Multi-Enzyme Membrane Electrodes for Determination of Creatinin and Creatine in Serum

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An enzyme electrode system for the determination of creatinine and creatine was developed by utilizing three enzymes: creatinine amidohydrolase (CA), creatine amidinohydrolase (CI), and sarcosine oxidase (SO). These enzymes were co-immobilized onto the porous side of a cellulose acetate membrane with asymmetric structure, which has selective permeability to hydrogen peroxide. Two kinds of multi-enzyme electrodes were constructed by combining a polarographic electrode for sensing hydrogen peroxide and an immobilized CA/CI/SO membrane or CI/SO membrane for creatinine plus creatine or creatine, respectively. The multi-enzyme electrodes responded linearly up to 100 mg of creatinine and creatine per liter in human serum. Response time was 20 s in the rate method and the detection limit was 1 mg/L. Only 25 μL of serum sample is required. Analytical recoveries, precisions, and correlations with the Jaffé method were excellent, and the multi-enzyme electrodes were sufficiently stable to perform more than 500 assays. No loss of activity of immobilized enzymes was observed after nine months of storage at 4 °C in air.

Additional Keyphrase: immobilized enzymes

Determination of creatinine and creatine in biological fluids is of significant value for diagnosis of renal, muscular, and thyroid function. Many of the currently used assay procedures for creatine and creatinine are based on the Jaffé alkaline picrate method. Several enzymatic methods have recently been investigated to increase specificity (1-3). However, they are laborious, costly, and relatively imprecise.

Here we propose a new enzyme electrode system for the determination of creatinine and creatine in biological fluids. The following three enzymes are used in the present method: creatinine amidohydrolase (CA; EC 3.5.2.10) (4), creatine amidinohydrolase (CI; EC 3.5.3.3) (5), and sarcosine oxidase (SO; EC 1.5.3.1) (6). These enzymes were co-immobilized onto the porous side of a cellulose acetate membrane with asymmetric structure. The skin layer of the asymmetric membrane has selective permeability to hydrogen peroxide. Two kinds of immobilized enzyme membranes were prepared, one containing three enzymes (CA, CI, and SO) and the other containing two (CI and SO). By combining these enzyme membranes with polarographic electrodes for sensing hydrogen peroxide, we constructed two types of enzyme electrodes, a creatinine electrode and a creatine electrode. With this dual enzyme electrode system, one can measure creatinine and creatine simultaneously. The schemes of the multi-enzyme reactions and electrochemical oxidation of the resulting hydrogen peroxide are as follows:

\[
\text{Creatinine} + \text{H}_2\text{O} \rightarrow \text{creatinine} \\
\text{Creatine} + \text{H}_2\text{O} \rightarrow \text{sarcosine} + \text{urea} \\
\text{Sarcosine} + \text{O}_2 + \text{H}_2\text{O} \rightarrow \text{formaldehyde} + \text{glycine} + \text{H}_2\text{O}_2 \\
2\text{H}_2\text{O}_2 \rightarrow 4\text{H}^+ + 2\text{O}_2 + 4\text{e}^- \text{(anode)} \\
4\text{H}^+ + \text{O}_2 + 4\text{e}^- \rightarrow 2\text{H}_2\text{O} \text{(cathode)}
\]

The hydrogen peroxide is detected with a polarographic electrode. Here, we describe the enzymatic properties of the immobilized multi-enzyme membranes and present the determination of creatinine and creatine in human serum by the enzyme electrode method as an alternative to the Jaffé method.

Materials and Methods

Apparatus

We used a Model 200-10 spectrophotometer (Hitachi, Ltd., Tokyo 105, Japan) to determine enzyme activity colorimetrically. Figure 1 shows a schematic diagram of the multi-enzyme electrode system. A home-made differential circuit was used for the rate method. We also determined creatinine and creatine in human serum by using the immobilized multi-enzyme membrane with a Model 23A Glucose Analyzer (Yellow Springs Instrument Co., Yellow Springs, OH 45387).

Reagents

CA and CI, both from Pseudomonas sp., were obtained from Biochemical Operations Division, Toyobo Co., Ltd., Osaka 530, Japan. SO from Corynebacterium sp. was purchased from Seishin Pharmaceutical Co., Ltd., Chiba 278, Japan. Crystalline bovine serum albumin was purchased from Sigma Chemical Co., St. Louis, MO 63178. Glutaraldehyde

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1 Nonstandard abbreviations: CA, creatinine amidohydrolase; CI, creatine amidinohydrolase; SO, sarcosine oxidase.

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(200 g/L aqueous solution), grade for electron microscopy, was purchased from Eiken Chemical Co., Ltd., Tokyo 113, Japan. Acetyl cellulose, type 394-30, was purchased from Tennessee Eastman, Kingsport, TN 37662. Distilled water was used throughout. For the multi-enzyme electrode we used potassium phosphate buffer (50 mmol/L) containing 5 mmol of disodium magnesium ethylenediaminetetraacetate (EDTA), 1 mmol of sodium hypophosphite, and 0.2 mmol of sodium azide per liter. The pH was adjusted to 7.5. Validate and Validate A (Warner-Lambert Co., Morris Plains, NJ 07950) were used as a control serum. Creatinine Reagent “Eiken” was purchased from Eiken Chemical Co., Ltd., Tokyo 113, Japan. Acetyl cellulose, type 394-30, was purchased from Tennessee Eastman, Kingsport, TN 37662.

Procedures

Preparation of immobilized multi-enzyme membrane. A cellulose acetate membrane with asymmetric structure, having permeability for hydrogen peroxide, was prepared as reported previously (7). In brief, a polymer solution consisting of 39.6 g of acetyl cellulose (type 394-30), 0.4 g of polyvinylacetate, 600 mL of acetone, and 400 mL of cyclohexane was cast 150 μm thick on a glass plate. The glass plate was placed in a dust-free environment at room temperature for 1 min. The membrane, which forms a skin layer, was carefully placed in n-hexane for a few hours, then dried in air. The 11-μm-thick asymmetric membrane so obtained was peeled from the glass plate in distilled water and then wound onto a glass roll. The membrane was unwound and spread onto a polyester film support, 40 μm thick. Before coupling with enzymes, the membrane was treated with γ-aminopropyltriethoxysilane as follows: A mixture of 100 μL of γ-aminopropyltriethoxysilane, 30 μL of acetic acid, and 200 μL of distilled water was spread over a 10 × 40 cm area of the porous side of the asymmetric membrane. After drying in air, the membrane was cured at 90–100 °C for 1 h. Then the membrane was immersed in 0.1 mol/L sodium hydroxide for 10 min at room temperature, then thoroughly rinsed with distilled water.

The phosphate buffer mixture (150 μL) containing 5 mg (1000 U) of CA, 15 mg (105 U) of Cl, 10 mg (38 U) of SO, and 5 mg of bovine serum albumin was mixed quickly with 50 μL of 40 g/L glutaraldehyde. Without delay, the mixed solution was spread over a 50 × 65 mm area of the porous side of the asymmetric membrane (the side treated with γ-aminopropyltriethoxysilane). On standing at 4 °C for 1 h in air, the enzymes crosslinked with the membrane. Next the enzyme membrane was treated with 50 mmol/L potassium phosphate buffer, containing only glycine (1 mol/L), at 4 °C for 16 h. Finally, the co-immobilized multi-enzyme membrane was treated with a mixture of glycerol and 50 mmol/L potassium phosphate buffer (50/950 by vol). The immobilized multi-enzyme (CA/Cl/So) membrane so obtained was covered with porous polycarbonate membrane and dried at 4 °C in air.

The immobilized CI/So membrane was prepared similarly.

Measurement of enzyme activity. The measurement of CA activity is based on the Jaffé reaction:

\[ \text{Creatine} + \text{H}_2\text{O} \rightarrow \text{creatinine} + \text{H}_2\text{O} \]

\[ \text{Creatine} + \text{picric acid} \rightarrow \text{orange dye} \]

The orange dye (molar absorptivity = 4.65 × 10³ cm²/mmol) has an absorption maximum at 580 nm.

To determine the activity of CA, we incubated 0.1 mL of 0.1 mol/L creatine in 50 mmol/L potassium phosphate buffer (pH 7.5) for 5 min at 37 °C, then added 0.1 mL of CA solution in the same buffer. After exactly 10 min of incubation at 37 °C, we transferred 0.1 mL of sample from the reaction mixture into 0.9 mL of ice-cold water and without delay added 1.0 mL of 1 mol/L sodium hydroxide solution and 1.0 mL of a 10 g/L picric acid solution. The mixture was incubated for 20 min at 25 °C. The absorbance of the resulting solution was measured at 520 nm vs water. One unit (U) of activity is that which causes the formation of 1 μmol of creatinine pикrate per minute under the given conditions.

The Michaelis constant (K_m) for creatine formation with CA was determined by the α-naphthol/diacetyl method (4), with 50 mmol/L potassium phosphate buffer solution.

The measurement of CI activity is based on the following reactions:

\[ \text{Creatine} + \text{H}_2\text{O} \rightarrow \text{creatinine} + \text{H}_2\text{O} \]

\[ \text{Urea} + 2 \text{p-dimethylaminobenzaldehyde} \rightarrow \text{yellow dye} + 2\text{H}_2\text{O} \]

The yellow dye (molar absorptivity = 3.21 × 10² cm²/mmol) formed from urea and p-dimethylaminobenzaldehyde has an absorption maximum at 435 nm.

The activity of CI was determined as follows. We incubated 0.9 mL of 0.1 mol/L creatine solution in 50 mmol/L potassium phosphate buffer (pH 7.5) for 5 min at 37 °C, then added 0.1 mL of CI solution, in the same buffer, to the solution. After exactly 10 min of incubation at 37 °C, we added 2.0 mL of a solution of 2.0 g of p-dimethylaminobenzaldehyde in 100 mL of dimethyl sulfoxide and 15 mL of concentrated HCl. After incubating this mixture for 20 min at 25 °C, we measured the absorbance at 435 nm vs water. One unit (U) of activity is that which causes the formation of 1 μmol of yellow dye per minute under the given conditions.

The measurement of SO activity is based on the following reactions:

\[ \text{Sarcosine} + \text{H}_2\text{O} + \text{O}_2 \rightarrow \text{glycine} + \text{formaldehyde} + \text{H}_2\text{O}_2 \]

\[ \text{Formaldehyde} + 4\text{-amino-3-hydrizino-5-mercappedo-1,2,4-triazole} + \text{OH}^- \rightarrow \text{tetrazine dye} \]

The tetrazine dye (molar absorptivity = 3.72 × 10⁴ cm²/mmol) has an absorption maximum at 550 nm.

To measure SO activity, we mixed 0.3 mL of 0.1 mol/L sarcosine aqueous solution and 0.1 mL of 0.3 mol/L potassium phosphate buffer (pH 7.7) and incubated the mixture for 3 min at 37 °C. We then added 0.1 mL of SO solution to the mixture, incubated at 37 °C for exactly 10 min, and finally added 1.0 mL of 5 mol/L potassium hydroxide and 1.0 mL of 4-amino-3-hydrizino-5-mercappedo-1,2,4-triazole (6 g/L) in 0.5 mol/L aqueous HCl. After letting this mixture stand for 10 min at room temperature, we added 1.0 mL of sodium periodate solution (7.5 g/L), with stirring, and measured the absorbance at 550 nm vs water. One unit (U) of activity is that which causes the formation of 1 μmol of formaldehyde per minute under the given conditions.

Creatinine and creatine assay with the multi-enzyme membrane electrode. Creatinine and creatine in standard solutions were determined as follows. An electrode probe was covered with either the CA/Cl/So membrane or the CI/So membrane and immersed in the cell containing 10 mL of the potassium phosphate buffer. After 5 min at 32 °C, 0.5 mL of the standard solution was injected into the cell, with stirring. The current generated in the polarized electrode is proportional to the hydrogen peroxide formed as a result of the enzyme reactions.
Total creatinine (creatinine + creatine) and creatine in human serum were determined according to the manual for the YSI Glucose Analyzer 23A, except that the CA/CI/SO or CI/SO membranes were used instead of a glucose oxidase membrane, the temperature of the cell was maintained at 32 °C, and the electrode current increase was recorded 1 min after sample injection. Because the CA/CI/SO membrane is sensitive to both creatinine and creatine in human serum, we determined creatinine concentrations by subtracting the creatine value from the total creatinine value.

Creatinine was determined in the rate mode based on the Jaffé reaction as follows.

We heated 2.0 mL of aqueous sodium hydroxide (4 g/L) for 5 min at 37 °C, then added 50 μL of serum sample. After exactly 3 min of incubation at 37 °C, 1 mL of aqueous picric acid (5 g/L) was added and the mixture placed in a spectrophotometer cell maintained at 37 °C. After exactly 2 min, we measured the change in absorbance at 550 nm vs water. Standards were 50, 100, 150, and 200 mg/L aqueous creatinine solutions.

To determine creatine, we used the end-point mode and the creatine reagent “Eiken.” This method is based on the Jaffé reaction and deproteinization with picric acid.

We mixed 0.5 mL of serum sample and 4.0 mL of aqueous picric acid (6 g/L). After 10 min, we centrifuged the mixture at 3000 rpm for 10 min, then heated 3.0 mL of the supernate at 100 °C for 90 min, hydrolizing the creatine to creatinine. After cooling the supernatant, the volume was corrected to 3.0 mL with a distilled water, then 1.0 mL of disodium phosphate (17 g/L) was added. After 30 min, we measured the absorbance at 530 nm vs water and calculated total creatinine (50 mg/L aqueous creatinine was the standard in this case). Next we determined creatinine in the same sample without boiling the supernate. The creatine value was obtained by subtracting the value for creatinine from that for total creatinine.

**Results**

**Analytical Variables**

Properties of each immobilized enzyme were compared with those of the corresponding free enzyme. The activity (and the activity yield) of each enzyme in the resulting immobilized enzyme membrane were 1.14 U/cm² (1.6%), 0.11 U/cm² (1.5%), and 0.013 U/cm² (0.5%) for CA, CI, and SO, respectively.

The optimum pH of SO was shifted 2.5 pH units to the basic side by immobilization. The range of optimum pH of the immobilized CA was broad, from pH 6 to 9. The optimum temperature of CA was increased by 10–25 °C by immobilization. The pH stability of none of the enzymes was affected by immobilization, being almost identical for free and immobilized enzymes within pH 6–10.

The thermal stability of CA was improved by immobilization, being stable below 70 °C in the phosphate buffer. Both CA and SO were stable below 37 °C under the same conditions, and their stabilities were not affected by immobilization.

$K_m$ and $V_{max}$ values were calculated from a Lineweaver-Burk plot. $K_m$ values of the immobilized enzymes were greater than those of free enzyme by about eightfold for CA and fivefold for CI, but for immobilized SO was only about one-third of that of the free enzyme. When compared at equal apparent activity, the $V_{max}$ value of each free enzyme agreed roughly with that of immobilized enzyme.

These properties of the three enzymes are summarized in Table 1.

Two types of enzyme electrodes were constructed, a combination of either the CA/CI/SO or the CI/SO membrane and a polarographic electrode for sensing hydrogen peroxide. Figure 2 shows response curves of the CA/CI/SO membrane electrode for creatinine. In the end-point method, addition of creatinine solution caused a rapid increase in current, which reached a steady state within 2 min; this current was directly proportional to the creatinine concentration. In the rate mode, addition of creatinine solution caused a rapid increase in reaction rate, reaching a maximum within about 20 s, and this increase was directly proportional to the creatinine concentration. In both techniques, calibration curves for the creatinine standard solution were excellent, being linear with concentration up to 100 mg/L. The CA/CI/SO membrane electrode responded to both creatine and sarcosine as well as the CI/SO membrane electrode did. In a typical run, responses of the CA/CI/SO membrane electrode to equimolar concentrations of creatinine, creatine, and sarcosine were in the ratio of 0.62, 0.59, and 1.00.

**Creatinine and Creatine in Human Serum**

We used enzyme electrodes to determine creatinine and creatine in human serum. Within-day precision (Table 2) was determined by analyzing three human sera.

Repeate assays (n = 10) of sample 2 (Table 2) during 10 days showed the between-day CV to be 8.4% for a mean creatinine concentration of 22 mg/L and 11.5% for a mean creatine concentration of 10 mg/L.

We used normal human serum to assess analytical recovery. Creatinine or creatine was added to a serum sample at five different concentrations to give final concentrations of 18.0 to 37.8 mg/L for creatinine, and 24.6 to 104.6 mg/L for creatine. The analytical recoveries ranged from 103 to 119% for creatinine (average, 109.6%) and from 91 to 109% for creatine (average, 102.2%).

A comparison of the present method with the Jaffé method is shown in Figure 3. Creatinine and creatine were determined simultaneously in 55 patients' serum samples by the present method. On the other hand, using the Jaffé method, we determined creatinine and creatine separately, that is, creatinine in 55 samples with the rate mode and creatine in 21 samples with the end-point mode.

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**Table 1. Properties of Free and Immobilized Enzymes**

<table>
<thead>
<tr>
<th></th>
<th>Units</th>
<th>CA</th>
<th>CI</th>
<th>SO</th>
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<tbody>
<tr>
<td>Activity $^b$</td>
<td>mg/L</td>
<td>236</td>
<td>6.3</td>
<td>4.2</td>
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<tr>
<td>K$_m$ $^b$</td>
<td>mmol/L</td>
<td>35</td>
<td>13.5</td>
<td>6.7</td>
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<tr>
<td>V$_{max}$ $^b$</td>
<td>mmol/L</td>
<td>278</td>
<td>64.9</td>
<td>2.4</td>
</tr>
<tr>
<td>Optimum pH (37 °C)</td>
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<td>7</td>
<td>7</td>
<td>7.5</td>
</tr>
<tr>
<td>Optimum temp. (pH 7.5)</td>
<td></td>
<td>55</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>pH stability $^c$</td>
<td></td>
<td>7.5–9.5</td>
<td>6–8</td>
<td>6–10</td>
</tr>
<tr>
<td>Temp. stability $^d$</td>
<td></td>
<td>&lt;45</td>
<td>&lt;37</td>
<td>&lt;37</td>
</tr>
</tbody>
</table>

$^a$ Upper value of each pair is value for free enzyme; lower value is for immobilized enzyme. $^b$ At 37 °C, pH 7.5. $^c$ After 16 h at 4 °C. $^d$ After 30 min at pH 7.5.
Fig. 2. Response curves of CA/Cl/So membrane electrode to creatinine in the end-point method (left) and the rate method (right).
Creatinine, mg/L: (1) 20, (2) 40, (3) 60, (4) 80, (5) 100

Table 2. Within-Day Precision with Three Human Sera (n = 10 Each)

<table>
<thead>
<tr>
<th>Creatinine</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
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<tr>
<td>Concentration (mg/L)</td>
<td>7.5</td>
<td>21.7</td>
<td>8.2</td>
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<tr>
<td>SD (mg/L)</td>
<td>0.5</td>
<td>0.3</td>
<td>1.0</td>
</tr>
<tr>
<td>CV (%)</td>
<td>7.2</td>
<td>1.3</td>
<td>11.7</td>
</tr>
</tbody>
</table>

Creatinine
| Concentration (mg/L) | 5.3 | 10.9 | 8.5 |
| SD (mg/L) | 0.4 | 0.6 | 0.4 |
| CV (%) | 7.6 | 5.7 | 4.8 |

Sample 1 is a normal control serum, Validate; sample 2 is an abnormal control serum, Validate-A; sample 3 is a normal fresh serum.

Fig. 3. Correlation between results by the enzyme electrode method and the Jaffé method
Creatinine (●): n = 55, r = 0.985, y = 1.078x - 2.6; creatine (O): n = 21, r = 0.982, y = 1.101x - 2.5

We investigated the durability of the CA/Cl/So membrane electrode by making repeated determinations of control human serum. As shown in Figure 4, the apparent activity of the enzyme electrode decreased very slowly. The apparent enzyme activity of the enzyme electrode was well retained: 83, 91, and 105% of the initial activity of CA, Cl, and So, respectively, was still present after 500 assays over 11 days. Moreover, we saw no loss of individual enzyme activity of the CA/Cl/So membrane after nine months of storage at 4 °C in air.

Discussion
The enzymes were co-immobilized by the crosslinking method. The conditions for this co-immobilization were optimum when the amounts of CN, Cl, So, bovine serum albumin, 40 g/L glutaraldehyde solution, and buffer solution were 5 mg (1000 U), 15 mg (105 U), 10 mg (38 U), 5 mg, 50 μL, and 150 μL, respectively. The solution of the three enzymes began to gel within about 1 min after glutaraldehyde was added at 4 °C. The response of the CA/Cl/So membrane electrode to creatinine was best when the total concentration of proteins such as enzymes and albumin immobilized on a membrane was 1.4 mg/cm². At greater concentrations, the activity per unit area of the resulting immobilized enzyme membrane increases slightly, but the responsiveness of the enzyme membrane electrode was lessened, presumably because the diffusion of substrates or reaction products through the membrane is retarded by a thick layer of proteins.
Guided by the pH profiles for each enzyme immobilized on the membrane, we adjusted the pH of the common buffer solution for the determination of creatinine or creatine to pH 7.5. The dipotassium magnesium EDTA in the buffer mixture is an activating agent for CA; it also stabilizes CI. Magnesium-free EDTA inhibits CA. Sodium hypophosphite, a mild reducing agent, is included as a protecting agent for CA and CI, both of which have sulfhydryl groups that probably play an important role in enzymic action. Generally, mercaptoethanol, dithiothreitol, reduced glutathione, and the like have been used as protecting agents for these enzymes, but were unsuited for the present enzyme electrode because they not only are unstable but also would interfere with the electrode reactions. Sodium azide inhibits the catalase contaminant of SO (catalase consumes hydrogen peroxide, thus causing a negative interference in the analysis of creatinine or creatine). The present buffer solution appears to confer excellent stability on the CA/CI/SO membrane electrode, as shown in Figure 4.

The response time of the CA/CI/SO membrane electrode—2 min in the end-point method and 20 s in the rate method—was influenced by the rate of diffusion of substrates into the co-immobilized enzyme layer, sequential enzymic reaction rates, and the rate of diffusion of resulting hydrogen peroxide to the electrode surface. Therefore, the response time can be shortened by use of a thinner enzyme layer and supporting membrane. The cellulose acetate membrane with an asymmetric structure is highly permeable to hydrogen peroxide. This supporting membrane is well suited for an enzyme membrane electrode when a polarographic electrode for sensing hydrogen peroxide is used.

The CA/CI/SO membrane electrode measured greater electrode currents for creatinine than for creatine. For example, the measured electrode currents were in the ratio of 1.00, 0.59, and 0.62 for equimolar solutions of sarcosine, creatine, and creatinine. Perhaps there is a reversible reaction of creatine to creatinine with CA. The equilibrium constant of the reaction from creatine to creatinine is 1.3 at 30°C and pH 7.5 in 10 mmol/L phosphate buffer solution (4).

When we used the multi-enzyme electrodes to determine creatinine and creatine in human serum, total creatinine (creatinine + creatine) was determined with the CA/CI/SO membrane mounted on an electrode of the YSI Glucose Analyzer 23A, then the concentration of creatine in the same sample was similarly determined by use of the CI/SO membrane. Total assay time was 100 s, response time being 60 s and recovery time on rinsing with buffer solution being 40 s.

The first enzyme electrode for creatinine was investigated by Rechnitz and co-workers (2, 8). Their creatinine enzyme electrode was constructed by trapping soluble creatininas in a cellophane membrane and the ammonia-gas-permeable membrane surface of the Orion 95-10 ammonia electrode. The second enzyme electrode for creatinine, reported by Guibault et al. (9), was constructed by crosslinking a creatininas directly onto the surface of ammonia-selective electrode. The main disadvantages of these enzyme electrodes are their long recovery time and low sensitivity. Our multi-enzyme electrode system has a relatively short response time, a short recovery time, and high sensitivity, as well as excellent durability and stability.

Our multi-enzyme electrode system appears to be a promising and attractive tool for clinical analysis as an alternative method of the Jaffé method. The present enzyme electrode method offers a rapid, economical procedure for simultaneous determination of creatinine and creatine in human serum.

References