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Note

Electrophoretic separation and thiomercurimetric monitoring of ionogenic thiols

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The thiomercurimetric monitoring of thiols has been successfully applied in high-performance liquid and gas chromatography[1]. In this paper, the selective detection of ionogenic thiols after their separation by means of disc electrophoresis on polyacrylamide gel is reported.

Several types of apparatus for continuous separation and elution by electrophoresis have been described[2–4]. They operate according to the same principles and differ in some details of the construction of the elution chambers and the types and means of fixing the membranes. Our assembly consists of an electrophoretic apparatus, a new version of an elution chamber and a thiomercurimetric detector. The free blue dithiofluorescein released in the reaction of the *o*-hydroxymercuribenzoic acid–dithiofluorescein complex with thiols is monitored by means of a flow photometer connected with a recorder. Test separations have been carried out using thioglycolic acid, 2-mercaptopropionic acid, N-acetylcysteine and glutathione.

EXPERIMENTAL

Reagents and materials

The following were used: acrylamide (Merck, F.R.G.), N,N′-methylenebisacrylamide (Bis) (BDH, U.K.), thioglycolic acid (TG) (Fluka, Switzerland), 2-mercaptopropionic acid (2-MPA) (Fluka), N-acetylcysteine (Ac-Cys) (Fluka), reduced glutathione (GSH) (Reanal, Hungary), *o*-hydroxymercuribenzoic anhydride (POCh, Poland), dithiofluorescein (POCh), antifoam emulsion (Schuchardt, F.R.G.), ammonium persulphate, N,N,N′,N′-tetramethylenediamine (TEMED), boric acid (BA) and triethanolamine (TEA).

Indicator solution

The *o*-hydroxymercuribenzoic acid (HMB) solution was prepared by mixing 3.2 g of *o*-hydroxymercuribenzoic anhydride with 10 g of triethanolamine disolved in 50 ml of water and diluting to 1 l. In another vessel, 0.2 g of dithiofluorescein and 5 g of triethanolamine were dissolved in 50 ml of water and the solution was filtered. To 20 ml of the HMB solution were added 40 g of triethanolamine and the mixture was diluted to 150 ml with water. To this solution was slowly added dithiofluorescein

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solution until a distinct blue-red colour was obtained, then the mixture was diluted to 1 l with water. The solution obtained was about 10^{-4} *M* with respect to the HMB–dithiofluorescein complex, the pH being 9.5. The indicator solution should be stored in darkness at 0–5°C.

Buffer solutions were prepared from triethanolamine and boric acid; their compositions are given with the experimental results. Prior to the introduction of the indicator solution and eluting buffer to the pumps, they were degassed on an ultrasonic bath with antifoam emulsion added.

Equipment

The apparatus for electrophoresis and monitoring of thiols is shown in Fig. 1. Gel tube I-2 and elution chamber I-3 were cooled by water using thermostat VIII. Cooling jacket I-1 consisted of two screwed connecting tubes (QS-24) and two screw caps I-4 (SQ-24/12; Jobling, U.K.). Internal cooler I-6 and cooling coil I-9 were used for cooling the buffer solutions in the electrode reservoirs I-5 and I-8, respectively.

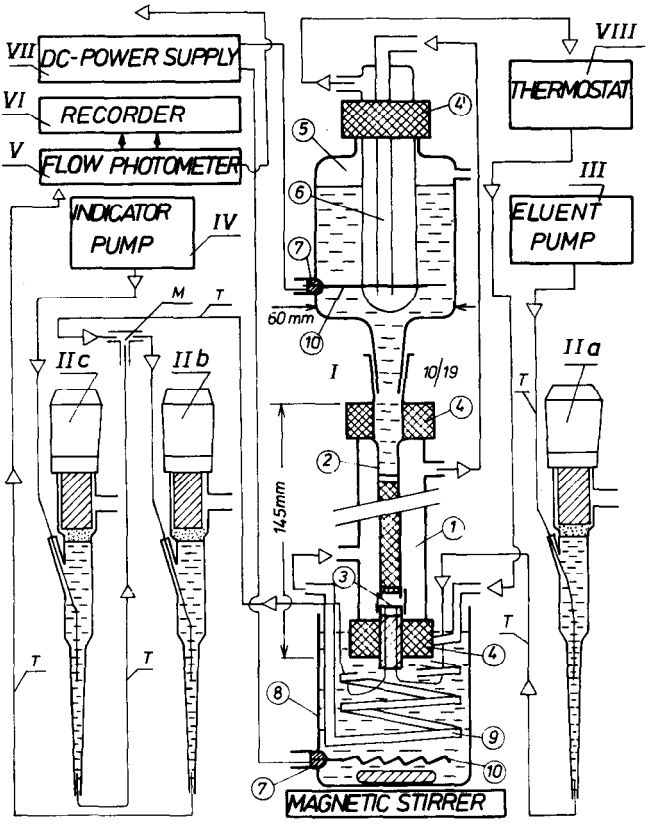

Fig. 1. Assembly for electrophoresis and monitoring of thiols. I, Electrophoresis apparatus: 1, cooling jacket; 2, separation gel tube; 3, elution chamber; 4, screw caps; 5, upper electrode buffer reservoir; 6, cooler, 7, Wood's alloy; 8, lower electrode buffer reservoir; 9, cooling coil; 10, platinum electrodes. IIa, b and c, degassing units; III, eluent pump; IV, indicator pump; V, flow photometer; VI, recorder (TZ-4100, Czechoslovakia); VII, d.c. power supply (Z-5001, Poland); VIII, ultrathermostat. The thiomercurimetric detector consists of IV → IIc → IIb → V → VI.

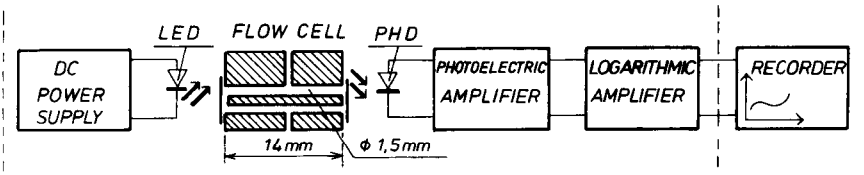

Fig. 2. Schematic diagram of the flow photometer.

Two pulseless syringe pumps III and IV taken a Type 302 liquid chromatograph (Poland) delivered the eluting and indicator solutions. Degassing units IIa, b and c with capillary endings were made from TFG/18 Rotaflo (Jobling). Pump III delivered the eluting buffer through the degassing unit IIa into the elution chamber I-3. Pump IV delivered the indicator solution through the degassing unit IIc. The buffer from the elution chamber was mixed with the indicator solution at point m. The combined solutions passed through the degassing unit IIb into the flow cell of the photometer V. Resolution results were obtained in the form electropherograms registered on the recorder VI.

The photometer (Fig. 2) used for registering the dithiofluorescein bands was equipped with a Type H flow cell. The light source was an electroluminescence diode (LED) emitting yellow light of wavelength 590 nm. The light receiver was a photodiode (PHD) with an operating spectral range of 450–100 nm. The photometer had a band range of 2–0.1 a.u.f.s.; 1 a.u.f.s. corresponds to 10 mV at the exit from the logarithmic amplifier.

Details of the construction of the elution chamber are shown in Fig. 3. The retaining gel consists of a mixture of 25% (w/v) of glass beads and 20% (w/v) of acrylamide–N,N'-methylenebisacrylamide (5%) in a buffer containing 30 g of BA

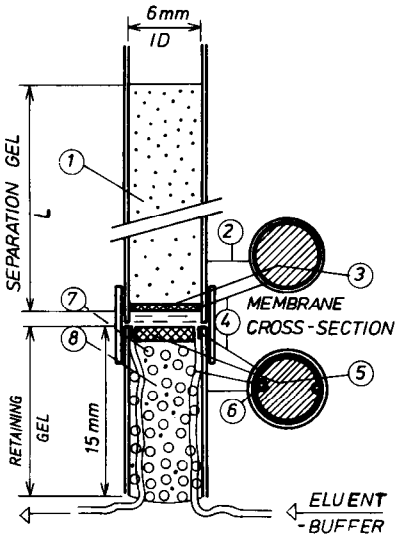

Fig. 3. Design of the elution chamber. 1, Separation gel; 2, thin-walled glass tube; 3, sintered-glass disc, upper membrane; 4, glyptal resin; 5, sintered-glass disc, lower membrane; 6, PTFE capillary tubes, 0.4 mm I.D.; 7, glass beads; 8, polyacrylamide gel.

and 10 g of TEA per litre. All polymerizations were carried out in the presence of TEMED and ammonium persulphate.

RESULTS

Figs. 4 and 5 show the results of calibrating the monitoring system. The calibration was performed as follows: on a short column of polyacrylamide gel were introduced with a Hamilton syringe samples of thioglycolic acid prepared in a buffer–glycerine solution (1:1, v/v). The samples were introduced under the surface of the electrode buffer after removing the upper reservoir I-5 (Fig. 1). The electrophoretic signals obtained allowed a calibration graph to be plotted (Fig. 5).

Figs. 6 and 7 show representative electrophoretic separation of a test mixture containing thioglycolic acid, 2-mercaptopropionic acid, acetylcysteine and glutathione. The test mixture was slightly saturated with uncontrolled amount of hydrogen sulphide prior to submitting the samples to electrophoretic separations, because of the possibility of the pre-concentrations of thiols from real samples through mercaptides and the recovery of free thiols by the action of hydrogen sulphide. All separations were carried out under the following constant conditions: detector range, 1 a.u.f.s.; recorder sensitity, 10 mV; chart speed, 0.01 mm sec^{-1}; and temperature of the cooling water, 15°C.

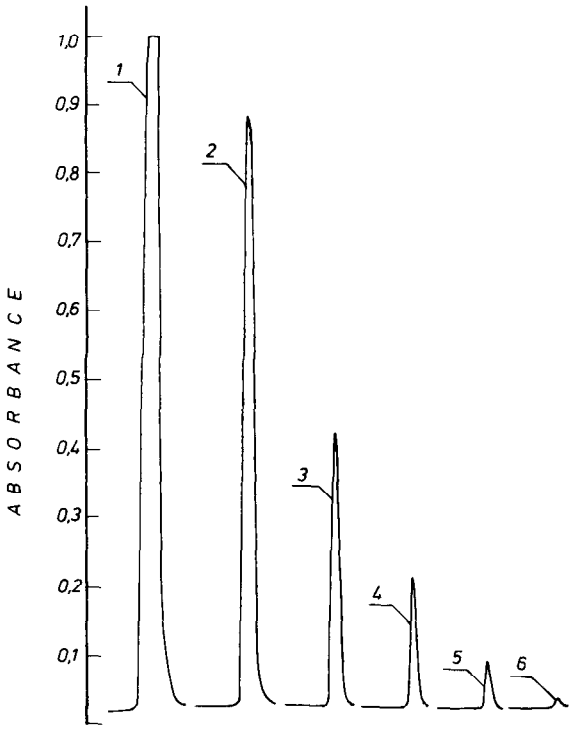

Fig. 4. Standardization of the detector by means of thioglycolic acid. Peak numbers and μmoles of TGA: (1) 0.5; (2) 0.25; (3) 0.125; (4) 0.0625; (5) 0.0312; (6) 0.0156. Sample volume, 20 μl; 15% polyacrylamide gel, length 2 cm; separation and electrode buffers, 10 g/l of TEA + 30 g/l of BA (pH 6.9).

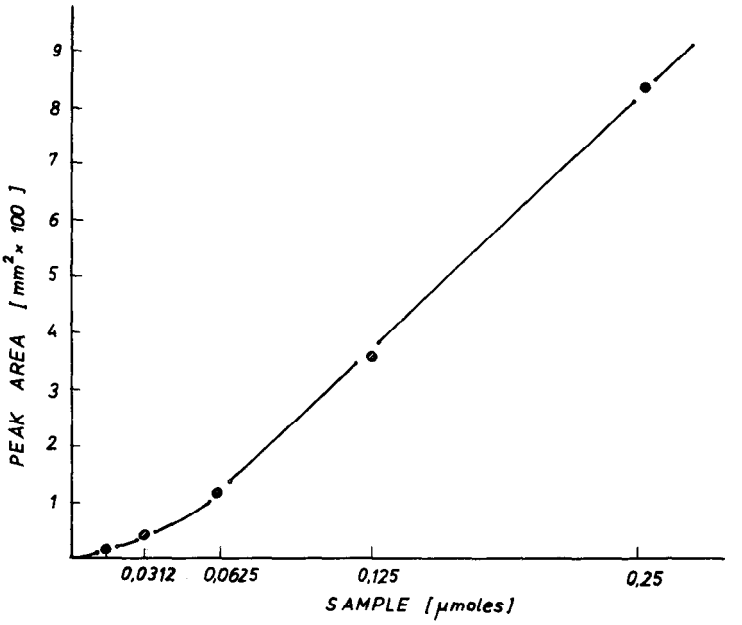

Fig. 5. Calibration graph for thioglycolic acid.

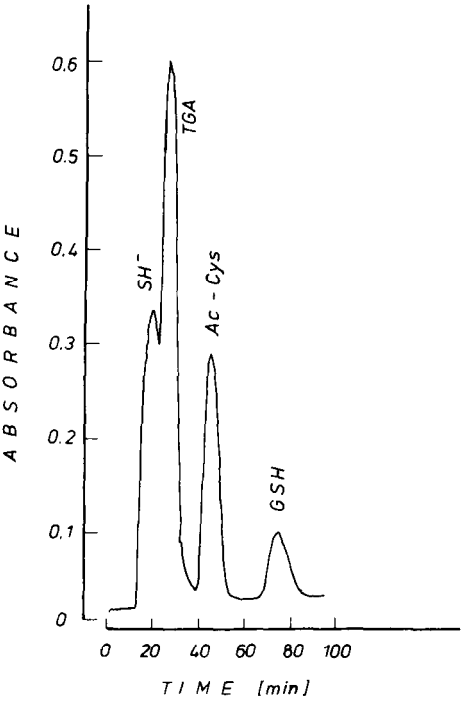

Fig. 6. Electrophoretic separation of the test mixture on 15% polyacrylamide gel (length 5 cm). Thioglycolic acid (0.14 μmole, N-acetylcysteine (0.09 μmole), glutathione (0.1 μmole) and hydrogen sulphide; sample, 20 μl; separation and electrode buffers, 20 g/l of TEA, 20 g/l of BA (pH 7.8); 350 V, 3.4 mA.

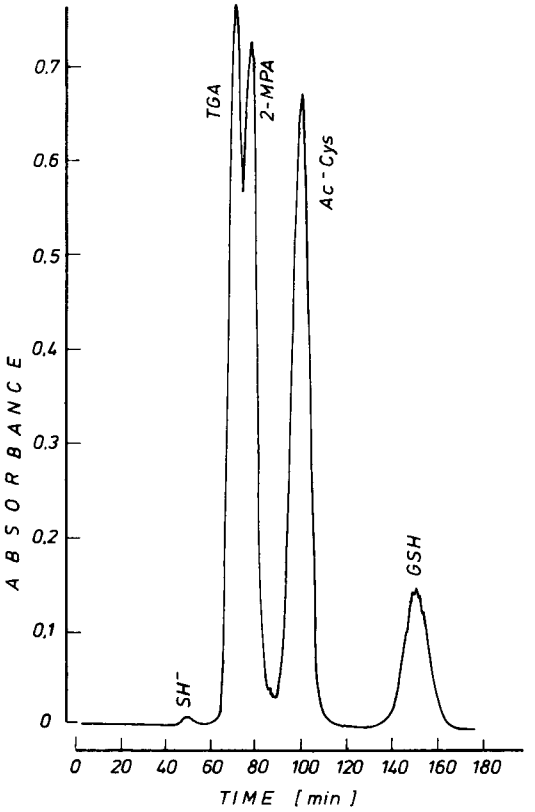

Fig. 7. Electrophoretic separation of the test mixture on 20% polyacrylamide gel (length 5 cm). Thioglycolic acid (0.12 μmole), 2-mercaptopropionic acid (0.12 μmole), N-acetylcysteine (0.15 μmole), glutathione (0.15 μmole) and hydrogen sulphide; sample, 15 μl; separation and electrode buffers, 10 g/l of TEA, 45 g/l of BA (pH 6.5); 405 V, 4.8 mA.

DISCUSSION

For continuous separation and elution by electrophoresis, major attention has been paid to the sealing membranes and increasing the mechanical resistance of the elution chamber. The application of a light-emitting diode has simplified the construction of the flow photometer, which suggests the possibility of using such diodes as light sources in similar types of equipment. A disadvantage of analytical system based on flow photometers is the occurrence of gas bubbles in stream of liquid passing through the flow cell of the photometer. To eliminate this inconvenience, three-stage degassing of solutions has been used (Fig. 1, IIa, b and c). The dead volume of the detector increased slightly only in unit IIb. The calibration results suggest the use of an internal standard.

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