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# Automated Microanalysis of Creatinine by Coupled Enzyme Reactions

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## ABSTRACT

Hydrogen peroxide was generated from creatinine by the sequential enzyme reactions of creatinine amidohydrolase, creatine amidinohydrolase and sarcosine oxidase. Hydrogen peroxide was, in turn, used stoichiometrically for the condensation of 4-aminoantipyrine and *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-*m*-toluidine catalyzed by horse-radish peroxidase, resulting in the formation of quinone dye with maximum absorbance at 546 nm. The optimized assay conditions for the enzymatic determination of creatinine in a HITACHI 7250 autoanalyzer was established. This system, which requires less than 5 μl of sample, was found to be the most economical for laboratories equipped with autoanalyzers.

***Key words:*** *Creatinine, Creatinine amidohydrolase, Creatine amidinohydrolase, Sarcosine oxidase*

Determination of the serum creatinine level is important in the assessment of muscle disorder and renal gromenular function. In the clinical diagnostic determination of creatinine in serum and urine, the Folin method[2] based on the Jaffé reaction[5] has been most commonly used. However, serum contains non-specific chromogens that will be false-positive in the Jaffé reaction, resulting in a 15 - 20%[11] overestimation of serum creatinine content. Therefore, the data obtained by the Jaffé reaction method must be interpreted with caution, especially when diseases such as diabetes mellitus or renal insufficiency are in question. To overcome the disadvantage of the Jaffé reaction method, more specific enzymatic measurement systems have been developed[1]: the creatinine deiminase system measures $NH_3$, and the creatinine amidohydrolase system detects the reduction of NADH catalyzed by a combination of creatinine amidohydrolase[3,6,10] (creatininase, EC 3.5.2.10), creatine kinase (EC 2.7.3.2), pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.1.27) or the generation of $H_2O_2$ produced by three creatinine catabolic enzymes:creatininase, creatine amidinohydrolase[6,7,15] (creatinase, EC 3.5.3.3) and sarcosine oxidase[4,8,9,13] (EC 1.5.3.1). However,the Folin method was more popular than any other method until the automated enzymatic determination of serum creatinine in a Greiner G-300 autoanalyzer was invented by the authors[12]. This system requires 60 μl of serum, and the enzyme reactions involved are as follows:

$$\text{creatinine} + H_2O \xrightarrow{\text{creatininase}} \text{creatine}$$

$$\text{creatine} + H_2O \xrightarrow{\text{creatinase}} \text{sarcosine} + \text{urea}$$

$$\text{sarcosine} + O_2 + H_2O \xrightarrow{\text{sarcosine oxidase}} \text{glycine} + \text{HCHO} + H_2O_2$$

$$\text{4-AA} + 2H_2O_2 + \text{EHSPT} \xrightarrow{\text{peroxidase (EC 1.11.1.7)}} \text{quinone dye}^{14}$$

4-AA: 4-aminoantipyrine
EHSPT: *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-*m*-toluidine

Although the creatininase-creatinase-sarcosine oxidase system is now widely employed, the existence of interfering substances in serum samples pose some problems: the effect of endogenous creatine and sarcosine, and the interference of ascorbic acid[12] and bilirubin[16] in the peroxidase reaction.

This paper shows how the system was modified to cope with these problems, being applicable to an autoanalyzer on a decreased scale.

## MATERIALS AND METHODS

*Materials*

Moni-Trol I and II were the products of American Dade, containing 1.0 and 5.5 mg/dl creatinine, respectively. Interferance-check A was purchased from IRC Japan, containing 208 mg/dl conjugated-bilirubin. All other materials were commercial products of analytical grade.

*Enzymes*

The following enzymes were products of Toyobo Co.: creatininase isolated from *Pseudomonas* species (240.0 U/mg); creatinase (12.5 U/mg) from *Actinobacillus* species; sarcosine oxidase (8.4 U/mg) from *Arthrobactor* species; ascorbate oxidase from cucumber *Cucumis* species (266.0 U/mg, EC 1.10.3.3); and horse-radish peroxidase (111.0 U/mg).

*Assay solutions*

Solution A was an aqueous solution of 11.9 mg/ml *N*-2-hydroxy-ethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 1.0 mg/ml polyoxyethylene nonylphenylether, 1.50 mg/ml NaOH, 0.2 mg/ml sodium azide, 4.0 mg/ml KCl and 0.38 mg/ml *N*-ethyl-*N*-(2-hydroxy- 3-sulfoproryl)-*m*-toluidine sodium salt dihydrate (EHSPT).

Solution B was an aqueous solution of 11.9 mg/ml HEPES, 1.0 mg/ml polyoxyethylene nonylphenylether, 1.50 mg/ml NaOH, 0.2 mg/ml sodium azide, 4.00 mg/ml KCl and 21.2 μg/ml potassium ferrocyanide.

The standard solution contained 5 mg/dl creatinine in 0.0054 N aqueous HCl.

Enzyme solution A contained 55 U of creatinase, 8 U of sarcosine oxidase, 5 U of peroxidase, 20 U of ascorbate oxidase and 1.25 mg sucrose in 1 ml of solution A.

Enzyme solution B contained 250 U of creatininase, 10 mg of 4-aminoantipyrine (4-AA), 1.25 mg of sucrose, 0.238 mg of HEPES and 5.5 μl of 1 N NaOH in 1 ml of solution B.

Enzyme solutions were prepared before each use.

*Assay procedures*

The HITACHI 7250 autoanalyzer has the following features. Model 7250 employs a turntable-discrete method and was installed with two reagent dispensers. The reaction is completed in 10 min at 37 ± 0.1°C. Three to 20 μl of the sample is required and the amount of each reagent is confined between 50 and 350 μl. The photometer can perform either duplicate or single wavelength measurement between 340 and 800 nm. The rate analysis, end-point analysis and sample-blank analysis are eligible.

Two hundred-fifty μl of enzyme solution A was added to 5 μl of serum and incubated for 5 min at 37°C. The sample was replaced with distilled water in the blank. Sixty μl of enzyme solution B was added and the whole was incubated for another 5 min at 37°C. The absorbance at 546 nm was measured. The creatinine standard solution was treated in the

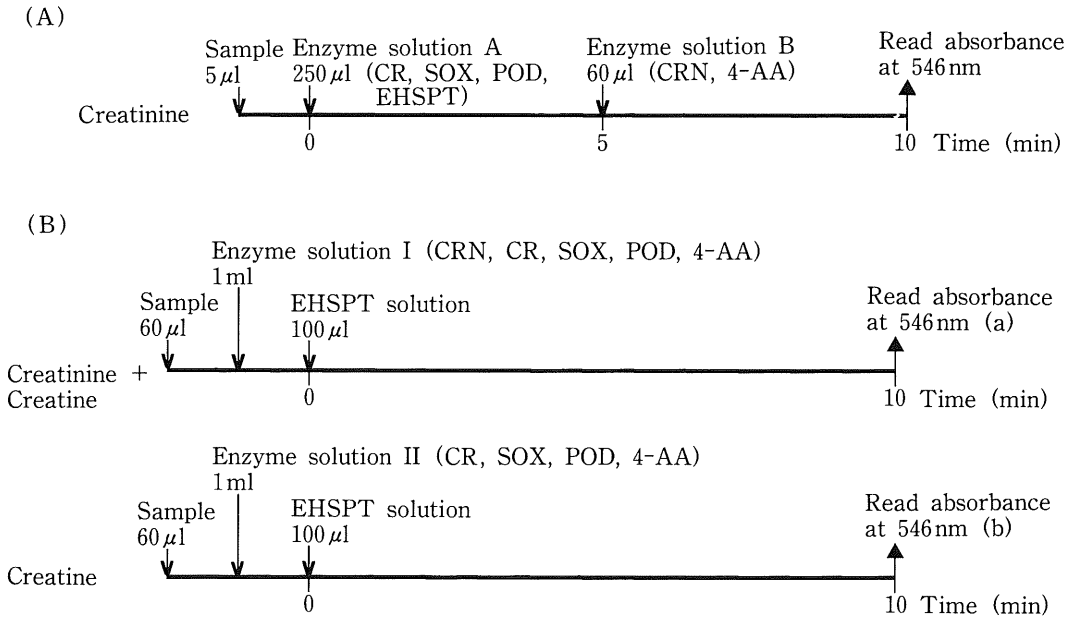


**Fig. 1.** Flow diagrams of the present and conventional methods.
A, the present method in HITACHI 7250 autoanalyzer; B, the conventional method in Greiner G-300 autoanalyzer[12]. CRN, creatininase; CR, creatinase; SOX, sarcosine oxidase; POD, peroxidase.

same way as the samples. A flow diagram of this analyzer is shown in Fig. 1 in comparison with the conventional one.

The creatinine content in the sample was calculated by the following equation:

$$\text{Creatinine in sample (mg/dl)} = \frac{A_{sample} - A_{blank}}{A_{standard} - A_{blank}} \times 5$$

where $A_{sample}$, $A_{standard}$ and $A_{blank}$ represent the absorbances of sample, creatinine standard (5 mg/dl) and blank, respectively.

## RESULTS

*Application to autoanalyzer*

In the conventional method using Greiner G-300 (Fig. 1B), two lines of enzymatic reactions were carried out for each sample: one line was used for the determination of the total amount of creatinine and creatine (a), the other for the determination of creatine (b). The creatinine content was expressed by (a) - (b). However, the single line of enzyme reactions depicted in Fig. 1A was found to be satisfactory for the determination of creatinine content (Fig. 2). Endogenous creatine and sarcosine were converted to $H_2O_2$ which was completely removed from the reaction mixture by incubating the sample in enzyme solution A containing creatinase, sarcosine oxidase and peroxidase for 5 min at 37°C. The development of quinone dye responsible for the creatinine content was triggered by the addition of enzyme solution B (creatininase). In the range of 15 - 150 mg/dl creatinine, the equilibrium was reached within 5 min. Furthermore, the absorbance of quinone dye formed was found to be proportional to the amount of creatinine. Based on these results, procedures for the creatinine determination shown in Fig. 1A were adopted for the microanalysis of creatinine in the HITACHI 7250 autoanalyzer.

By employing this system, the sample volume could be reduced to as little as 5 μl, and from the point of view of economy, it is far superior to the conventional method in which 60 μl of the sample is used.

The results obtained by the present system correlated well with those obtained by the conventional method (Fig. 3). The linear regression equation is expressed as Y = 0.9855 X + 0.0423 where X and Y represent the results obtained by the conventional method and the modified one, respectively.

*Optimized assay conditions for the determination of serum creatinine*

When this system was applied to serum samples, the interference of ascorbate and bilirubin with quinone dye formation was noted. The former can be suppressed from the serum sample by the addition of ascorbate oxidase to enzyme solution A as in the case of endogenous creatine and sarcosine. The effect of 20 mg/dl ascorbic acid on the determination of creatinine was negligible in the presence of 20

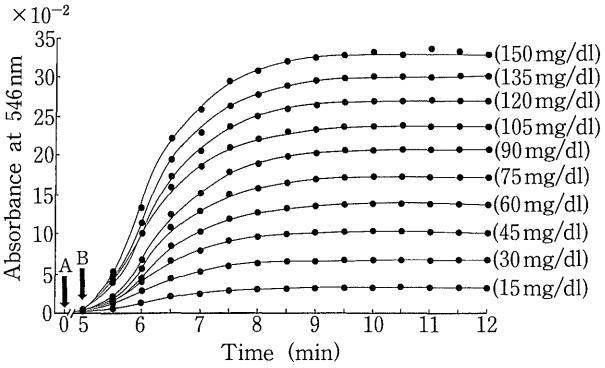


**Fig. 2.** Time course of quinone dye formation after the addition of enzyme solution B.
The mixture of creatinine solution (5 μl) and enzyme solution A (250 μl) was incubated for 5 min. After the addition of enzyme solution B (60 μl), the absorbance at 546 nm was measured every 30 sec. A, creatinine + enzyme solution A; B, enzyme solution B.

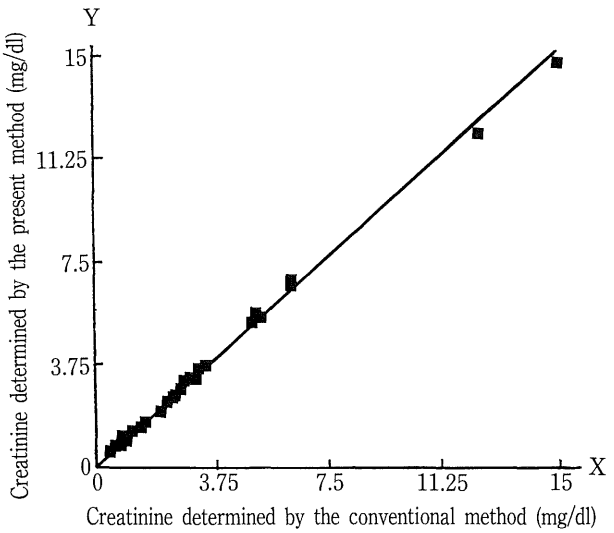


**Fig. 3.** Correlation between the data obtained by the present and conventional methods.

U/ml or more of ascorbate oxidase (Table 1).

Potassium ferrocyanide was used to suppress the negative effect of bilirubin. A mixture of 9 ml of Moni-Trol I and 1 ml of Interference Check A (IRC Japan), which contains 208 mg/dl conjugated-bilirubin, or distilled water was tested for its creatinine content in the presence of various amounts of potassium ferrocyanide in enzyme solution B. As shown in Table 2, the creatinine content estimated in the presence of 21.12 μg/ml potassium ferrocyanide compared favorably to that of the bilirubin-free control (98.8%).

The compositions of the two enzyme solutions were finally established as shown in "Materials and Methods" section.

*Within-run reproducibility*

Using Moni-Trol I and II (American Dade) which contain 1.0 mg/dl and 5.5 mg/dl creatinine, respec-

**Table 1.** Reversal of the effect of ascorbate on the creatinine determination by ascorbate oxidase

| Ascorbate oxidase (U/ml) | Creatinine determined (%) | |
|---|---|---|
| | Moni-Trol I | Moni-Trol I + 20 mg/ml ascorbic acid |
| 0 | 100 | 52.3 |
| 5 | 100.1 | 77.5 |
| 15 | 100.0 | 98.2 |
| 20 | 99.3 | 99.1 |

**Table 2.** Reversal of the effect of bilirubin on the creatinine determination by potassium ferrocyanide

| Potassium ferrocyanide (μg/ml) | Creatinine determined (%) | |
|---|---|---|
| | Moni-Trol I + $H_2O$ (9:1) | Moni-Trol I + Interference check A (9:1) |
| 0 | 100 | 71.7 |
| 5.28 | 100.0 | 73.8 |
| 10.56 | 100.0 | 95.2 |
| 21.12 | 100.0 | 98.8 |

**Table 3.** Within-run reproducubility in the creatinine determination

| Sample | Creatinine determined (mg/dl) | Statistical data |
|---|---|---|
| Moni-Trol I | 1.0, 1.0, 1.0, 1.0, 1.0,<br>1.0, 1.0, 1.0, 1.0, 1.0,<br>1.0, 1.0, 1.0, 1.0, 1.0,<br>1.0, 1.0, 1.0, 1.0, 1.0 | n = 20<br>mean = 1.0 mg/dl<br>SD = 0.0 mg/dl<br>CV = 0.0 % |
| Moni-Trol II | 5.5, 5.5, 5.4, 5.4, 5.5,<br>5.4, 5.5, 5.6, 5.5, 5.5,<br>5.5, 5.5, 5.4, 5.4, 5.4,<br>5.5, 5.4, 5.4, 5.4, 5.5 | n = 20<br>mean = 5.48 mg/dl<br>SD = 0.052 mg/dl<br>CV = 0.95 % |

tively, the within-run reproducibility was tested. As shown in Table 3, the respective CV values were 0.0 and 0.95%.

## DISCUSSION

The serum creatinine level was determined enzymatically by the creatininase-creatinase-sarcosine oxidase system using a HITACHI 7250 autoanalyzer. This system requires only 5 μl of serum instead of the 60 μl needed for the conventional method. The final reaction volume was also scaled down from 2160 μl to 315 μl. In addition, the determination of creatinine can be done on a single line of enzyme reactions whereas conventionally it has been done on duplicate lines.

The correlation coefficient of the results obtained by the present method and those by the conventional method was 0.9986 with the linear regression equation being Y = 0.9855X + 0.0423 (n = 168). Thus, the new method seems superior to the conventional one in terms of economy with no loss of reliability when compared with the conventional method.

The effect of endogenous creatine and sarcosine can be suppressed by incubating the serum with creatinase and sarcosine oxidase for 5 min at 37°C before the addition of creatininase to the reaction mixture. Likewise, the interference of ascorbate with peroxidase reaction can be reversed by ascorbate oxidase in enzyme solution A. The interfering effect of bilirubin on peroxidase reaction becomes negligible when potassium ferrocyanide is present in enzyme solution B. The creatinine levels of normal adult serum samples (n = 94) tested by this method ranged between 0.46 and 0.97 mg/dl. The same samples were estimated for their creatinine levels by the Jaffé reaction method with the results being 0.50 - 1.29 mg/dl.

Thus, the present method will be economical and useful in clinical laboratories, especially those equipped with automated analyzing apparatus.

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