and SS groups in organic compounds were sensitively detected in NH_3–NH_4Cl (0.24 *M*)–Co^{2+} ($5 \cdot 10^{-4}$ *M*) solution by differential-pulse polarography (DPP). A new type of flow-through cell was developed and used to combine the polarograph with a high-performance liquid chromatograph. The experimental conditions for polarography and chromatography were studied and the effectiveness of the cell was also evaluated. The chromatogram obtained showed that this combined system works very well for the detection of thiols. The sensitivity was similar to that given by UV detection but was not handicapped by the wavelength. As a negative potential was used, the coexistence of other species did not cause large interferences.

INTRODUCTION

Thiols and corresponding disulphides constitute an important group of organosulphur compounds owing to their abundance and properties[1], but biochemical and environmental studies have been limited because of shortcomings in the existing analytical techniques[2]. For the determination of thiols, the usual method applied is chromatography with UV or fluorescence detection. The main disadvantage of UV spectrophotometric detection is its limited wavelength range and that of fluorimetric detection is that it detects only fluorescent substances. On the other hand, the linking of electrochemical detection with high-performance liquid chromatography (HPLC) has advantages for the measurement of various sulphur-containing compounds[3]. Shea[4] applied polarographic detection with a platinum electrode covered with thin mercury membrane to hydrophilic thiols in sediment pore water or marine water, the detection limit being 2 pmol. However, the electrode has to be checked every day and the mercury membrane peels off even in weakly alkaline

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solution. A dual-electrode detector was developed by Laura and Shoup[5]. Both thiols and disulphides can be detected by using the series mode. However, complete conversion of disulphides has to be ensured, which is difficult.

SH and SS groups can be sensitively detected by measuring the "Brdička current" in NH_3/NH_4Cl solution containing Co^{2+} or Ni^{2+} ions[6], but so far it has been seldom used with HPLC, mainly because of the difficulty of making a suitable flow-through cell for HPLC. The object of this study was to develop a new type of flow-through cell which is well suited for combining polarography with HPLC for the application of Brdicka current detection to the determination of SH groups.

EXPERIMENTAL

Chemicals

L-Cysteine, reduced glutathione, thiomalic acid, α-thioglycerol and mercaptoethanol were obtained from Tokyo Kasei (Tokyo, Japan), ammonia solution and solvents (HPLC grade) from Kanto (Tokyo, Japan), cobalt dichloride from Showa (Tokyo, Japan) and the ion-pair reagent, sodium trichloroacetate, from Nacalai Chemicals (Kyoto, Japan). The water used for the preparation of the mobile phase and electrolyte was deionized by passage through a mixed-bed resin in a Milli-Q laboratory water purification system (Millipore).

Equipment

The HPLC–differential pulse polarographic (DPP) system consisted of an SSC-3100-J pump (Senshu, Tokyo, Japan) and either a Model SSC-3000A-2 UV detector (Senshu) or a P-1100 polarographic analyser (Yanagimoto, Kyoto, Japan) equipped with a P-1000ST dropping mercury electrode (DME) stand. The controls were set as follows: current range, 5 μA/V; modulation amplitude, 50 mV; drop time, 1 s; and potential scan rate (when required), 5 mV/s.

Polarographic peaks were measured with a three-electrode system consisting of a platinum metal electrode, a saturated calomel reference electrode (SCE MR-P2) and a DME as the working electrode. The electrode characteristics were m (*i.e.* flow-rate of mercury) = 1.989 mg/s, pulse interval 50 ms and pulse sampling time 20 ms. All measurements were made at room temperature.

Peristaltic pumps were used to send carrier solution and Co^{2+} mixing solution into the flow-through cell via thin PTFE tubing (0.5 mm I.D.) and wide glass tubing (4 mm I.D.), respectively. The columns used were Capcell SG-ODS (250 mm × 4.6 mm I.D.), obtained from Shiseido (Tokyo, Japan), Senshu Pak ODS-N-1251 and Senshu Pak ODS-H-1251 (250 mm × 4.6 mm I.D.), obtained from Senshu and TSKgel-120T (200 mm × 4.6 mm I.D.), obtained from Tosoh (Tokyo, Japan).

Construction of flow-through cell

An effective flow-through cell must have a small effective determination volume[7], in other words, the volume around the DME should be as small as possible. As shown in Fig. 1, the newly designed flow cell was made with glass tubing of 4 mm I.D. There are three holes in the cell through which three electrodes (working, reference and counter) were inserted. From one end of the cell PTFE tubing of 0.5 mm I.D. was inserted exending to a position very close to the DME. Sample solution was introduced

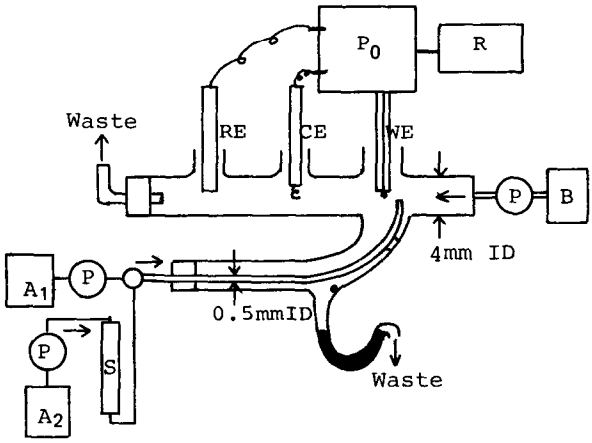

Fig. 1. Flow-through cell and HPLC–DPP system. A_1, B = NH_3–NH_4Cl–CH_3OH–Co^{2+} solution; A_2 = NH_3–NH_4Cl–CH_3OH solution; Po = polarograph; S = separation column; P = pump; RE = reference electrode; CE = counter electrode; WE = working electrode.

through this thin tubing and when it flowed out of the outlet (1 ml/min) it immediately diffused to the surface of the mercury drop and an electrode reaction occurred. The sample solution was carried away from the DME and removed from the cell by the carrier solution (2.5 ml/min) which was flowing in through the wide glass tubing which came from another inlet of the cell. Accumulated mercury supports the liquid line, which ensures the flow of carrier solution through the electrodes.

Because the outlet of thin tubing was very close to the electrode drop and because the inside diameter of the tubing was very small, the electrode reaction in fact occurred in a very small effective determination volume (*ca.* 3 μl). The volume could be hypothetically calculated using three values, the maximum radius of drop (0.33 mm), the distance between the drop and the tubing outlet (2.0 mm) and the radius of the tubing outlet (0.25 mm). In addition, as the glass tubing had an I.D. of 4 mm, turbulence did not occur.

Because the sample solution flows out upwards to the DME, there was a larger surface area for receiving diffusing sample ions than in the previous design, which received sample ions diffused from only one side. The structure was beneficial for enhancing the intensity of current detected.

This flow cel could be easily made in the laboratory and is believed to be applicable to other electrodes such as the hanging mercury drop electrode or the mercury membrane- covered platinum electrode. Moreover, the flow cell could be easily connected to the HPLC column.

HPLC–DPP procedure

Mobile phase solution (solution A_2) (1.0 ml/min), carrier solution (solution B) (2.5 ml/min) and Co^{2+} mixing solution (solution A_1) (0.5 ml/min) are pumped into the flow-through cell to obtain a stable baseline, then the sample solution is injected into the mobile phase. After separation, the compounds immediately diffuse to the surface of DME and the Brdička current is recorded. Waste solution and mercury are drained

from the other two outlets of the cell. Because the concentrations of common components of solutions A_1, A_2 and B are identical, the electrode reaction is not affected by the heterogeneous mixing.

RESULTS AND DISCUSSION

Brdička current and Brdička reaction

The Brdička current is a catalytic hydrogen evolution current given by SH and SS groups in NH_3–NH_4Cl solution in the presence of Co^{2+}. It is characterized by double waves with a special peak form in a negative potential range and by the extreme sensitivity for the determination of SH and SS groups. The Brdička current of dithiouracil in the DPP mode is shown in Fig. 2.

The Brdička reaction can be represented as follows[8]:

$$[Co^{2+}(RS^-)_2] + 2e \rightarrow [Co^0(RS^-)_2]^{2-} \text{ (electrode reaction)}$$
$$[Co^0(RS^-)_2]^{2-} + 2NH_4^+ \rightarrow [Co^0(RSH)_2]^0 + 2NH_3 \text{ (chemical reaction)}$$
$$[Co^0(RSH)_2]^0 + 2e \rightarrow [Co^0(RS^-)_2]^{2-} + H_2 \text{ (electrode reaction)}$$

In the Brdička reaction, the second electrode reaction produces the double waves. $[Co^0(RSH)_2]^0$ acts as a catalyst and NH_4^+ provides protons. For the investigation of the Brdička reaction, we studied seven kinds of SH-containing compounds. They showed two potential ranges for producing a Brdička current, -1.20 to -1.30 V and -1.50 to -1.70 V (potentials corresponding to the top of the peaks). No compound was detected in the range -1.35 to -1.45 V. This result coincided with that for proteins containing SH groups. The detection potential of the thiols are given in Table I.

pH and concentrations of NH_4^+ and Co^{2+} ions

As discussed, the formation of active $[Co^0(RSH)_2]^0$ by the reaction between $[Co^0(RS^-)_2]^{2-}$ and NH_4^+ ions lead to the production of NH_3, which can partially escape from the solution. As a result, the reaction can proceed continuously. The Brdička reaction in fact occurs in a weakly alkaline solution and a certain concentration of NH_4^+ is required. The effect of pH an $[NH_4^+]$ on the Brdička reaction were investigated and the results are shown in Fig. 3. It can be seen that the strongest

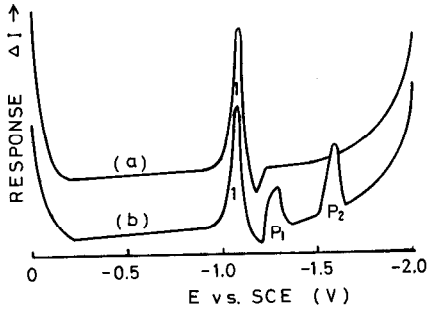

Fig. 2. Differential-pulse polarograms of dithiouracil obtained batchwise. (a) 0 *M*; (b) $1 \cdot 10^{-5}$ *M*. I = Reduction current of Co^{2+}; P_1, P_2 = Brdička current of dithiouracil.

TABLE I

DETECTION POTENTIALS (E_p) AND PEAK HEIGHTS (P) FOR SOME THIOLS

Thiols	E_{p1} *(V vs SCE)*	E_{p2} *(V vs SCE)*	P_1 *(mm)*	P_2 *(mm)*
Dithiouracil	−1.30	−1.60	36	121
Thionalide	−1.25	−1.50	150	88
Thiomalic acid	−1.25	−1.55	17	38
L-Cysteine	−1.30	−1.59	79	98
α-Thioglycerol	n.d.[a]	−1.70	0	84
2-Meraptoethanol	−1.20	−1.60	15	55
Glutathione	−1.25	−1.60	80	23

[a] Not detected.

Brdička current is obtained when the pH is 9.47 and $[NH_4^+]$ is 0.265 M. The results were obtained by detecting thionalide; other thiols showed small differences in the intensity of the current at the same concentration but gave similar curves. Fig. 3 also shows the effect of Co^{2+} concentration on the intensity of the Brdička current. Similar to reports[6,8] on proteins containing SH groups, the current intensity increased with increase in the concentration of Co^{2+}. At Co^{2+} concentrations above $5 \cdot 10^{-4}$ M, the current intensity showed no noticeable increase. A Co^{2+} concentration of $5 \cdot 10^{-4}$

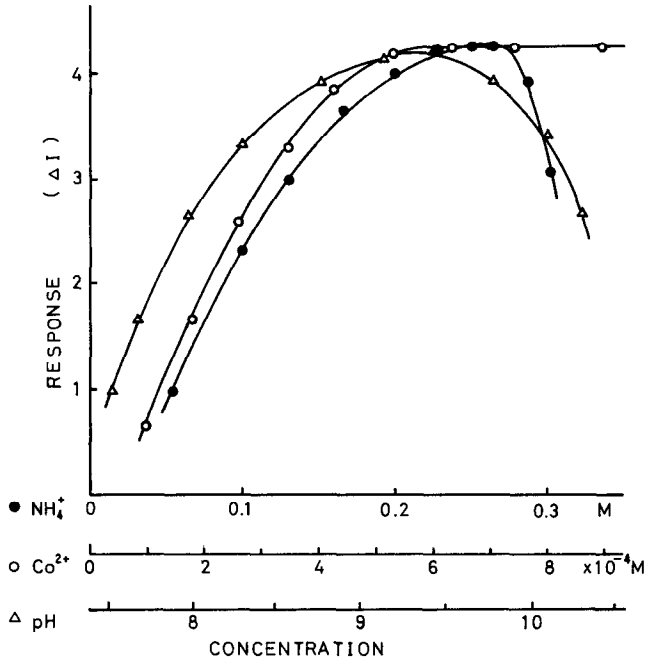

Fig. 3. Effects of (●) $[NH_4^+]$, (○) $[Co^{2+}]$ and (△) pH on response of thionalide. $1 \cdot 10^{-5}$ M thionalide in 20% ethanol solution.

M was therefore selected for use. As with pH and $[NH_4^+]$, the effects of $[Co^{2+}]$ for all the thiols studied are very similar.

The detection limit for thionalide was $2 \cdot 10^{-9}$ *M* under the optimum experimental conditions.

Separation of thiols

In studies of the separation of thiols by HPLC, most workers have concluded that reversed-phase columns and an acidic pH were appropriate[3,9–13]. Accordingly, we selected reversed-phase columns for thiol separation. In this case, the pH has to be considered, because a pH of 9.47 was needed for electrochemical detection.

To investigate the applicability of the HPLC–DPP analytical system, we chose several thiols with different retention times. We first used a general ODS column coupled with a UV detector, and found that even though only water was used as the mobile phase, thiols could be separated very well. Mixing of the eluent with Co^{2+} solution was carried out after separation because Co^{2+} has strong absorption properties which may lead to peak tailing. The results showed that Co^{2+} greatly affected the Brdička reaction and reliable polarograms could not be obtained. Accordingly, the electrolyte solution was used as the mobile phase.

The addition of Co^{2+} ions was carried out after separation and was found to have no effect on the Brdička reaction. Because an alkaline mobile phase was used, a general ODS column could not be used, so a Capcell SG-ODS column, which can be used over a wide pH range (2–10), was used. With this column and mobile phase, good separations were obtained. The capacity factors (k') decreased very little in comparison with those obtained with water as the mobile phase and a general ODS column.

Improvement of separation and peak shapes

Methanol and an ion-pair reagent [trichloroacetate (TCA)] were added to the mobile phase and their effects on k' and the Brdička reaction were studied. It was found that even a very small amount of methanol (0.1%) increased k' considerably, but at methanol concentrations above 0.5% k' began to decrease. The addition of methanol had no effect on the Brdička current. A concentration of 0.5% v/v) methanol was therefore selected. The ion-pairing reagent TCA had no effect on the separation or the Brdička reaction of thiols so it was not subsequently employed.

The effect of flow-rate was als investigated. In our HPLC–DPP flow system, with three flow lines the most important is the flow-rate of the mobile phase because the tubing for the HPLC system and the tubing inserted in the cell are very narrow (I.D. 0.25 and 0.5 mm). A small increase in flow-rate will therefore lead to a large increase in linear velocity, which has considerable effects both on mixing of the eluent with Co^{2+} solution and diffusion of the sample to the electrode surface. It was found that at mobile phase flow-rates above 1.2 ml/min the Brdička current decreased substantially and the peak width increased. At flow-rates below 1.0 ml/min, good diffusion of sample to the electrode could not be obtained, which led to a decrease in the intensity of the Brdička current. Hence a mobile phase flow-rate of 1.0 ml/min was selected and, to correspond with this flow-rate, 0.5 and 2.5 ml/min were selected for the Co^{2+} mixing solution and carrier solution, respectively.

A chromatogram of an aqueous solution containing thiomalic acid, L-cysteine, α-thioglycerol and 2-mercaptoethanol is shown in Fig. 4. The chromatogram shows

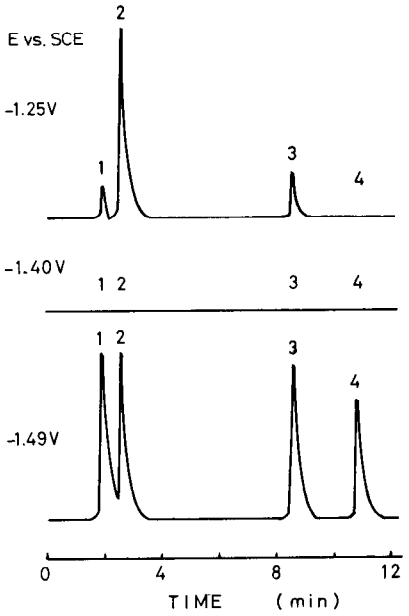

Fig. 4. Chromatograms obtained with the HPLC–DPP system with detection potentials of −1.25 V (top), −1.40 V (centre) and −1.49 V (bottom). Peaks: 1 = thiomalic acid; 2 = L-cysteine; 3 = α-thioglycerol; 4 = 2-mercaptoethanol. Concentration of each thiol, $1 \cdot 10^{-5}$ *M*; Sample size, 10 l. Column: Capcell SG-ODS (250 mm × 4.6 mm I.D.). Mobile phase (A_2): NH_3–NH_4Cl (0.24 *M*)–CH_3OH (0.5%), 1.0 ml/min. Co^{2+} mixing solution (A_1): NH_3–NH_4Cl (0.24 *M*)–CH_3OH (0.5%)–Co^{2+} ($1.5 \cdot 10^{-4}$ *M*), 0.5 ml/min. Carrier solution (B): NH_3–NH_4Cl (0.24 *M*)–CH_3OH (0.5%)–Co^{2+} ($5 \cdot 10^{-4}$ *M*), 2.5 ml/min.

a good separation of these four thiols. It is interesting that a change in detection potential leads large changes in the chromatogram. When the potential was −1.49 V, all four thiols could be detected. When −1.25 V was used, the peak height of L-cysteine did not change, but those of thiomalic acid and α-thioglycerol decreased considerably and the Brdička current of 2-mercaptoethanol could not be detected. When a detection potential of −1.40 V was used, no Brdička current for any of the thiols could be detected.

DPP can be used as a more selective method than other polarographic modes. Very small changes in the of detection potential have a large effect on the peak height and the intensity of the Brdička current. Although it is difficult to obtain the maximum Brdička current for each thiol in the sample mixture at a fixed detection potential, the latter can be adjusted to obtain the strongest Brdička current for a particular thiol.

The chromatograms shown in Fig. 5 were obtained by UV (254 nm) detection and with the HPLC–DPP system. Studies showed that glutathione has no absorption at 254 nm, but at a detection potential of −1.25 V it could be detected by the HPLC–DPP system. This means that the HPLC–DPP system can detect thiols or disulphides which have no UV absorption but give a Brdička current. Of course, at this potential (−1.25 V), mercaptoethanol could not be detected, as discussed with regard to Fig. 4. A detection potential of −1.49 V was also used, but glutathione gave a very small peak, the Brdička current intensity at −1.25 V being only one quarter of that at −1.49 V.

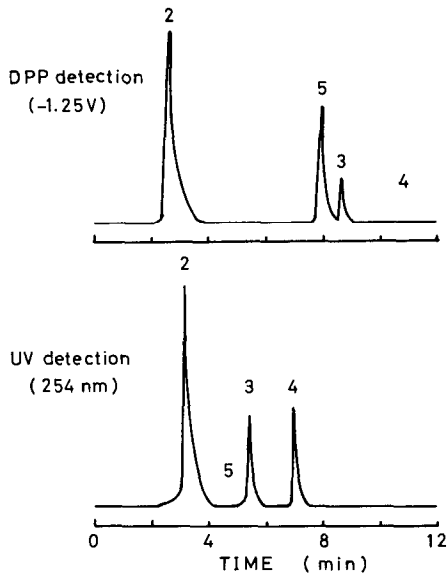

Fig. 5. Chromatograms of thiols with (top) DPP and (bottom) UV detection. Peaks: 2–4 as in Fig. 4; 5 = glutathione. Experimental conditions for DPP detection as in Fig. 4. For UV detection: column, TSK gel-120 T (250 m × 4.6 mm I.D.); mobile phase, CH_3OH (1%), 1.0 ml/min; concentration of each thiol, $1 \cdot 10^{-5}$ *M*; sample size, 10 μl.

CONCLUSION

In this basic investigation of the use of a Brdička current measurement cell in HPLC detection, it was clearly shown that thiols can be detected selectively, and the sensitivity is comparable to that of UV detection. Further, it was shown that the Brdička current can also differentiate thiol by selection of an appropriate applied potential. This system is expected to have wide application in biological, clinical and environmental chemistry.

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