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Determination of cysteine by capillary zone electrophoresis with end-column amperometric detection at a gold/mercury amalgam microelectrode without deoxygenation

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Abstract

Capillary zone electrophoresis was employed for the determination of cysteine using an end-column amperometric detection with a gold/mercury amalgam microelectrode, at a constant potential of 0.02–0.06 V vs. saturated calomel electrode. In this procedure deoxygenation is not necessary. The electrochemical characteristics at the microelectrode, the effect of the concentration of the buffer and the separation voltage across the capillary on the migration time and separation efficiency, and the dependence of the injection voltage and time on the detection signal, the separation efficiency and coulometric efficiency has been investigated. The calibration plot was found to be linear over four orders of magnitude and the limit of detection was $5.8 \cdot 10^{-8}$ mol/l (or 14. 5 amol). The method was applied to the determination of cysteine in human plasma, blood and urine.

Keywords: Electrochemical detection; Detection, electrophoresis; Electrodes; Cysteine; Amino acids

1. Introduction

Cysteine is an important amino acid in protein structure. It has been determined by using liquid chromatography–electrochemistry with a mercury, gold or a chemically modified electrode containing cobalt phthalocyanine at a carbon paste electrode [1–3]. As the mercury electrode is used, cysteine reacts with mercury at low oxidation potential and the determination of cysteine is therefore very selective [1].

Capillary zone electrophoresis (CZE) has been introduced as a highly efficient separation technique for ionized solutes [4–6]. Amperometric detection with a microelectrode is one of the most sensitive detection techniques for CZE separation [7–9]. O'Shea et al. [10] used an off-column amperometric detector to determine cysteine with a gold/mercury amalgam microelectrode in 0.01 mol/l 2-(*N*-morpholine)-ethanesulfonic acid (pH 5.5). In their experiments, for deoxygenation, argon was bubbled through the solution for 20 min prior to run and passed into the anodic buffer as well as passed over the cathodic buffer. The technique of detection of cysteine has two disadvantages: first, this kind of amperometric detector needs a conductive joint between the separation capillary and the detection capillary, and the working microelectrode must be inserted into the detection capillary. Secondly, the

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buffer has to be deaerated prior to and during each run.

In this paper we will present the results of end-column amperometric detection of cysteine in Na_2HPO_4–NaH_2PO_4, by CZE using a gold/mercury amalgam microelectrode without a conductive joint or deoxygenation. This method has been used to determine cysteine in human plasma, blood and urine.

2. Experimental

2.1. Apparatus

The instrumentation was as described previously [11]. In cyclic voltammetry, a commercial polarograph (Model 83-2.5, Ningde Analytical Instruments, China) coupled with an x–y recorder (Model 3086-11, Yokogawa Hokuskin, Japan) was used in connection with a cell, using potentiostatic control of the electrode potential by means of a three-electrode system. In CZE, a reversible high-voltage power supply (Beijing Institute of New Technique, China) provided a variable voltage of 0–30 kV across the capillary with the outlet of the capillary at ground potential. Fused-silica capillaries (300 μm O.D.×25 μm I.D.) were purchased from Yongnian Optical Conductive Fiber Plant, China. The amperometric detection at a constant potential with CZE was performed using the end-column approach with a microcurrent voltammeter (Model 901-PA, Ningde Analytical Instruments, China). The detection cell and detector were housed in a faradaic cage in order to minimize the interference from external sources of noise. Electrochemical detection was carried out with a three-electrode system, which consisted of a gold/mercury amalgam microelectrode as the working electrode, a saturated calomel electrode (SCE) as the reference electrode and a coiled Pt wire as the auxiliary electrode, which also served as the ground for the potential drop across the capillary.

The gold/mercury amalgam microelectrode was constructed using a 100 μm diameter, ca. 1.5 cm long gold wire, which was carefully inserted through a ca. 9 cm×1 mm I.D.×2 mm O.D. glass capillary until it protruded approximately 1 cm from the end and sealed using epoxy resin. The gold wire was connected to a copper lead via a mercury junction. A piece of fused-silica capillary (8 mm×150 μm O.D.) was put outside the fine gold wire and epoxy resin was applied to the two ends of the fused-silica capillary to seal the fine gold to it under a microscope. The gold wire was cleaned with 1:1 (v/v) HNO_3, water, ethanol and double distilled water, and was dipped into pure mercury for approximately 2 min. These gold/mercury amalgam electrodes were washed with double distilled water.

2.2. Reagents and solutions

A 0.01 mol/l stock solution of L-cysteine was prepared by dissolving an appropriate amount of L-cysteine (content>98.5%, Shanghai Kangda amino-acid factory, China) in 0.02 mol/l Na_2EDTA of pH 3 and was stored at 4°C in a refrigerator. Dilute solutions were obtained by serial dilution of the stock solution with water. Other reagents were of analytical grade. All solutions were prepared with double distilled water.

2.3. Procedure

In cyclic voltammetry, after the solutions was deaerated with pure nitrogen, the cyclic CZE voltammograms were recorded. In CZE, the gold/mercury amalgam electrode was cemented onto a microscope slide, which was placed over an xyz micromanipulator and glued in place, so that the end containing the exposed gold/mercury amalgam protruded from the edge of the slide. The position of the gold/mercury amalgam electrode was adjusted (under a microscope) against the end of capillary, so that the electrode and the capillary were in contact. The other end of the capillary was inserted into a plastic syringe tip (the metal needle was removed before) and glued in place with a small amount of epoxy glue. Before each run, the capillaries were flushed with double distilled water for 2 min and then with the corresponding separation electrolyte by means of a syringe. During the experiments the separation voltage was applied across the capillary and the detection potential was applied at the working electrode. After the electroosmotic current reached a constant value (after about 5 min), the electromigration injection was carried out and the

electropherogram was recorded. The separation electrolyte in the capillary was replaced after five runs. All potentials were measured vs. SCE.

2.4. Preparation of human plasma, blood and urine samples

2.4.1. Plasma sample

Fresh human blood (2 ml) was collected in a 5-ml centrifuge tube and centrifuged at 1722 *g* for 20 min to separate erythrocytes. A 800-μl volume of the supernatant liquid and the same volume of precipitating agent solution of protein containing 2 mol/l $HClO_4$ and $4 \cdot 10^{-3}$ mol/l Na_2EDTA were mixed in another centrifuge tube. The mixture was allowed to stand for 10 min to precipitate the proteins. The sample was centrifuged at 1195 *g* for 10 min. In 200 μl of the supernatant liquid, 20 μl of 1 mol/l NaOH was added and the solution was adjusted to pH 7. Then 300 μl of 0.02 mol/l Na_2HPO_4–NaH_2PO_4 buffer (pH 7) was added. After the sample solution was homogenized,, the sample solution was injected into the CZE system by electromigration.

2.4.2. Blood sample

A 1-ml volume of a solution containing $4 \cdot 10^{-3}$ mol/l Na_2EDTA and 2 mol/l $HClO_4$ and 1 ml of fresh blood were combined in a 5-ml centrifuge tube, causing the erythrocytes to lyse and protein to precipitate. The tube was vortexed briefly. After standing for 10 min to precipitate proteins, the mixture sample was centrifuged for 10 min at 1195 *g*. A 20-μl volume of 1 mol/l NaOH was added into 200 μl of the supernatant liquid to adjust the solution to pH 7. Then 300 μl of 0.02 mol/l Na_2HPO_4–NaH_2PO_4 was added prior to injection into the CZE system by using electromigration.

2.4.3. Urine sample

A 100-μl volume of a solution containing $4 \cdot 10^{-3}$ mol/l Na_2EDTA_4 and 2 mol/l $HClO_4$ and 500 μl of fresh urine were combined in a 5-ml centrifuge tube, causing the proteins to separate. After standing for 5 min to precipitate proteins, the sample was centrifuged for 10 min at 1195 *g*. The supernatant liquid of 100 μl was adjusted to pH 7 by 1 mol/l NaOH and 500 μl of 0.01 mol/l Na_2HPO_4–NaH_2PO_4 (pH 7) was added. After homogenizing, the sample solution it was injected into the CZE system by electromigration.

3. Results and discussion

3.1. Characteristics of the gold/mercury amalgam microelectrode.

The gold/mercury amalgam microelectrode was constructed by a 100 μm diameter, ca. 1.5 cm long gold wire, which was carefully inserted through a ca. 9 cm×1 mm I.D×2 mm O.D. glass capillary until it protruded approximately 1 cm from the end. Using a microscope, epoxy resin was then applied to the junction of the glass capillary to seal the fine gold wire to it (Fig. 1a). The gold wire was connected to a copper lead via a mercury junction (Fig. 1b). In order to protect the glass capillary, during cyclic voltammetry a glass tube was put outside the glass capillary, which was bonded to the glass tube at the two ends of the glass tube using epoxy resin (Fig. 1c). During CZE a piece of fused-silica capillary (8 mm×150 μm I.D.) was put outside the fine gold

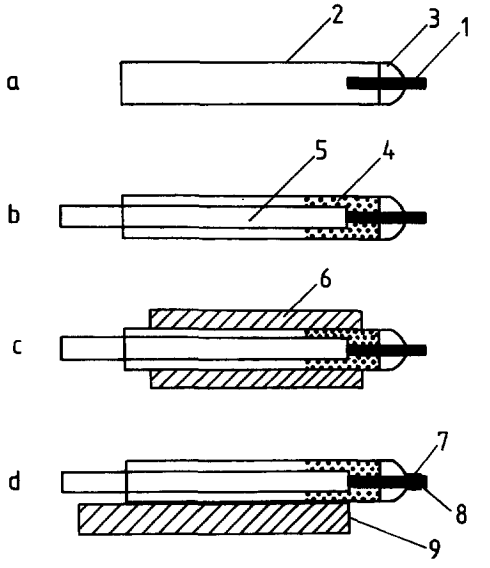

Fig. 1. Manufacturing process of the gold microelectrode: 1, gold wire; 2, glass capillary; 3, epoxy resin; 4, mercury; 5, copper wire; 6, glass tube; 7, fused-silica capillary; 8, gold/mercury amalgam; 9, microscope slide.

wire and epoxy resin was applied to the two ends of the fused-silica capillary to seal the fine gold to it under a microscope (Fig. 1d). The gold electrode was wiped lightly with emery paper (Number 06, Shanghai Emery Wheel Factory, China) first. The gold electrode was then washed with 1:1 (v/v) HNO_3, water, ethanol and double distilled water. The gold/mercury amalgam was prepared by dip-coating, in which the gold wire was dipped into the pure mercury for approximately 2 min. These gold/mercury amalgam electrodes were washed with double distilled water again.

After the microelectrode was prepared, a period of equilibration was necessary during which the response of the microelectrode was unstable and the background current was noisy. It was found that if a voltage of −0.1 to 0.1 V is applied to the fresh microelectrode, the period of equilibration can be shortened from 12 h reported in literature [10] to 10 min in our experiments. If the electrode is exposed to air for longer time, its surface will be oxidized and the electrochemical characteristics will deteriorate. Cyclic voltammograms for cysteine with a freshly prepared microelectrode, with a microelectrode exposed in air for 10 h, and with a microelectrode used for longer time are illustrated in Fig. 2. After the microelectrode has been used over ten or more runs, its electrochemical noise gets larger and the signal of the response is getting smaller (Fig. 2, curve 3). Dark spots on the surface of the microelectrode can be observed under the microscope, because the electrode is contaminated by impurities. In this case if 0.1 V potential is applied to the microelectrode for 2 min in the supporting electrolyte and then the microelectrode is immersed in double distilled water overnight, the microelectrode can be used again. Usually, the lifetime of the microelectrode is approximately 1 week. Then the microelectrode has to be renewed. There are two methods to renew the microelectrode. The first is dip-coating after removing the old mercury film which can be accomplished by immersing the microelectrode in concentrated nitric acid for 2 s, and then reapplying Hg as described in Section 2. The second is dip-coating after washing the microelectrode which can be performed by inserting the microlectrode in doubly distilled water for several hours and then immersing the microelectrode in pure mercury for 2 min. With the first method there is a small diminution in response current which may be attributed to the decrease in microelectrode size resulting from the removal of substrate gold. Therefore, the second method is used.

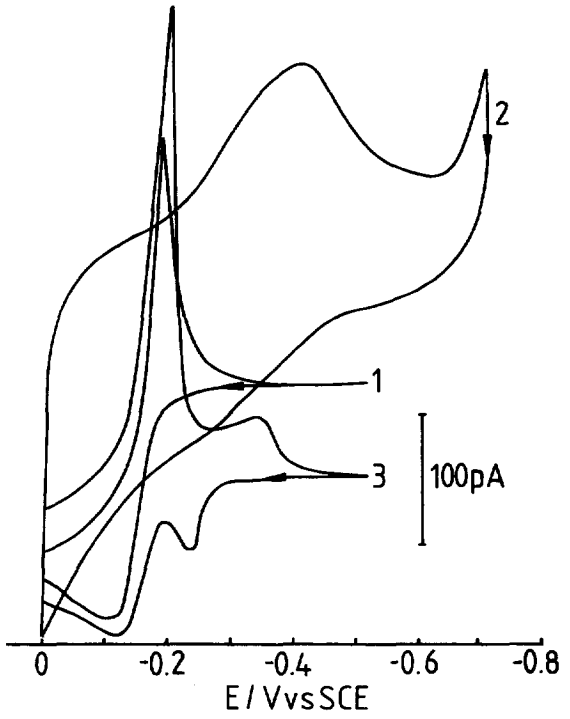

Fig. 2. Cyclic voltammograms of cysteine at the gold/mercury amalgam microelectrode; 0.02 mol/l Na_2HPO_4–citric acid (pH 2.4); $2 \cdot 10^{-4}$ mol/l cysteine; $v = 100$ mV/s. 1, The fresh electrode; 2, the electrode exposed in air for 10 h; 3, the electrode contaminated.

3.2. Effect of oxygen

Since the oxidation of cysteine is catalyzed by trace metals and occurs faster at high pH, all stock solutions were prepared in a buffer of pH 3 containing 0.02 mol/l Na_2EDTA. It was also found that the cysteine did not oxidize during the CZE run because of the short run time and the presence of Na_2EDTA.

Usually, oxygen in the solution is reduced at the mercury electrode, which produces the detection noise. It can be seen that when the potential is more negative than 0 V, the reduction current of oxygen is

observed; the more negative the potential, the higher the current is. When the potential is more positive than 0 V, the current is zero. Therefore, when the detection potential more positive than 0 V is applied, the deoxygenation is not necessary. It makes the procedure much simpler.

3.3. Cyclic voltammograms of cysteine

The typical linear sweep cyclic voltammograms of cysteine in solutions of different pH are shown in Fig. 3. In solutions of Na_2HPO_4, with NaH_2PO_4 or citric acid at different pH, cysteine can be oxidized at the gold/mercury amalgam microelectrode. If the detection potential is more positive than 0 V the cysteine can be determined in all three solutions. Of these solutions the 0.02 mol/l Na_2HPO_4–NaH_2PO_4, (pH 7) buffer is the best for the detection signal, detection limit and separation efficiency. In the following sections, therefore, this solution is chosen.

3.3.1. Optimum conditions of CZE amperometric detection

The length of the microelectrode is an important parameter which influences the signal of detection obtained in the end-column amperometric detector, which is dependent on the coulometric efficiency, Q_e, of the oxidation reaction. Coulometric efficiencies are calculated as the fraction of the moles of analyte detected over the moles of analyte injected. The amount of analyte detected is estimated from peak areas.

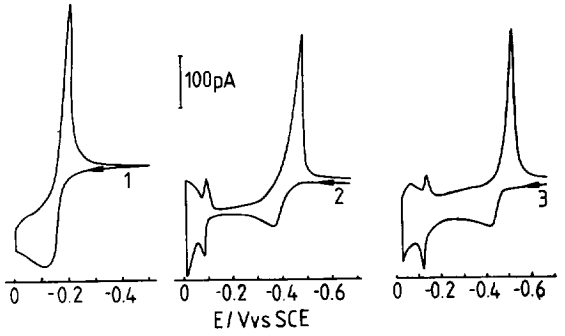

Fig. 3. Cyclic voltammograms of cysteine in solutions of different pH: 1 = 0.02 mol/l Na_2HPO_4–citric acid (pH 2.4); 2 = 0.02 mol/l Na_2HPO_4–NaH_2PO_4 (pH 7); 3 = 0.02 mol/l Na_2HPO_4–NaH_2PO_4 (pH 8); v = 100 mV/s.

The peak current, i_p, and the electric charge q detected on the electropherograms and the coulometric efficiencies Q_e at different length of the microelectrode, L, are listed in Table 1. When $L>0.2$ mm, i_p, q and Q_e maintain constant. When a disk electrode ($L=0$ mm) was used, i_p and Q_e are only $\frac{1}{16}$ and $\frac{1}{12}$ of the values of $L>0.2$ mm, respectively. The noise of detection also depends on the length of the microelectrode. The noise currents, i_n, and the ratio of signal to noise, i_p/i_n, are listed in Table 1, too. The noise current increases with increasing the length of the microelectrode. However, the maximum of the signal-to-noise ratio lies at $L=0.2$ mm. So the length of $L=0.2$ mm is chosen in subsequent experiments.

In end-column amperometric detection, the peak current detected is critically dependent on the proper alignment of the microelectrode with the bore of the capillary. In order to guarantee the reproducibility, before each experiment the alignment must be checked with following steps:

First the alignment was made under the microscope. Then the elute curve of $2\cdot10^{-5}$ mol/l cysteine was recorded. The place of the microelectrode was adjusted by using the laboratory-made *xyz* micromanipulator again. The elute curve was recorded again. The manipulation went on until the steady baseline, the best peak shape and the highest peak current were obtained.

Effect of the concentration of the buffer, c_B, on the migration time, t_m, the number of theoretical plates, N and i_p in Na_2HPO_4–NaH_2PO_4, is shown in Fig. 4, t_m, N and i_p increase with increasing c_B. However,

Table 1
Peak currents detected, i_p, and electric charge, q, on the electropherograms, and coulometric efficiencies, Q_e, noise current, i_n, and signal-to-noise ratio, i_p/i_n, at different lengths of the microelectrode, L

L (mm)	i_p (pA)	$10^{10}q$ (C)	Q_e (%)	i_n (pA)	i_p/i_n
1.0	405	6.07	62.2	1.5	270
0.5	396	5.96	61.1	0.8	490
0.2	400	6.03	61.8	0.7	571
0.1	330	4.95	50.7	0.65	508
0.05	178	3.03	31.0	0.5	356
0	25	0.51	5.2	0.4	63

0.005 mol/l NaH_2PO_4–Na_2HPO_4 (pH 7); $2\cdot10^{-5}$ mol/l cysteine; separation voltage, 25 kV; capillary, 35 cm × 25 μm I.D., injection 0.5 kV for 10 s; detection potential 0.02 V.

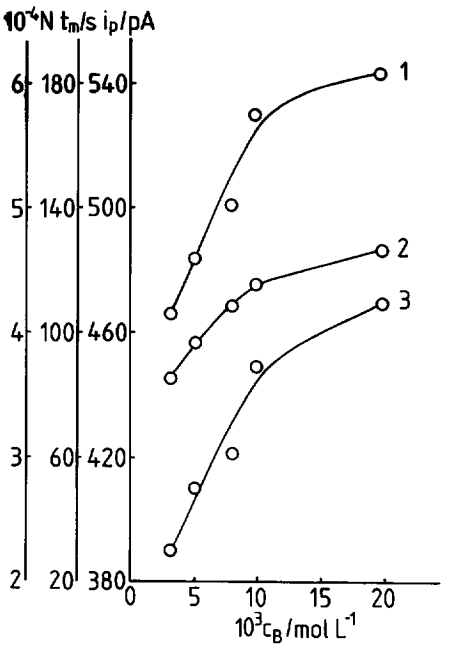

Fig. 4. Dependence of (1) the migration time, (2) the number of theoretical plates and (3) the peak current detected on the concentration of the buffer; $2 \cdot 10^{-5}$ mol/l cysteine; separation voltage, 20 kV; capillary, 40 cm×25 μm I.D.; injection, 0.5 kV for 10 s; detection potential, 0.02 V.

when $c_B > 1 \cdot 10^{-2}$ mol/l, their increase becomes slower. In our experiments $1 \cdot 10^{-2}$ mol/l Na_2HPO_4–NaH_2PO_4 was used because of short t_m, high i_p and large N.

The separation voltage, V_s, exerts an influence on t_m, N [5]. It was found that there were the linear dependences of V_s on $1/t_m$ and N; t_m is dependent on L. There is good linearity with zero intercept between t_m and L^2. The separation efficiency is hardly dependent on the length of the separation capillary. In our experiments a length of the separation capillary of 35–40 cm was selected. The detected peak current, i_p, is dependent on the detected potential, E_d. When $E_d < 0$ V, the i_p is smaller, when $i_p > 0.02$ V, the increase of i_p is slower. When an E_d of 0.09 is applied, the baseline of detection current is getting bad and noise is getting larger and the shape of the peak on the electropherogram also becomes worse. Thus, an E_d of 0.02–0.06 V is chosen in our experiments.

3.4. Injection

Injection was by electromigration. The parameters i_p, q (the electric charge detected) and N depend on the injection voltage, V_i, and injection time, t_i. It can be found that the i_p and q are proportional to V_i and t_i, which means that the signal detected is directly proportional to the amount injected. The separation efficiency decreases with increasing V_i and t_i. It is because the increase of the quantity injected makes the volume injected increase, which leads to zone broadening. Therefore, an injection voltage lower than 1.0 kV and an injection time shorter than 10 s were selected in our experiments.

3.5. Reproducibility, limit of detection and linear range

The response for a series of ten injections of $5 \cdot 10^{-6}$ mol/l cysteine resulted in a relative standard deviation of 1.9% for t_m, 4.2% for i_p and 5.3% for q, respectively. The limit of detection is $5.8 \cdot 10^{-8}$ mol/l, or 14.5 amol (according to a signal-to-noise ratio of 2), which was estimated from the electropherograms obtained for $1 \cdot 10^{-7}$ mol/l cysteine. A linear relationship holds between the peak current detected or the electric charge detected and a concentration in the range $1 \cdot 10^{-7}$–$1 \cdot 10^{-4}$ mol/l cysteine. Least-squares treatment of these data yielded a slope of 24 pA/μmol l^{-1} with correlation coefficient of 0.997.

3.6. Comparison of electrophoretic characteristics of cysteine in different buffers

The electrophoretic characteristics of cysteine are listed in Table 2. It was found that in 0.01 mol/l Na_2HPO_4–NaH_2PO_4 buffer of pH 7.0 the migration time is slightly longer than that at pH 8.0, separation efficiency is largest and the limit of detection is lowest. Therefore, 0.01 mol/l Na_2HPO_4–NaH_2PO_4 of pH 7.0 is most suitable for the determination of cysteine.

3.7. Determination of cysteine in human plasma, blood and urine

Cysteine concentration in human plasma can be readily determined by using this CZE–electrochemi-

Table 2
Electrophoretic characteristics of $1 \cdot 10^{-5}$ mol/l cysteine in different buffers

Buffer	t_m (s)	$10^{-4} N$	i_p (pA)	LD
Na_2HPO_4–citric acid (pH 2.4)	384	3.27	432	1×10^{-7}
Na_2HPO_4–NaH_2PO_4 (pH 7.0)	136	4.55	450	6×10^{-8}
Na_2HPO_4–NaH_2PO_4 (pH 8.0)	122	4.16	380	5×10^{-7}

Buffer concentration, $1 \cdot 10^{-2}$ mol/l; other conditions as in Table 1. LD, Limit of detection.

cal detection system. Fig. 5 shows an electropherogram of a human plasma sample and an electropherogram of the plasma sample solution containing $1.96 \cdot 10^{-6}$ mol/l cysteine standard solution. The results obtained by using a calibration curve are listed in Table 3. The average content of cysteine of $9.8 \cdot 10^{-6}$ mol/l in plasma agrees with the value of $1.08 \cdot 10^{-5}$ mol/l obtained by using standard addition. The recovery is 95%. The value is in agreement with literature values [3,12], which were in the range $7 \cdot 10^{-6}$–$1.2 \cdot 10^{-5}$ mol/l cysteine of human plasma.

The results obtained for the blood sample by using the calibration curve are also listed in Table 3. It can be seen that the concentration of cysteine in blood is the same as in plasma, which means that cysteine exist mainly in plasma, not in the erythrocytes.

The electropherograms of human urine sample without and with the standard solution of cysteine are also shown in Fig. 5. The average concentration in the urine samples obtained by the using calibration curve is $2.24 \cdot 10^{-5}$ mol/l (see Table 3), which agrees with the value of $2.38 \cdot 10^{-5}$ mol/l by using the standard addition. The recovery is 96%. The value is in agreement with literature values [13], which were in the range $2–5 \cdot 10^{-5}$ mol/l cysteine in human urine.

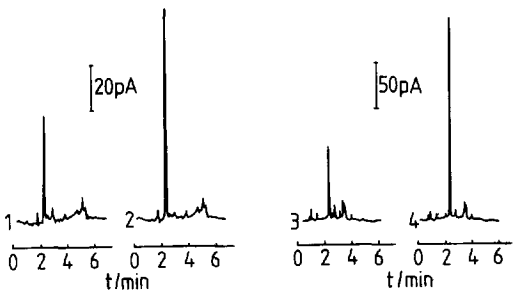

Fig. 5. Electropherograms of cysteine in samples of human plasma and urine. 1, Plasma sample; 2, the plasma sample $+1.96 \cdot 10^{-6}$ mol/l cysteine; 3, urine sample; 4, the urine sample $+6.40 \cdot 10^{-6}$ mol/l cysteine.

Acknowledgments

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Table 3
Results for determination of cysteine in samples of human plasma, blood and urine

Sample	Concentration (10^{-5} mol/l)	Average value (10^{-5} mol/l)	Relative standard deviation (%)
Plasma	1.02, 0.98, 0.94	0.98	4.3%
Blood	1.06, 1.02, 0.96	1.01	5.0%
Urine	2.25, 2.05, 2.41	2.24	8.0%

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