A “Reagentless” Fluorometric Method for Creatine Kinase Activity in Serum

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A “reagentless” fluorometric method is described for the analysis of serum creatine kinase (CK) activity. The method is based on the use of silicone rubber pads, upon which are placed all the reagents for assay of CK. The rate of formation of NADH fluorescence at 460 nm is measured and equated to CK activity. The method is simple, rapid, inexpensive, and as little as 3 µl of serum is needed.

Additional Keyphrase: reagents impregnated onto silicone rubber pads

Measurement of serum creatine kinase (CK; EC 2.7.3.2) activity is of great value in the diagnosis of myocardial and skeletal muscle diseases (1–3). The greater specificity of CK over that of other enzymes, such as lactate dehydrogenase and aspartate aminotransferase, for the detection of muscular dystrophy and myocardial infarction has attracted much interest in CK determinations. In a preliminary study by the Sigma Co. (4) it was noted that the average increase in CK activity after an infarction is almost 1000%.

We report here a “reagentless” micro-fluorometric method for the determination of serum CK. The basic reaction was first described by Oliver (5) and was subsequently improved by Rosalki (6) and Hess et al. (7). In this procedure, the “reverse” reaction of CK is coupled with two indicator reactions as follows:

\[
\text{Creatine phosphate + ADP } \xrightarrow{\text{CK}} \text{ creatine + ATP (1)}
\]

\[
\text{ATP + glucose } \xrightarrow{\text{hexokinase}} \text{ ADP + glucose-6-phosphate (2)}
\]

\[
\text{Glucose-6-phosphate + NAD } \xrightarrow{\text{GPD}} \text{ 6-phosphogluconate + NADH + H}^+ \ (3)
\]

The production of NADH is measured fluorometrically at an excitation wavelength of 340 nm and an emission wavelength of 460 nm.

In this procedure the “reagentless,” solid-surface fluorometric method previously described by Guillbault et al. for assay of cholinesterase (8), alkaline phosphatase (9), and LDH (10) was used: all the reagents and enzymes for an assay are immobilized, in lyophilized form, on the surface of an appropriate pad. The sample of the fluid containing the substance to be assayed is then added, and the fluorescence produced is measured by use of a filter fluorometer. Advantages of the system are speed (generally less than 1–2 min by an initial-rate method); no external reagents are needed, only the sample need be added; the dry reagent pads are stable for weeks or months; self-quenching interferences in fluorescence assays are minimized; the system is more selective and gives a large linear concentration range; and only 3–25 µl of sample is required. In obtaining the results reported here, we used, for convenience, the Worthington “Statzyme” CK kit, which contains all the enzymes, cofactors, and substrates for CK assay.

Materials and Method

Reagents and Solutions

“Statzyme” CK (Worthington Biochemicals Corp., Freehold, N. J. 07728; Cat. No. 7860), supplied in individually sealed vials, was stored in the refrigerator before use.

Fresh sera were stored at −20 °C before use and were not kept longer than three days. Their CK activities had been obtained spectrophotometrically with the CK “Statzyme” kit, at 30 °C. Reference sera were measured by the same procedure.

Equipment

An Aminco filter fluorometer, set on its side and equipped with the cell and cell holder described below, was used for all measurements. A Wratten 7-60 filter and a combination Wratten 47B and 48 filter were used as the primary and secondary filters, respectively. A Beckman 12.5-cm linear recorder was used to display the results obtained.

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Received May 15, 1973; accepted June 20, 1973.
The cell and cell holder (Figure 1) used was a modified version of that designed by Guilbault and Vaughn (9). The cell holder consisted of an Aminco cuvet adapter (Cat. No. 4-7330) held to constant temperature by circulating water. Black binders were placed on both sides of the two entrance and exit slits so that smaller slits, about two-thirds the length of the pad used, would restrict the radiation entering and leaving the cell cavity.

The cell is constructed of a cylindrical aluminum rod with a slot, approximately twice the length of the pad, located towards the end of the rod. The depth of the slot is such that the pad, with its contents, receives the full beam of incident radiation. The cell is painted a dull black to avoid scattered light.

A Virtis automatic freeze-dryer (Model No. 10-010) connected to a vacuum desiccator was used for all lyophilization.

A Hamilton 10-μl syringe (Microliter No. 701) and a Hamilton 50-μl syringe (Gastight No. 1705) were used to apply serum and water, respectively, to the pad.

**Preparation of silicone rubber pads.** Silicone rubber (Glass and Ceramic Adhesive, Dow-Corning, Midland, Mich. 48640; Cat. No. 3145) pads were prepared by pressing uncured silicone rubber between a glass plate and a stainless-steel mold (Figure 2), both of which were lined by a piece of glassine paper (Eli Lilly and Co., supplied by Sargent-Welch Sci. Co., Skokie, Ill. 60076). The surfaces in contact with the silicone rubber were pre-lubricated with a thin layer of Dow-Corning silicone stopcock grease. The silicone rubber was left in the mold at room temperature for two days, to cure. The cured strips were then removed, wiped, then washed briefly with concentrated KOH solution to remove the grease. The pads were then washed with water and dried at 80 °C for 1 h. The strips were cut to individual pads 6 mm in width. About 20 individual pads can be obtained from one strip.

A CK reagent solution was prepared by dissolving the contents of a Worthington CK "Statzyme" vial in 0.29 ml of ice-cold distilled water. Then 10 μl of this solution was applied to each pad with a syringe. At least 27 pads can be so prepared from the con-
tents of each vial, and 100 pads can be lyophilized at once. (Restrict the rate of evacuation initially with the ground joint stopcock of the desiccator to avoid splattering). After about 1 h of lyophilization, a white crystalline film of reagent appeared on each pad. The pads can be refrigerated until use, stored either in the same desiccator or in individual vials.

*Calibration curve and sample analysis.* A calibration curve for analysis was prepared by diluting a reference serum known to have an abnormally high CK value (about 560 U/liter) with water. The pads were removed from the refrigerator and were allowed to reach room temperature (about 30 min). Then 87 μl of water at 30 °C was added to each pad placed in the cell holder, followed by 3 μl of the serum sample. The holder was maintained at 30 ± 0.1 °C. The recorder was turned on, and the fluorescence at 460 nm (λex = 340 nm) was measured. In about 3 to 6 min the reaction rate would become linear, then the change in fluorescence per 5 min (ΔF/5 min) was measured and was plotted vs. serum CK activity to obtain the curve shown in Figure 3, which was linear from 0–540 U/liter.

For assay of CK activity in unknown serum, the same procedure as above was followed. The activity of the sample was read from the calibration plot. If the sample has an activity greater than 504 U/liter, use less sample (1–2 μl) with more water to make a total of 90 μl.

**Results and Discussion**

Reported herein is a fluorometric silicone rubber pad method for serum CK. It is specific, simple, rapid, and as little as 3 μl of serum sample is needed. All the reagents are immobilized as a crystalline film on the pad, so that no tedious reagent preparation is required. Commercially available CK “Statzyme” kit was used, out of convenience only; still the cost of analysis is less than 5 cents, only 1/24 the cost of each current spectrophotometric method for CK.

**Characteristics of the Reaction Curve**

Hess et al. (11) in an evaluation of the spectrophotometric method for CK (identical to the fluorometric method) noted a time lapse (Phase A) between the addition of serum sample and the onset of the linear reaction rate (Phase B). In general, the higher the CK activity, the shorter are Phase A and Phase B. Similar results were obtained in the fluorometric method. With 50 U/liter of CK, Phase A is about 6 min and Phase B about 12 min; with 500 U/liter, Phase A is less than 3 min and Phase B lasts about 5 min.

**Effect of Temperature and Serum Sample Size**

The effect of using a larger volume of serum sample, 5 μl, while keeping the 87 μl of water constant, was studied. The range of linearity of the calibration curve shortened as expected and a linear relationship was obtained only to 336 U/liter. This is equivalent to about 560 U/liter for a 3-μl sample.

If a temperature of 25 °C (room temperature) is used rather than 30 °C as described, a linear calibration curve can be obtained for 0–250 U/liter with a 5-μl sample.

**Stability of Pads**

The stability of the pads and the lyophilized “Statzyme” reagent were studied fluorometrically and spectrophotometrically. For this study two 10-ml samples of serum having activities of about 100 and 200 U/liter were obtained from the hospital. These were divided into several airtight vials and were stored in a freezer at −20 °C until use. The CK pads were prepared and stored in a refrigerator.

In the fluorescence method on each of the days indicated in Table 1, 3 μl of serum, having a CK activity of about 200 U/liter, was added to a pad at 30 °C.

For the spectrophotometric method, two CK “Statzyme” reagents were tested for stability. In one, the commercial reagent was stored as obtained in the refrigerator. In the second, the reagent was dissolved in 0.29 ml of water, then lyophilized for 1 h and stored in the refrigerator. In each case the stored reagent was diluted on each of the days shown in Table 1. Each reagent was reconstituted with 2.9 ml of water, 0.1 ml of serum (activity, about 100 U/liter) was added, and the change in absorbance, ΔA/5 min, was measured at 340 nm and 30 °C according to the procedure outlined in the Worthington manual (12).

Table 1 shows the stability of the freeze-dried pads for fluorometric assay and also the stability of the CK “Statzyme” reagent stored under these two conditions. In this investigation it is assumed that the CK activity in frozen serum is stable for at least a month (13). There is a conflicting report (11) on the stability of activity of CK in frozen serum. However, it is safe to conclude that the pads and the lyophilized CK pads and lyophilized CK ("Statzyme" Vials)

<table>
<thead>
<tr>
<th>Daysa</th>
<th>Pads (ΔF/5 min)</th>
<th>Daysb</th>
<th>Non-lyophilizedc</th>
<th>Lyophilized</th>
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<tr>
<td>0</td>
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<td>0</td>
<td>0.094</td>
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<td>5</td>
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<td>0.098</td>
<td>0.095</td>
</tr>
<tr>
<td>9</td>
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<td>20</td>
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</tr>
<tr>
<td>14</td>
<td>28.7</td>
<td>30</td>
<td>0.091</td>
<td>0.098</td>
</tr>
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</table>

*a Days after preparation of CK pads by lyophilization. Stability studied fluorometrically.
*b Days after preparation of CK Statzyme vial by lyophilization. Stability studied spectrophotometrically.
*c Commercial CK Statzyme vials used according to manufacturer's instructions.
lized CK reagent vials were stable for at least 32 days and probably for as long as three months with less than a ±5% activity loss.

Reproducibility

A serum of unknown CK activity was analyzed 15 times with the same batch of CK pads on a single day. A mean value of 189 U/liter was obtained (SD, 8.7 U/liter; CV, 4.6%).

Sample Analysis

Fresh sera were obtained from the Methodist Hospital in New Orleans and were assayed fluorometrically by the present method and spectrophotometrically by the Statzyme CK method. Results obtained by the two methods are compared in Table 2. The fluorometric method appears less sensitive at the lower range of CK values (less than 50 U/liter), but the results agree very well with those by the accepted spectrophotometric procedure at high values. Since clinicians are only interested in elevated CK values (CK activity increases by an average of 1000% after an infarction), this new method should be ideally suited to clinical analysis.

The financial assistance of the National Institute of Arthritis and Metabolic Diseases, NIH (Contract No. NIAMD-72-2216) is gratefully acknowledged. We also thank the Methodist Hospital, New Orleans, Louisiana, for providing samples of serum, and Dr. Peter M. Froehlich for helping with designing the steel mold used for the pads.

References