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Determination of Urea and Creatinine Concentrations in Urine Using Two-Electrode Sensor System Combined with Two-Compartment Cell

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The concentrations of urea and creatinine in human urine were simultaneously determined using a two-electrode sensor system equipped with a two-compartment cell. A small and a larg compartment, whose volumes were 0.5 and 19.5 ml, respectively, were separated by a glass filter (pore size < 0.25 mm). An amperometric creatinine-sensing electrode was inserted into the small compartment; urea-sensing, Ag/AgCl reference and auxiliary electrodes were inserted into the larg compartment. A drop of urine (5 μl), which contained a high concentration of urea (ca. 0.3 M) and a relatively low concentration of creatinine (ca. 10 mM), was injected into the small compartment. The creatinine concentration was first measured in the small compartment with a low dilution factor for the sample; then the sample diffused into the large compartment and the urea electrode response at a higher dilution factor could be obtained. The concentrations of the two components could be determined within 3.5 min.

1. Introduction

The simultaneous determination of the concentrations of urea and creatinine in human urine provides useful information for the diagnosis and treatment of kidney diseases. Urea is a major metabolic product of protein, and its concentration in urine depends on both glomerular filtration rate and renal function. On the other hand, the concentration of creatinine is less influenced by dietary changes and is a reliable and quick index for glomerular filtration rate. A high urea concentration and normal creatinine level is, for

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example, indicative of low renal function, and in response the patient is put on a low-protein diet.

For the accurate and rapid determination of the concentrations of the two components, the use of a two-electrode enzyme sensor system is a suitable approach. Most enzyme electrodes for determining urea concentrations are based on the hydrolysis of urease (EC 3.5.1.5). The hydrolytic reaction can be followed by monitoring the enzymatic reaction products: numerous works have been carried out on the fabrication of urease-based sensors using potentiometric pH-,[1–10] NH_4^+-[11,12] and NH_3-[13–15] sensing devices. Similarly, potentiometric creatinine sensors have been constructed by using creatinine deiminase (EC 3.5.4.21) associated with NH_3-sensing electrodes.[16–18] However, potentiometric methods in principle suffer from the disadvantages of low accuracy and reproducibility,[19,20] *e.g.*, a ±1 mV error corresponds to ±4% of the analyte concentration. Furthermore, the interference due to pH changes, endogenous ammonia and several cationic substances in the sample to be measured makes the systems inconvenient in clinical application. To avoid these drawbacks, amperometric enzyme sensors for urea[21,22] and creatinine[23–29] have been developed. A pair of amperometric sensors, one based on urea amidolyase (EC 3.5.1.45), pyruvate kinase (EC 2. 7.1.40) and pyruvate oxidase (EC 1.2.3.3) for urea[22] and another based on creatininase (EC 3.5.2.10), creatine amidinohydrolase (EC 3.5.3.3) and sarcosine oxidase (EC 1.5.3.1) for creatinine,[23–29] would be useful for the simultaneous determination of the concentrations of the two components.

In addition, the concentrations of urea and creatinine are very different: the concentration ranges of urea and creatinine are usually 200–500 and 6–12 mM, respectively. This suggests that their simultaneous determination using a simple measuring cell system equipped with two trienzyme electrodes is rather difficult. When a tiny amount of urine is added to the measuring cell, the creatine concentration in the cell is too low to measure the sensor response to the corresponding analyte precisely. In contrast, a low dilution factor of the sample results in a urea concentration too high to determine with the urea-sensing electrode. Hence we have introduced a cell divided into two compartments by a glass filter: urea- and creatinine-sensing electrodes are inserted into the compartments with larger and smaller volumes, respectively, and the sample is injected into the small compartment. The creatinine concentration is first measured in the small compartment with a low dilution factor for the sample; then the sample diffuses into the large compartment and the urea electrode response at a higher dilution factor can be obtained. This paper describes the preparation and use of the two-sensor/two-component cell system.

2. Materials and Methods

2.1 *Reagents*

The enzymes used were urea amidolyase (UA, EC 3.5.1.45, from *Candida* sp. 3.1 U mg^{-1}; Toyobo, Osaka), pyruvate kinase (PK, EC 2.7.1.40, from rabbit muscle, 380 U mg^{-1}; Sigma, St. Louis, MO), pyruvate oxidase (PyOx, EC 1.2.3.3, from *Aerococcus viridans*, 47 U mg^{-1}; Asahi Chemical Industry, Shizuoka), creatininase (CN, EC 3.5.2.10, from *Pseudmonus* sp. 256 U mg^{-1}; Toyobo), creatine amidonohydrolase (CA, EC 3.5.3.3, from *Actinobacillus* sp.12.8 U mg^{-1}; Toyobo) and sarcosine oxidase (SOx, EC 1.5.3.1, from *Bacillus* sp., 50 U mg^{-1}; Sigma). Photo-crosslinkable poly(vinyl alcohol) bearing the stilbazolium group[30] (SPP-H13; aqueous solution, 11%(w/v), pH 7) and poly

(dimethylsiloxane) (BY22-826; emulsion, 45%(w/v)) were obtained from Toyo Gosei Kogyo (Chiba) and Toray Dow Corning Silicone (Tokyo), respectively. Adenosine-5'-triphosphate (ATP), phosphoenolpyruvate (PEP), thiamine pyrophosphate (TPP), flavin adenine dinucleotide (FAD), urea and creatinine were obtained from Sigma. Other reagents used were of analytical reagent grade (Nacalai, Kyoto). F-kit (Boehringer Mannheim, Mannheim) for urea and Creatinine-test Wako (Wako, Osaka) were used for the spectrophotometic measurements of the two components. The kit for urea uses the enzyme pair of urease and glutamate dehydrogenase (EC 1.4.1.3), and that for creatinine is based on the reaction of the analyte with picrate to give a red-yellow complex.[31] Deionized, twice-distilled water was used throughout.

2.2 *Enzyme electrodes*

Platinum disk electrodes (diameter, 1.6 mm; Bioanalytical Systems, West Lafayette, IN) were polished with a 0.05-μm-diameter alumina slurry, rinsed with water, sonicated in water for 2 min, and then cleaned by electrochemical oxidation/reduction (–0.19 to +1.16 V vs Ag/AgCl (saturated with KCl), 0.1 V s^{-1}) in 0.5 M H_2SO_4 for more than 30 min. The platinum electrodes thus cleaned were dipped into an emulsion of poly(dimethylsiloxane), which was diluted to 5%(w/v) with water just before the dip-coating process. The electrode was allowed to dry for 4 h at room temperature and then placed under a vacuum (1 Pa at room temperature) for 1 h. The thickness of the poly(dimethylsiloxane) layer was ca. 20 μm.

Two kinds of trienzyme membranes were prepared using photo-crosslinked poly(vinyl alcohol) as the support. An UA/PK/PyOx membrane was prepared from a mixture of SPP-H13 and a solution (pH 7) containing UA (2%(w/v)), PK (1%(w/v)) and PyOx (1%(w/v)), 1:1 by weight. The mixture was placed on a PTFE plate and dried for 16 h at 4°C in the dark. The dried film (thickness ca. 30 μm) was removed from the plate and irradiated to crosslink the polymer with a fluorescent lamp (30 W) for 5 min on each side. Similarly, a CN/CA/SOx membrane (thickness in the dried state, 30 μm) was prepared from a mixture of SPP-H13 and a solution (pH 7) containing CN (1%(w/v)), CA (2%(w/v)) and SOx (1%(w/v)), 1:1 by weight. Each trienzyme membrane (3 mm × 3 mm) was placed on the poly(dimethylsiloxane)-coated electrode surface and covered with a polyester mesh (100 mesh, 2 cm in diameter). The mesh was held in place with a rubber ring so that the trienzyme membrane was in direct contact with the poly(dimethylsiloxane) layer. Each trienzyme electrode thus prepared was stored in 0.1 M potassium phosphate buffer solution (pH 7.0) containing 10 μM FAD at 4°C when not in use.

2.3 *Measuring cell and procedure*

A dual-potentiostat (Hokuto Denko) was used in an amperometric measuring system with two working electrodes, i.e., the UA/PK/PyOx-based, urea-sensing electrode, the CN/CA/SOx-based, creatinine-sensing electrode, an Ag/AgCl (saturated with KCl) reference electrode and a platinum auxiliary electrode. Figure 1 shows the arrangement of the two-component measuring cell system equipped with the two enzyme electrodes. A small cylindrical compartment (inner diameter, 9 mm; volume, 0.5 ml) and a large compartment (inner diameter, 40 mm; volume, 19.5 ml) were divided by a glass filter (pore size, < 0.25 mm). The amperometric creatinine-sensing electrode was inserted into the small compartment; the urea-sensing, Ag/AgCl reference and auxiliary electrodes were inserted into the large compartment.

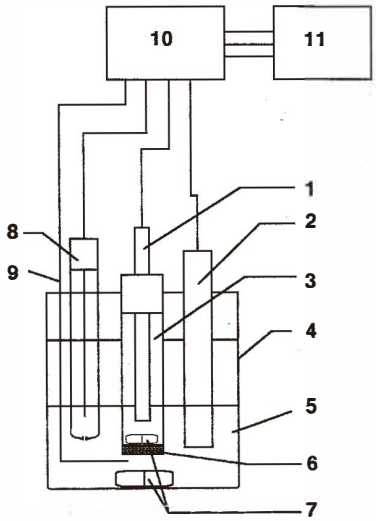

Fig. 1. Arrangement of the two-electrode sensor system equipped with the two-compartment cell: (1) creatinine-sensing trienzyme electrode; (2) urea-sensing trienzyme electrode; (3) small compartment; (4) larg compartment; (5) test buffer solution (6) glass filter attached to the bottom of the small compartment; (7) magnetic stirring bars; (8) Ag/AgCl reference electrode; (9) platinum auxiliary electrode; (10) dual potentiostat; (11) recorder.

The test buffer solution (total volume 20 ml) was an air-saturated 0.1 M phosphate buffer (pH 7.5) containing 1 mM ATP, 1 mM PEP, 5 mM $MgCl_2$, 5 mM $KHCO_3$, 0.6 mM TPP and 10 μM FAD. Here, ATP and PEP were cosubstrates for the UA- and PK-catalyzed reactions, respectively; Mg^{2+} was the activator of UA, PK, and PyOx; HCO_3^- was the activator of UA; TPP and FAD were the activators of PyOx. The pH of the solution, 7.5, was close to the optimal pH of the all the enzymes used. The solution in each compartment was stirred with a magnetic bar, and its temperature was kept at 25.0 ± 0.2°C. The potential of each enzyme electrode was kept at –0.4 V vs Ag/AgCl. The potential was sufficiently negative for the monitoring of oxygen concentration at the poly(dimethylsiloxane)-coated platinum electrode.[22,32,33] A drop of sample solution was injected into the small compartment, and the current responses on the two electrodes were recorded.

The current response for each trienzyme electrode was also examined using a conventional cylindrical cell (inner diameter, 40 mm; volume, 20 ml).

3. Results and Discussion

3.1 *Responses of urea- and creatinine-sensing electrodes in conventional cell systems*

The cathodic current on each trienzyme electrode decreased after the addition of the corresponding analyte in the cylindrical cell and reached steady state within 30 s. The magnitude of steady-state current decrease for 0.1 mM urea was 9.5 μA cm^{-2} and that for

0.1 mM creatinine was 6.1 μA cm^{-2}. The current decreases were proportional to the analyte concentration up to 0.35 mM for the urea-sensing system and up to 0.6 mM for the creatinine-sensing one. The detection limits were 3 and 5 μM for the urea- and creatine-sensing electrodes, respectively (signal-to-noise ratio for each electrode, 3). The relative standard deviation for 10 successive measurements of 0.1 mM corresponding analytes was ca. 2% for each electrode.

The cathodic detection of oxygen consumption through oxidase-catalyzed reactions is useful for the selective examination of the enzyme substrate in biological samples. That is, the cathodic measurements can be carried out without interference from oxidizable species such as L-ascorbic acid and uric acid, which exist in blood and urine samples in high concentrations. Actually, none of the trienzyme electrodes showed discernible current responses upon the addition of L-ascorbic acid and uric acid.

3.2 *Responses for two-electrode/two-compartment cell system*

Curves A and B in Fig. 2 show the current-vs-time curves for the urea- and creatinine-sensing electrodes, respectively, upon the addition of a urea/creatinine mixture to the small compartment. The mixture (5 μl) contained 0.2 M urea and 5 mM creatinine. Hence the concentrations of urea and creatinine immediately after injection into the small compartment (initial concentration) were 2 mM and 50 μM, respectively, since the volume of the small compartment was 0.5 ml, as described in the experimental section. The sample diffused toward the large compartment through the glass filter at the bottom of the small compartment, so that the concentrations of urea and creatinine in each compartment gradually increased to 50 and 1.25 μM, respectively (the total volume of the two compartments was 20 ml, as detailed in the experimental section). In accordance with the increase in the urea concentration in the large compartment, the current at the urea-sensing electrode

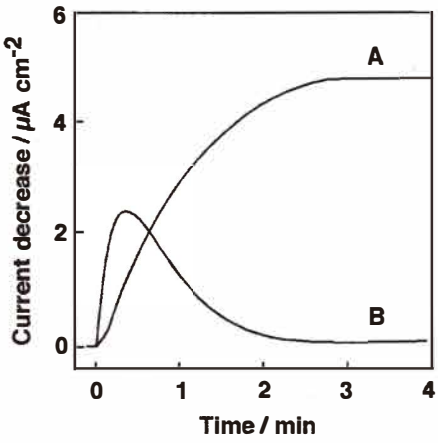

Fig. 2. Current time curves of (A) the urea-sensing trienzyme electrode and (B) the creatinine-sensing trienzyme electrode, upon the addition of a mixture of 2 mM urea and 50 μM creatinine. The concentration of each component was that immediately after injection into the small compartment.

gradually decreased to another steady state within 3.5 min. The magnitude of the steady-state current response, 4.8 μA cm^{-2}, coincided with that obtained in the conventional cell system upon the addition of 50 μM urea. On the other hand, the current on the creatinine-sensing electrode reached a minimum 15 s after the injection of the sample and reached steady state within 3.5 min: the gradual decrease in the creatinine concentration after the addition of the species to the small compartment results in the gradual decrease after the initial increase in the current response.

The steady-state current response for urea was independent of the creatinine concentration in the sample and similarly, the current response at the peak for creatinine was not influenced by the change in the urea concentration. Thus the urea and creatinine concentrations can be determined from the steady-state response of the urea sensor and the current change at the peak on the creatinine sensor. The time required for the determination of the two components was 3.5 min. The relative standard deviation of the steady-state response on the urea-sensing electrode was 2.1%, for 10 successive measurements of 2 mM (initial concentration) urea. For 10 successive measurements of 50 μM (initial concentration) creatinine, the relative standard deviation of the response on the creatinine-sensing electrode at the peak was 2.9%. Figure 3 shows calibration curves for urea and creatinine: curve A shows the relationship between the initial concentration of urea in the smaller chamber and the steady-state current response; curve B, that between the initial concentration of creatinine in the small chamber and the current response at the peak. The current responses were proportional to analyte concentrations up to 15 mM for the urea-sensing electrode and up to 1 mM for the creatinine-sensing one. Since the final dilution factor was

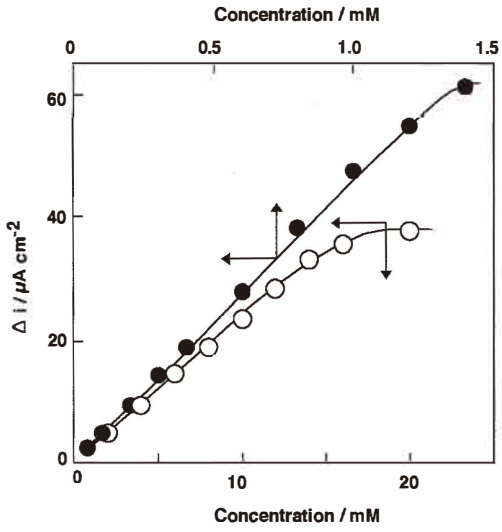

Fig. 3. Calibration graphs for urea and creatinine: (A) relationship between the urea concentration immediately after injection into the small chamber and the steady-state current response on the urea-sensing trienzyme electrode; (B) relationship between the creatinine concentration immediately after injection into the small chamber and the current response at the peak on the creatinine-sensing trienzyme electrode.

40 times larger than the initial one, the linear range for measuring the urea concentration was markedly extended to a higher region than in the case of the conventional cell system.

When a mixture containing 2 mM urea and 50 μM creatinine was added to a conventional cylindrical cell with two trienzyme electrodes, the urea concentration could not be measured exactly; the urea concentration was much higher than the measurable region for the urea sensor, 3 μM – 0.35 mM. On the other hand, the addition of a mixture containing 50 μM urea and 1.25 μM creatinine did not bring about a discernible current response for the creatinine-sensing electrode. This two-electrode/two-compartment cell system has proved to be useful for the simple and accurate determination of the concentrations of two components in a sample that contains a high concentration of urea and a much lower concentration of creatinine.

3.3 *Determination of concentrations of two components in urine and stability of two-electrode system*

Table 1 gives the results for the determination of urea and creatinine concentrations in urine samples. The samples (volume, 5 μl) were used without any pretreatment; the initial dilution factor in the small chamber and the final dilution factor were 100 and 4000, respectively. The results were compared with those obtained by spectrophotometric measurements. The agreement was excellent for both urea and creatinine: the correlation coefficients between this method and the corresponding spectrophotometric method for the five samples given in Table 1 were 0.984 for urea and 0.957 for creatinine.

The long-term stability of the two-electrode system was examined: a mixture containing urea and creatinine, whose initial concentrations were 2 mM and 50 μM, respectively, was examined 5 times a day, every day for 3 weeks. The steady-state response for urea did not change for 10 days. The electrode response for urea gradually decreased after 10 days to ca. 40% of the initial value on the 21st day. The loss of UA activity was principally responsible for the reduction in urea response.[22] On the other hand, the creatinine response at the peak did not change for 15 days and the magnitude of the response on the 21st day was > 80% of the initial value.

The determination of urea and creatinine concentrations in human urine has successfully been carried out using a two-electrode sensor system equipped with a two-compart-

Table 1
Comparison of results obtained for urea and creatinine concentrations in human urine by different methods.

Sample	Urea concentration/mM		Creatinine concentration/mM	
	Proposed Method	Spectrophotometric Method	Proposed Method	Spectrophotometric Method
1	289	280	12.8	13.1
2	227	219	13.4	13.0
3	310	315	10.2	10.7
4	245	237	9.7	9.5
5	270	277	11.1	11.8

ment cell. Although, the system with the UA/PK/PyOx-based, urea-sensing electrode requires the addition of expensive reagents such as ATP, PEP and FAD, the high selectivity and the usefulness of the system compensates for this expense.

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