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A Fluorophotometric Determination of Serum Creatinine and Creatine using a Creatinineamidohydrolase-Creatineamidinohydrolase-Sarcosine Oxidase-Peroxidase System and Diacetyldichlorofluorescin[1]

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A sensitive method for the fluorophotometric assay of creatinine and creatine was established by using a novel enzyme system, creatinineamidohydrolase-creatineamidinohydrolase-sarcosine oxidase-peroxidase system. Diacetyldichlorofluorescin, which has been regarded as a stable storage form of dichlorofluorescin, was found to react readily with peroxide in the presence of peroxidase in a buffer, (pH 7.7) to give intense fluorescence. This reagent was employed for the estimation of hydrogen peroxide generated by the action of the above enzyme system on creatinine and creatine. The present method is highly sensitive and only 20 μl of serum is required for each assay. Ascorbic acid, bilirubin and uric acid, which often affect enzymatic assay methods that utilize peroxidase, did not interfere with the present method. Excellent recovery from human serum was observed for both creatinine and creatine. The results obtained by the present method showed a good correlation with those obtained by the Jaffé method.

Keywords——serum creatinine; creatinine determination; creatine determination; enzymatic analysis; creatinineamidohydrolase; creatineamidinohydrolase; sarcosine oxidase; diacetyldichlorofluorescin; fluorometry; peroxidase

Measurements of serum creatinine and creatine provide significant diagnostic information regarding renal and muscular function. The Jaffé reaction has commonly been used for the estimation of creatinine,[3] but this method is not specific and requires a comparatively large amount of serum. Moreover, this method cannot be directly applied to the determination of creatine. Assay of "true creatinine" has therefore been attempted by using chromatographic separation[4] or enzymatic reaction.[5,6] In a typical enzymatic method, creatine, produced by the hydrolysis of creatinine with creatinineamidohydrolase (creatininase), is acted upon by creatine kinase, and then pyruvate kinase and lactate dehydrogenase; the overall result is a change in the NADH concentration, which can be measured by spectrometry or fluorometry.[6]

Recently, Suzuki isolated creatineamidinohydrolase (creatinase)[7] and sarcosine oxidase[8] from *Flavobacterium* sp. U-188 and *Colinebacterium* sp. U-96, respectively. This paper describes a fluorometric method for the determination of serum creatinine and creatine, empolying these newly obtained enzymes coupled with peroxidase (POD). In addition, diacetyldichlorofluorescin[9] was used as a sensitive fluorescence reagent for the peroxide generated.

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1) This work was supported by a grant-in-aid for scientific research (No. 221220) from the Ministry f Education, Science and Culture, Japan.
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Materials and Methods

Creatininase (EC 3.5.2.10; 50.8 U/mg), creatinase (EC 3.5.3.3.; 7.5 U/mg) and sarcosine oxidase (EC 1.5.3.1; 2.5 U/mg) were gift of Mr. Masaru Suzuki, Noda Institute of Industrial Sciences. POD (EC 1.11.1.7; 100 U/mg), Preciset® Creatinine as a standard solution of creatinine, and Creatinine Color Test, a commercial kit (Jaffé method), were purchased from Boehringer Mannheim GmbH (Mannheim). Hyland Q-Pak® Chemistry Control Serum I was obtained from Travenol Laboratories Inc. (Illinois). *p*-Chloromercuribenzoic acid was purchased from Wako Pure Chemical Industries Ltd. (Osaka). The other chemicals used were of reagent grade. Diacetyldichlorofluorescin was prepared according to the method of Keston and Brandt[10] from dichlorofluorescein, which was obtained from Tokyo Chemical Industry Co. Ltd. (Tokyo).

Diacetyldichlorofluorescin Stock Solution——Diacetyldichlorofluorescin (10 mg) is dissolved in 100 ml of ethanol. This stock solution is stable for at least six months in a refrigerator.

Diacetyldichlorofluorescin Reagent——A 3 ml portion of the above stock solution is diluted with 50 mM phosphate buffer, pH 7.7, to make 100 ml. This reagent is stable for about 1 week in a refrigerator.

Enzyme Solution——Enzyme solution I is prepared by dissolving 11.8 mg of creatininase, 20 mg of creatinase, 12 mg of sarcosine oxidase and 3 mg of POD in 50 mM phosphate buffer, pH 7.7, to make 50 ml. Enzyme solution II is prepared by dissolving 20 mg of creatinase, 12 mg of sarcosine oxidase and 3 mg of POD in 50 mM phosphate buffer, pH 7.7, to make 50 ml. Enzyme solution III is prepared by dissolving 12 mg of sarcosine oxidase and 3 mg of POD in 50 mM phosphate buffer, pH 7.7, to make 50 ml. Each enzyme solution should be prepared immediately before use.

Assay Procedure——System I: To 20 μl of serum are added 2.5 ml of diacetyldichlorofluorescin reagent and 1.0 ml of the enzyme solution I, and the mixture is incubated at 37° for 30 min. System II: To 20 μl of serum are added 2.5 ml of diacetyldichlorofluorescin reagent and 1.0 ml of the enzyme solution II, and the mixture is incubated under the same conditions as system I. System III: To 20 μl of serum are added 2.5 ml of diacetyldichlorofluorescin reagent and 1.0 ml of the enzyme solution III, and the mixture is incubated under the same conditions as system I. The reaction of system I, II, or III is terminated by adding 0.5 ml of 2% sodium carbonate solution containing 1 mM *p*-chloromercuribenzoic acid.[11] Fluorescence intensity is measured at excitation and emission wavelengths of 337 nm and 517 nm with a Shimadzu RF-510 spectrofluorophotometer.

The serum creatinine concentration is obtained from the fluorescence intensity difference between systems I and II. The serum creatine concentration is obtained from the fluorescence intensity difference between systems II and III.

Results

The excitation spectra obtained by the reaction of the diacetyldichlorofluorescin reagent with H_2O_2 in the presence of POD show excitation maxima at 337 nm and 503 nm, and give a

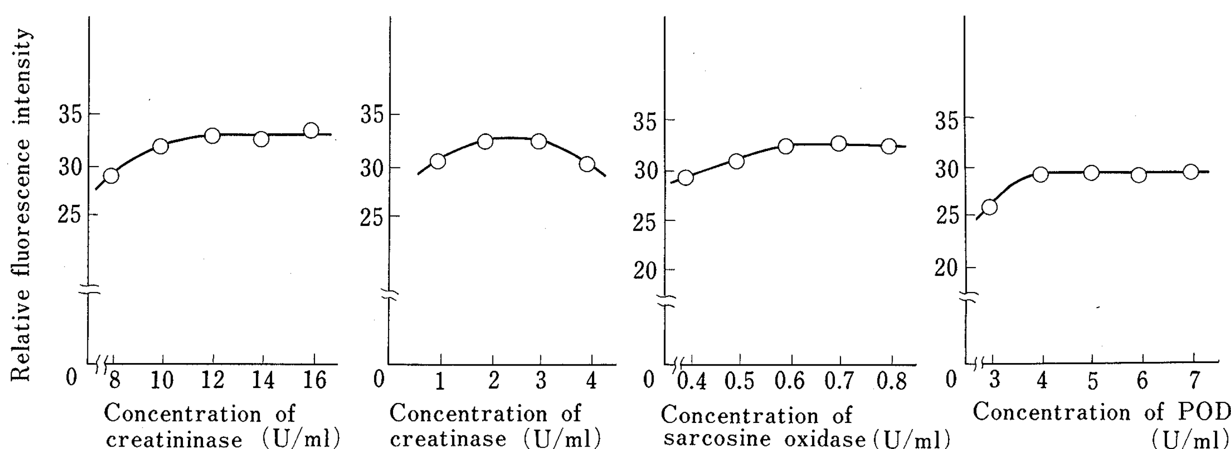

Fig. 1. Effects of Enzyme Concentration of the Fluorescence Intensity

A 20 μl portion of aqueous solution containing 1.2 μg of creatinine and the enzyme solution I was used for each examination. The abscissa shows the concentrations of each enzyme examined in the enzyme solution I. The concentrations of reagents and enzymes other than the enzyme examined were those in the standard procedure.

10) R. Brandt and A.S. Keston, *Anal. Biochem.*, **11**, 6 (1965).
11) M. Suzuki, *Medical Technology*, **7**, 945 (1979).

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single fluorescence maximum at 517 nm. The excitation maximum at 337 nm is used in the standard procedure because excitation at 503 nm may affect the measurement of fluorescence.

Figure 1 shows the fluorescence intensity from creatinine plotted against the enzyme concentrations. Maximum fluorescence was observed at 12 U of creatininase, 2.0 U of creatinase, 0.6 U of sarcosine oxidase and 4.0 U of POD in the reaction mixture.

The fluorescence intensity increased with increasing pH, but the optimum pH values for creatininase, creatinase, sarcosine oxidase and POD are 6.5,[7] 7.7,[7] 7.7[8] and 7.0,[12] respectively. Accordingly, pH 7.7 was adopted in the standard procedure.

The fluorescence intensities of creatinine and creatine incubated with systems I and II reached plateaus in 60 min and 40 min, respectively. In order to limit the reaction time,

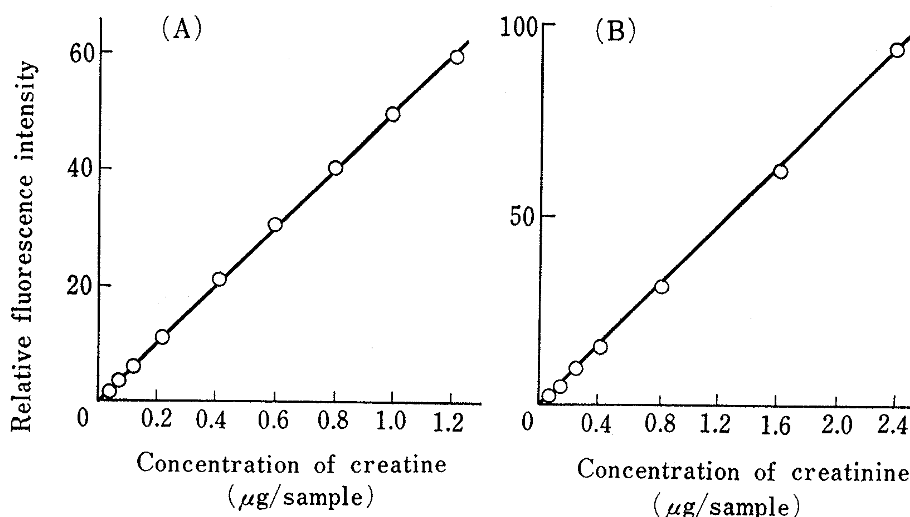

Fig. 2. Standard Curves for Creatine (A) and Creatinine (B)

TABLE I. Recovery of Creatinine and Creatine added to Serum (20 μl)

No.	Creatine added	Recovery (%)	Creatine added	Recovery (%)
1	0.12 μg/sample (0.6 mg/dl)	103.8	0.6 μg/sample (3.0 mg/dl)	100.0
2		103.4		96.4
3		100.0		95.6
4		100.0		95.2
5		100.0		95.2
6		100.0		95.2
7		100.0		94.8
Average		101.0		96.1
C.V. (%)		2.42		1.88

No.	Creatinine added	Recovery (%)	Creatinine added	Recovery (%)
1	0.24 μg/sample (1.2 mg/dl)	102.0	1.2 μg/sample (6.0 mg/dl)	96.3
2		94.9		95.2
3		98.9		96.6
4		98.7		99.0
5		95.6		100.5
Average		98.0		97.5
C.V. (%)		2.92		2.22

12) G.G. Guilbault and D.N. Kramer, *Anal. Chem.*, **36**, 1662 (1964).

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the incubation is terminated by addition of *p*-chloromercuribenzoic acid solution, which inhibits sarcosine oxidase.

Standard curves for creatine and creatinine are illustrated in Fig. 2 (A) and (B), respectively. The plots are linear in the range of 0.03 μg to 2.4 μg fo rcreatine and 0.06 μg to 2.4 μg for creatinine, and the slopes decrease above these upper limits. The standard plots illustrated in Fig. 2 cover the practically important range from normal to abnormally high concentrations

Table II. Interference by Several Compounds when added to 0.20 μg/sample of Creatinine

Compound	Weight of compound added (μg)	Found as creatinine (μg)	Recovery of creatinine (%)
Ascorbic acid	0.40	0.20	100.0
Bilirubin	0.1	0.19	95.0
	1.0	0.19	95.0
Glucose	200	0.20	100.0
	2000	0.21	105.0
Glycine	0.2	0.19	95.0
	2.0	0.20	100.0
Heparin	0.02	0.19	95.0
Thiamine	0.2	0.20	100.0
Urea	72	0.21	105.0
	720	0.20	100.0
Uric acid	0.6	0.20	100.0
	3.0	0.20	100.0
$CuSO_4$	0.08	0.19	95.0
	0.40	0.20	100.0
$FeCl_3$	0.96	0.20	100.0
	9.60	0.20	100.0
NaF	2.0	0.20	100.0

Table III. Interference by Several Compounds when added to 0.12 μg/sample of Creatine

Compound	Weight of compound added (μg)	Found as creatine (μg)	Recovery of creatine (%)
Ascorbic acid	0.40	0.12	100.0
Bilirubin	0.10	0.12	100.0
	1.0	0.12	100.0
Creatinine	0.24	0.12	100.0
	2.40	0.12	100.0
Glucose	200	0.12	100.0
	2000	0.12	100.0
Glycine	0.40	0.12	100.0
	4.0	0.12	100.0
Heparin	0.02	0.12	100.0
Urea	72	0.12	100.0
	720	0.12	100.0
Uric acid	0.60	0.12	100.0
	3.0	0.12	100.0
$CuSO_4$	0.08	0.12	100.0
	0.40	0.12	100.0
$FeCl_3$	0.96	0.12	100.0
	9.60	0.12	100.0
NaF	2.0	0.12	100.0

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of both substances in 20 µl of serum. Excellent recoveries of creatine and creatinine from human serum (Hyland Q-Pak® Control Serum I) were obtained, as shown in Table I.

Tables II and III summarize the interference from several compounds with fluorescence, examined by measuring the fluorescence intensity from creatinine and creatine in the presence of these compounds. None of the compounds tested affect either assay.

Figure 3 shows the correlation between the serum concentration of creatinine obtained by the present method and that obtained by the Jaffé method (Creatinine Color Test) from 20 patients. The calculated correlation coefficient was 0.9925, and the linear regression equation was $Y=0.9650X+0.03360$.

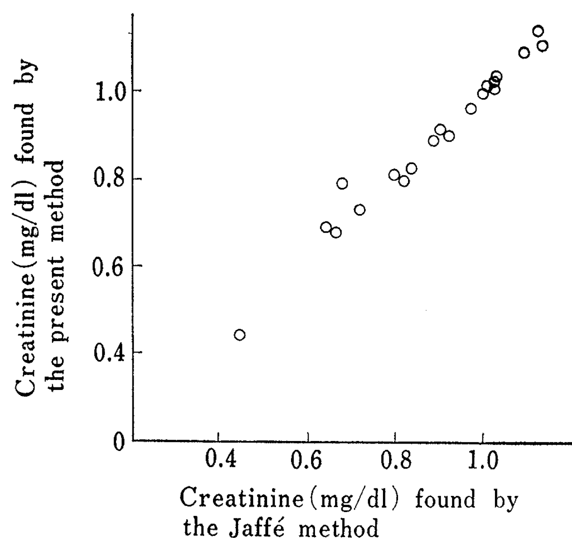

Fig. 3. Correlation between the Results obtained by the Present Method (Y) and those of the Jaffé Method (X) for the Determination of Creatinine Concentration in Serum

$Y=0.9650X+0.03560$, $r=0.9925$, $n=20$.

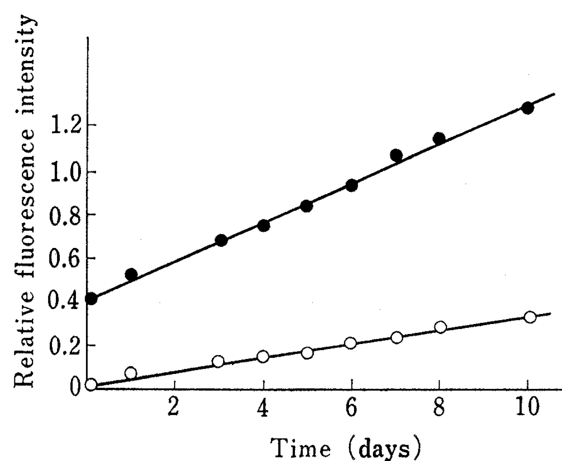

Fig. 4. Stability of Diacetyldichlorofluorescin and Alkali-treated Diacetyldichlorofluorescin at 4°

—○—: To 3 ml of ethanolic diacetyldichlorofluorescin solution (100 µg/ml) was added 50 mM phosphate buffer, pH 7.7, to make 100 ml.
—●—: To 3 ml of ethanolic diacetyldichlorofluorescin solution (100 µg/ml) was added 12 ml of 10 mM NaOH, and the mixture was allowed to stand for 20 min then diluted with 50 mM phosphate buffer, pH 7.7, to make 100 ml.

Discussion

Moss *et al.*[6] reported a kinetic method for determining serum creatinine by using creatininase, coupled with creatine kinase, pyruvate kinase and lactate dehydrogenase. However, their method was based on the decrease in the absorbance of NADH and it required 250 µl of serum for a single assay. Recently, Chen[13] proposed a fluorometry based on the same principle but giving better sensitivity. However, their method requires a special technique in the assay procedure since it employs a silicone rubber pad for the reaction.

In the present study, a novel enzyme system was introduced into the assay of creatinine and creatine. The creatininase–creatinase–sarcosine oxidase system coupled with POD and diacetyldichlorofluorescin permitted the determination with only 20 µl of serum (without deproteinization) due to its high sensitivity and selectivity.

Keston and Brant[9] have shown that diacetyldichlorofluorescin serves as an excellent fluorescence reagent for the assay of peroxide in the presence of POD. Diacetyldichlorofluorescin was treated with alkali before use.[9] Although diacetyldichlorofluorescin is a very stable compound, it becomes unstable after alkali treatment and gives a high blank value due

13) S.P. Chen, S.S. Kuan, and G.G. Guilbault, *Clin. Chim. Acta*, **100**, 21 (1980).

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to autoxidation. In the present method, diacetyldichlorofluorescin gives intense fluorescence without alkali treatment, and the reagent blank value is substantially reduced. The reagent may be simultaneously hydrolyzed and oxidized by the enzyme system.

Figure 4 shows the fluorescence intensity of diacetyldichlorofluorescin and alkali-treated diacetyldichlorofluorescin in the phosphate buffer, pH 7.7, plotted against the time of standing at 4°. This figure indicates that intact diacetyldichlorofluorescin is far more stable than the alkalitreated compound.

All the enzymes used are stable in the solid state for 1 month in a refrigerator. Creatininase is also rather stable in solution, whereas creatinase and sarcosine oxidase are unstable in solution, and the latter enzymes should be dissolved immediately before use. It would be useful in the future to study methods for stabilizing these enzymes.

The present method is not interfered with by ascorbic acid, bilirubin, or uric acid, which all affect the peroxide-POD system (Tables II and III). The effects of these substances are cancelled out by taking the difference of the fluorescence intensity between two enzyme systems containing POD.

There is an excellent correlation between the serum creatinine concentrations observed by the present method and by the Jaffé method.

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