Journal of Chromatography, 455 (1988) 336–343
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 837

Note

Simultaneous determination of thiols and corresponding disulphides in mixtures by high-performance liquid chromatography with polarographic detection using a Dual-Tast Analyser

BARBARA DĘBOWSKA and KRYSTYNA SAMOCHOCKA*
Department of Chemistry, Warsaw University, Pasteura 1, 02-089 Warsaw (Poland)
and
MAREK PRZASNYSKI* and DANUTA SYBILSKA
Institute of Physical Chemistry, Polish Academy of Sciences, Kasprzaka 44, 01-224 Warsaw (Poland)
(First received May 16th, 1988; revised manuscript received July 18th, 1988)

Reduced glutathione (GSH), cysteine (CSH) and the corresponding disulphides, *i.e.*, oxidized glutathione (GSSG) and cystine (CSSC) form important biological systems. Their rôle in living processes has stimulated the interest of analysts and various procedures have recently been reported for their determination; they are mainly chromatographic methods. Due to the scarcity of sensitive detectors for disulphides, the latter are usually determined as thiols after precolumn reaction, thus the most of the elaborated methods do not distinguish thiols from disulphides. Very often, however, such determinations are not sufficient and more specific data are desirable, *i.e.*, describing the equilibria between thiols and disulphides. The following two different approaches have recently been designed to resolve this problem by high-performance liquid chromatography (HPLC) with electrochemical detection.

Allison *et al.*[1,2] used an amperometric detector with dual thin-layer mercury–gold amalgam electrodes. After chromatographic separation of all species, disulphides were converted into the corresponding thiols at an upstream electrode and were then detected as thiols at a downstream electrode via their oxidation of the mercury surface. The upstream electrode serves as a postcolumn reactor so only the downstream electrode current need be monitored.

We have applied polarographic detection with a DC-Tast polarograph at two suitably chosen potentials of the dropping mercury electrode (DME)[3]. Species of interest (cysteine and cystine) were separated on a reversed-phase (RP) column (according to the ion-pair mechanism) and the first cystein eluted was detected at the DME (at +0.25 V *vs.* silver–silver chloride) according to the anodic reaction

$$2CSH + Hg \rightarrow Hg(CS)_2 + 2H^+ + 2e^- \quad (1)$$

* Present address: 40, Powsińska Str., 02-903 Warsaw, Poland.

0021-9673/88/$03.50 © 1988 Elsevier Science Publishers B.V.

while subsequently eluted cystine was reduced after a rapid shift of the potential of the DME to −0850 V *vs.* silver–silver chloride, the reaction:

$$CSSC + 2H^+ + 2e^- \rightleftarrows 2CSH \quad (2)$$

For quantitation of the species, both currents, *i.e.*, oxidation and reduction, should be recorded. Due to the continuously renewed mercury surface, the reproducibility of this simple method is satisfactory and its sensitivity comparable to that attained with chemical derivatization and fluorescence detection[4]. The main disadvantage arises from the change in potential during the chromatogram. Besides the difficulties of automatization, it implies the need for chromatographic separation of both species, which should be better than baseline separation. To avoid this inconvenience, for the present work we applied a specially constructed supplementary apparatus, a Dual-Tast. This enables automatic measurements of both anodic and cathodic currents on each mercury drop.

EXPERIMENTAL

Reagents

L-Cysteine puriss was obtained from Fluka (Buchs, Switzerland) and L-cystine, reduced and oxidized glutathione from Reanal (Budapest, Hungary). All other reagents were of analytical reagent or laboratory grade and were used without further purification.

Apparatus

Chromatographic experiments were performed using a Type 302 liquid chromatograph equipped with a Type PP2 flow-through detector, a Type PPW1 DC-Tast polarograph coupled with a Dual Tast Analyser. All the above instruments were constructed at the Institute of Physical Chemistry of the Polish Academy of Sciences (Warsaw, Poland). The DC-Tast mode presents several advantages as compared with the DC one, particularly with respect to detection in flow-through systems[3,5]. These advantages can sometimes be enlarged by coupling a DC-Tast polarograph with a Dual-Tast Analyser. A more detailed description of the apparatus mentioned above was given previously[5]. In the present work a simplified scheme of measurements is given. Chromatograms were registered with a Type TZ 4200 dual pen recorder (Laboratorni Přistroje, Prague, Czechoslovakia). Stainless-steel columns (250 mm × 4 mm I.D. and 100 mm × 4.6 mm I.D.) slurry packed with 10-μm LiChrosorb RP-8 (Merck, Darmstadt, F.R.G.) were used for separation.

The Dual-Tast Analyser performs automatic measurements of the current on each mercury drop at two different potentials suitably chosen and at the delay times chosen in advance. Detection is performed at the naturally dropping electrode.

The key principles of the measurements are shown in Fig. 1. Fig. 1A shows the changes in potential applied to the DME *versus* time, t. Two different potentials, E_1 and E_2, chosen for tast measurements in this work, correspond to the oxidation and the reduction of thiols and disulphides at the DME, according to eqns. 1–4 and the polarograms exemplified in Fig. 2.

The corresponding changes in current due to the electrode reaction of thiols and

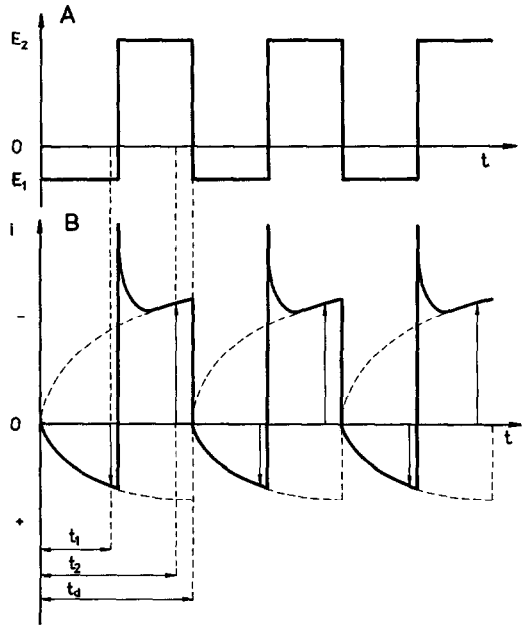

Fig. 1. Measurement scheme using a DC-Tast polarograph equipped with a Dual-Tast Analyser. (A) Changes of potential *versus* time; (B) corresponding changes of the current due to the electrode reaction of thiols and disulphides.

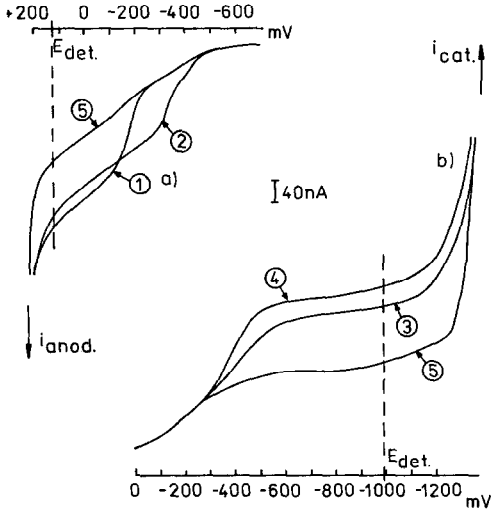

Fig. 2. Polarographic curves of (a) $2 \cdot 10^{-5}$ *M* reduced glutathione (1), $2 \cdot 10^{-5}$ *M* cysteine (2) and supporting electrolyte (5) and (b) 10^{-5} *M* cystine (3), 10^{-5} *M* oxidized glutathione (4) and supporting electrolyte (5) 0.03 *M* phosphate buffer pH 3.5. Reference electrode: SCE.

disulphides are shown in Fig. 1B; t_d is the natural drop lifetime, t_1 and t_2 are the delay times for the first (at E_1) and second (at E_2) measurements respectively counted from the drop fall. The full line corresponds to the current flowing through the detector when potentials E_1 and E_2 are applied successively to each mercury drop. The dotted line represents the current flowing when only one potential, namely E_1 or E_2, is applied. The measured values of the current are marked in Fig. 1B with arrows.

The first tast measurement of the current is performed at the potential E_1 after the delay time t_1. Then the potential of the DME is automatically adjusted to E_2 and after the delay time t_2 (counted from the drop fall) the second measurement is carried out.

The sampling time (*ca.* 5 ms) is not shown in Fig. 1 because this value is very small compared with t_1, t_2 and t_d (*ca.* 2 s). The times t_1 and t_2 should be as long as possible under the following conditions: (a) $t_1 < t_2 < t_d$; (b) $(t_2 - t_1)$ should be longer than the recharging time of the double layer after the change of potential; (c) the time necessary for the detection of the drop fall $(t_d - t_2)$ should be at least 50 ms.

In order to detect the drop fall the sinusoidal voltage ($U_0 \sin\omega t$, $U_0 = 20$ mV, $f = 3.5$ kHz, not shown in Fig. 1) is superimposed just after the second measurement of the current at E_2.

The disappearance of the alternating current with drop fall changes the electrode potential to E_1.

In the case of a reversible polarographic process, a shadow chromatographic peak of the same retention as the main component was detected at the second potential (E_2). This is due to the compound produced at the electrode at the first potential (E_1) if E_2 corresponds to the electrode reaction of the compound produced.

Procedure

All measurements were carried out at 25 ± 0.2°C and at a constant flow-rate of 0.8 or 1.0 ml/min.

The aqueous mobile phase containing 0.03 *M* phosphate buffer of various pH and various concentrations of D-camphor-10-sulphonic acid (CSA) (Merck, Schuchard) as a pairing ion and methanol were deareated with argon. Samples (5 μl) injected on the column contained oxygen or were deoxygenated by the simple procedure described by Lloyd[6]. Chromatograms were obtained by monitoring simultaneously two currents: anodic (at +0.250 V *vs.* silver–silver chloride) and cathodic (at −0.850 V *vs.* silver–silver chloride). In order to avoid the appearance of small additive peaks due to the products of the reversible reactions 2 and 3

$$GSSG + 2H^+ + 2e^- \rightleftarrows 2GSH \tag{3}$$

it was necessary to hold the DME first at a potential of +0.250 V corresponding to the irreversible reactions 1 and 4

$$GSH + Hg \rightarrow GSHg + H^+ + e^- \tag{4}$$

and then change it to −0.85p V. In the case of the opposite sequence (first potential −0.850 V, second +0.250 V) it was possible to detect the trace amounts of the products of the reversible reduction reactions 2 and 3, *i.e.*, CSH and GSH respectively in the second step at +0.25 V.

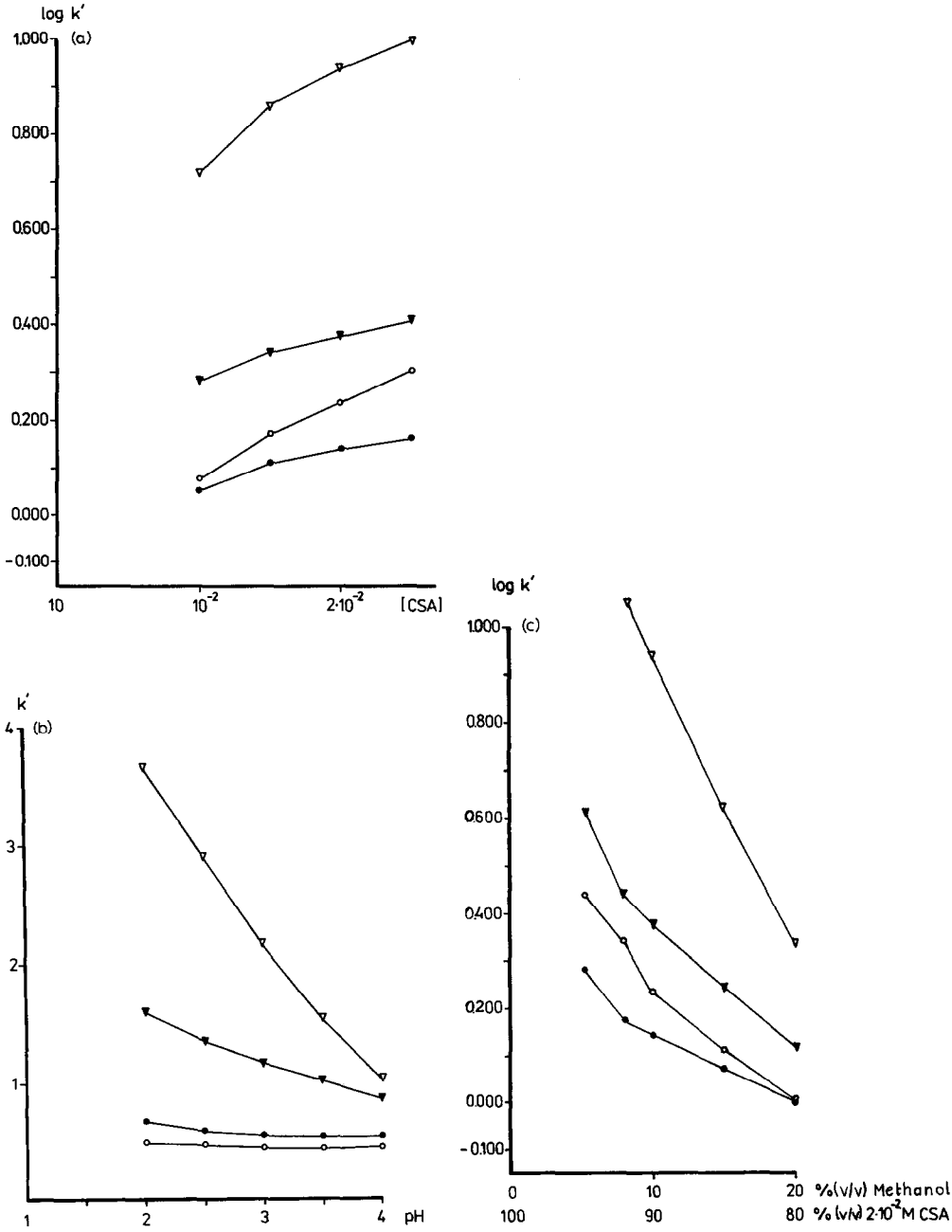

Fig. 3. (a) Plots of the logarithms of capacity factors, k', of cysteine (●), cystine (○), reduced glutathione (▼) and oxidized glutathione (▽), *versus* CSA concentration. Stationary phase: 10-μm LiChrosorb RP-8. Mobile phase: 0.03 *M* phosphate buffer with 10% (v/v) methanol, pH 2. (b) Plots of k' *versus* pH of the mobile phase (0.03 *M* phosphate buffer). Stationary phase: 10-μm LiChrosorb RP-8. (c) Plots of the logarithms of k' *versus* % of methanol in the mobile phase: $2 \cdot 10^{-2}$ *M* CSA in 0.03 *M* phosphate buffer pH 2. Stationary phase: 10-μm LiChrosorb RP-8.

Polarographic curves of pure cysteine, cystine and reduced and oxidized glutathione were obtained with a saturated calomel electrode (SCE) as the reference elctrode, and for the detection in chromatography a silver–silver chloride electrode was used, $V_{Ag/AgCl} - V_{SCE} = 139$ mV.

RESULTS AND DISCUSSION

Fig. 3 shows the changes in capacity factors of investigated solutes with pH and CSA and methanol concentrations. Each k' value was determined with a relative standard deviation of 0.3%.

It has already been stated that better than baseline separation of CSH and CSSC can be achieved at high CSA concentration and at low pH, *i.e.*, at $2 \cdot 10^{-2}$ *M* CSA and pH 2.0[3]. However, as the plots in Fig. 3 demonstrate, under these conditions the retention of GSH and GSSG strongly increases, prolonging the analysis time and worsening the detectability. Small amounts of methanol may suppress this effect to some extent, but the Dual-Tast Analyser was designed to avoid these difficulties. To exemplify how it operates, two chromatographic systems using mobile phases of different compositions (I and II) were chosen: I, $2 \cdot 10^{-2}$ *M* CSA, 0.03 *M* phosphate buffer pH 2 and 10% (v/v) methanol in water; II, 0.03 *M* phosphate buffer pH 3.5–methanol (90:10).

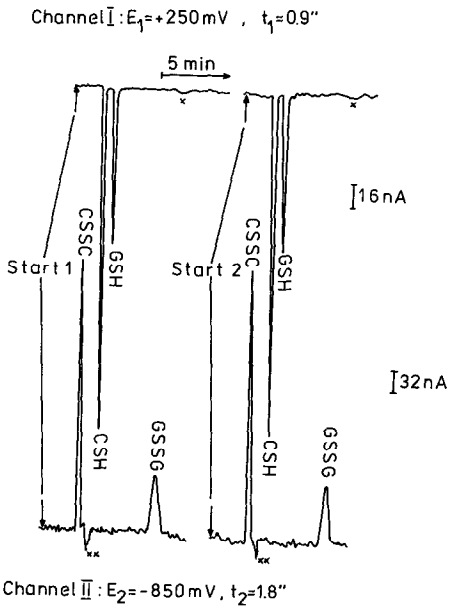

Fig. 4. Two consecutive chromatograms of a mixture of $2 \cdot 10^{-4}$ *M* CSH, $2 \cdot 10^{-4}$ *M* GSH, 10^{-4} *M* CSSC and 10^{-4} *M* GSSG. Mobile phase: $2 \cdot 10^{-2}$ *M* CSA in 0.03 *M* phosphate buffer pH 2 with 10% (v/v) methanol. Column (100 mm × 4.6 mm I.D.): 10-μm LiChrosorb RP-8. Flow-rate: 1 ml/min. Samples deoxygenated. Drop time: $t_d = 2.15$ s. ×, Unidentified peak accompanying oxidized glutathione of retention equal to that of DSSC; × ×, unidentified peak accompanying reduced glutathione of retention equal to that of GSH.

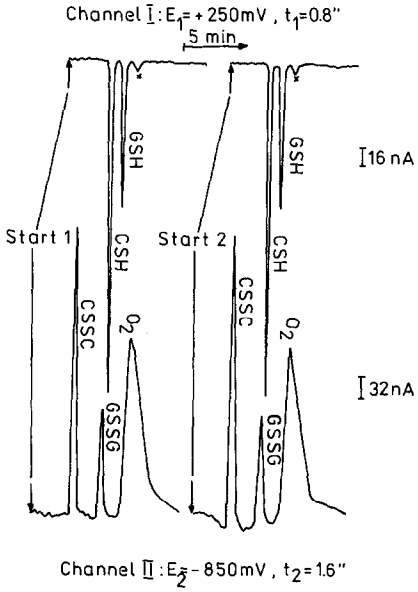

Fig. 5. Two consecutive chromatograms of a mixture of $2 \cdot 10^{-4}$ *M* CSH, $2 \cdot 10^{-4}$ *M* GSH, 10^{-4} *M* CSSC and 10^{-4} *M* GSSG. Mobile phase: 0.03 *M* phosphate buffer pH 3.5 with 10% (v/v) methanol. Column (250 mm × 4 mm I.D.); 10-μm LiChrosorb RP-8. Flow-rate: 0.8 ml/min. Samples containing oxygen. Drop time: t_d = 2.08 s.

The corresponding chromatograms demonstrating different resolutions, different analysis times and also different detectabilities are shown in Figs. 4 and 5. Mobile phase solution I results in almost baseline resolution of CSH and CSSC and complete separation of GSH and GSSG within *ca.* 10 min (Fig. 4). The samples should be deaerated because elution of oxygen is partly superimposed on the cystine peak. With mobile phase II, during *ca.* 6 min baseline separation of GSH and GSSG is observed but the resolution of CSH and CSSC is then poor (Fig. 5). Oxygen forms a separate peak and thus the sample must not be deaerated.

As seen in Figs. 4 and 5, each species can be determined both in the case of complete and poor resolution. The detection limits, defined as the amount of injected substance giving a signal twice that of the baseline noise, are collected in Table I.

TABLE I

DETECTION LIMITS OF INVESTIGATED THIOLS AND DISULPHIDES IN TWO CHROMATOGRAPHIC SYSTEMS

Compound	*Detection limit (pmol)*	
	System I	*System II*
Cysteine	4	4
Cystine	18	17
Reduced glutathione	9	10
Oxidized glutathione	82	43

A linear dependence of the peak height on concentration up to 10^{-3} *M* for CSH, GSH, GSSG and up to $5 \cdot 10^{-4}$ *M* for CSSC was found.

In all, the analysis time in system II presenting the poor resolution is almost half that of system I with complete separation. The detectability achieved for GSSG is also twice as good.

The Dual-Tast Analyser seems to be an efficient tool for rapid determinations of such redox systems as sulphides. It may also be useful in the case of other poorly resolved compounds differing significantly in their polarographic behaviours.

REFERENCES

1 L. A. Allison and R. E. Shoup, *Anal. Chem.*, 55 (1983) 8.
2 L. A. Allison, J. Keddington and R. E. Shoup, *J. Liq. Chromatogr.*, 6 (1983) 1975.
3 D. Sybilska, M. Przasnyski, B. Mysior and K. Samochocka, *J. Chromatogr.*, 283 (1984) 453.
4 J. D. H. Cooper and D. C. Turnell, *J. Chromatogr.*, 227 (1982) 158.
5 M. Przasnyski, J. Piecek and W. Kutner, *Nauch. Apparat., Scientific Instrumentation*, 2 (1987) 21–38.
6 J. B. F. Lloyd, *J. Chromatogr.*, 256 (1983) 323.