Improved Electrophoretic Separation of Creatine Kinase Isoenzymes

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Creatine kinase isoenzymes are separated electrophoretically on cellulose acetate, with use of an improved procedure for reactivation and visualization of enzymic activity. N-Acetyl cysteine is used as the reactivator and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide for visualization in a tetrazolium-coupled system. This test, both practical and reliable, is a suitable alternative to chromatographic or immunochemical assay.

Materials and Methods

Materials

Sera obtained from hospital patients were examined immediately or stored at −20 °C for not longer than 24 h. Total CK activity was measured in them according to the Rosalki method (7), with commercial reagents ("CPK activated," Boehringer, Mannheim, G.F.R.; and "Merckotest CK NAC-aktiviert," Merck, Darmstadt, G.F.R.). The reference interval for this assay is 10–100 U/L, at 30 °C. Aspartate aminotransferase (EC 2.6.1.1) and lactate dehydrogenase (EC 1.1.1.27) activity were measured according to 8 and 9, also with use of commercial reagents ("Spin Chem SGOT" and "Plus Chem LDH-P"); SKI, Sunnyvale, CA 94086). For lactate dehydrogenase isoenzyme electrophoresis I used cellulose acetate according to Dietz et al. (10), modified (11), with use of a commercial modification (Chemotron Chimica, 20189 Milano, Italy).

The CK-MB isoenzyme was determined by the immunological method according to Würzburg et al. (12), with commercial reagents ("Merckotest CK-MB NAC-aktiviert").

Procedure

For electrophoresis I used 2.5 × 17 cm cellulose acetate strips ("Chemegel," Chemotron Chimica) and barbital buffer (50 mmol/L, pH 8.6). I used 5 μL of serum, or 10 μL if measuring samples with total CK activity <100 U/L. At room temperature, separation was achieved in 50 min at 200 V.

For each run, 5 mL of a 10 g/L agar gel was melted in a bath of boiling water, cooled to about 45 °C, and mixed with 3 mL of substrate-reactivator-buffer solution. Per liter, this solution contained: diadenosine pentaphosphate 10 μmol, magnesium acetate 10 mmol, ADP 2 mmol, AMP 5 mmol, NADP+ 2 mmol, creatine phosphate 30 mmol, hexokinase (EC 2.7.1.1) > 2.5 kU; glucose-6-phosphate dehydrogenase (EC 1.1.1.49) > 1.5 kU; N-acetyl cysteine 20 mmol, imidazole buffer (pH 6.7) 0.1 mol, glucose 20 mmol, and disodium EDTA 2 mmol. (I used commercial reagents: "Merckotest CK-NAC aktiviert,"
reconstituted according to the manufacturer's instructions.)

The complete mixture was poured into 12.5 x 9.5 cm plastic trays or into Petri dishes 12-15 cm in diameter and allowed to solidify for 2-3 min. After electrophoretic separation was finished the cellulose acetate strips were removed, trimmed, and placed inverted onto the gel surface. The trays were kept at 25, 30, or 37 °C for 30 min, then the strips were removed and dipped into a freshly prepared staining solution consisting of 10 mg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide and 5 mg of phenazine methosulfate (both from Sigma Chemical Co., St. Louis, MO 63178) in 100 mL of distilled water. Violet bands on a white background appear at the location of CK isoenzymes after 1-2 min, and the strips can then be stored for several days in water/acetic acid solution (95/5 by vol). Relative activities for the various bands were estimated by densitometric scanning at 500-550 nm.

As an alternative, I used a substrate with a different reactivator ("CPK activated," Boehringer). After reconstitution according to the manufacturer's instructions, its composition per liter was: trithanolamine buffer (pH 7.0) 0.1 mmol, glucose 20 mmol, magnesium acetate 10 mmol, ADP 1.0 mmol, AMP 10 mmol, NADP* 0.6 mmol, glutathione 9 mmol, glucose-6-phosphate dehydrogenase 1.2 kU, hexokinase 1.2 kU, and creatine phosphate 35 mmol.

I tried as chromogen six tetrazolium salts besides the one already mentioned: 2,3,5-triphenyltetrazolium chloride, p-iodonitrotetrazolium violet, netotetrazolium chloride, thio-carbamyl nitro blue tetrazolium, nitro blue tetrazolium, and tetranitro blue tetrazolium (all from Sigma).

An isoenzyme control serum (Ortho Diagnostics, Raritan, NJ 08869) was included as a control with each series of electrophoretic separations and was used in assessing accuracy and precision.

Results

Technical Evaluation

When the enzyme reaction was carried out with either reactivator at 25, 30, and 37 °C, the best results (sharpest and strongest bands) were obtained at 30 and 37 °C; with these temperatures the results were valid for all fractions.

I compared the two reactivators, glutathione and N-acetyl cysteine, in the agar-substrate-buffer and found the latter to be the better (Figure 1): in all sera I examined, the densitometric tracing obtained with use of it showed near-baseline separation and better intensity and purity of the fractions, the latter being confirmed spectrophotometrically.

Twenty sera from our Coronary Care Unit showed a detectable CK₂ band only with the N-acetyl cysteine reactivated method, and not with the glutathione-reactivated one, a finding confirmed by spectrophotometric determination of total CK and CK-MB with the two reactivators. For sensitivity and precision tests, I therefore used only the method with N-acetyl cysteine.

I estimated the sensitivity of this method by means of calibration experiments with various dilutions of inactivated serum to which pure human CK had been added (Ortho Control Serum), applying different dilutions according to the isoenzyme being looked for. The bands were still both visible and detectable by the scanner with serum diluted to contain 8 U/L.

I evaluated the precision of the N-acetyl cysteine activated procedure with human sera of normal (26 U, SD 4 U), slightly above normal (180 U, SD 6 U), and pathologically high (890 U, SD 50 U) CK activity per liter. Ten tubes were set up and stored at −20 °C, and one was tested each day for 10 days; the tracings were repeated eight times a day, for each serum. The within-day CV ranged between 5 and 7%, and the day-to-day CV between 5 and 9%.

Using Isoenzyme Control Serum containing the three isoenzymes of CK in different proportions, I tested the correspondence with densitometric measurement. The results are summarized in Table 1.

No bands were visible when creatine phosphate was omitted from the incubation mixture.

Nonspecific bands are known to appear when tetrazolium salts are used ("nondehydrogenase"), so I checked the effect of volume of serum used, of the presence various concentrations of added bovine albumin (17.5, 35, and 70 g/L), of incubation duration, and of the phenazine methosulfate. In no case did I see any nonspecific bands.

Of the tetrazolium salts tested, only p-iodonitrotetrazolium violet, nitro blue tetrazolium, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide gave satisfactory results, the last being the best (Figure 2).

<table>
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<th>Table 1. Correspondence of Values for CK Isoenzymes (in Per Cent of Total CK Activity) as Measured by the Present Technique and by Five &quot;Kit&quot; Procedures</th>
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* Beckman Instruments, Fullerton, CA. ** Corning/ACI, Palo Alto, CA. † Gelman Instrument Co. Ann Arbor, MI. ‡ Helena Laboratories, Beaumont, TX. § Sigma Chemical Co., St. Louis, MO.  ± values are SD.
Diagnostic Evaluation

Sera from 203 normal subjects and 168 patients without myocardial infarction presented a more or less pronounced isoenzyme band in the β-γ globulin region of the protein tracing, due to CK3 (Figure 3, upper tracing).

Ninety-eight patients admitted to the Coronary Care Unit with a diagnosis of suspected acute myocardial infarction were tested for the presence of the CK2 band (Figure 3, middle). In 82 documented cases of infarction no false positives were obtained, and no false positives were seen in 16 documented cases without infarction. The CK2 band appeared very early after infarction (4-6 h after the clinical episode) and usually had disappeared from specimens collected 72 h later. Correlation between the immunoinhibition and N-acetyl cysteine methods was excellent.

Eight cases with total CK activity above normal and an immunologically detectable CK2 (range: 11-34 U/L) showed no CK2 by the present method; the clinical symptoms and the patients’ course documented the absence of infarction. In such cases an atypical band in the γ-globulin region was seen, along with the band due to CK3. One of these patients had uncompensated diabetes; another had rheumatoid arthritis. For the remaining cases a correlation with pathological state was not possible, because they did not present any clinically evident illness.

In 69 patients undergoing heart surgery, an obvious CK2 band was present in the serum immediately after the operation, and this rapid increase was followed by an equally rapid disappearance within 36 h.

In four cases of progressive muscular dystrophy, a sharply demarcated CK2 fraction was demonstrable.

In a case of congenital neuropathy (Werdnig–Hoffmann disease) with secondary muscular dystrophy, the CK1 band (migrating with albumin) was marked (Figure 3, bottom). In another girl affected by the same syndrome, an increased total CK was accounted for the CK3 fraction alone; the father’s pattern was normal, but the mother’s showed a marked CK1 band in addition to CK3.

Discussion

The described procedure based on the use of N-acetyl cysteine as reactivator and of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide as visualizing reagent allowed me to separate CK isoenzymes in a reliable and diagnostically useful way. The staining procedure I finally adopted for the visualization of CK isoenzymes was that already in use for alkaline phosphatase and arylesterase (13, 14).

The method has a good practicability (rapidity and handiness strictly related to the protein fractionation procedure) and the use of tetrazolium salts is simpler than fluorometry of NADPH. Besides, in electrophoretic fluorometry, bands due to other causes (native fluorescence of serum) can be mistaken for isoenzymes of CK (15–17).

Visual examination of the strips is satisfactory, but scanning allows quantitation.

With this method I could demonstrate the CK2 fraction in cases of muscle dystrophy and document the presence of CK1 both in a patient with neural disease and in another case in one of the parents. The presence of CK2 was consistently demonstrable in documented myocardial infarction and therefore the method is also useful here; although the immunoinhibition method may be more practical and speedy, in some cases detection of CK2 with the immunological method was not correlated with clinical and other laboratory data. In the electrophoretic separation, in fact, CK2 fraction was absent and anomalous bands migrating between CK2 and CK3 were present. Such discrepancy is reasonable, because the immunological method is based on immunoinhibition of the typical M subunit and therefore does not allow one to discriminate between MB and BB hybrids or to recognize totally or partly atypical MM fractions.

The intermediate band I observed between CK3 and CK2...
has as yet no clearcut diagnostic meaning. Others have observed similar atypical bands without reaching a satisfactory interpretation (3, 18–20). An important fact to be considered is that CK comes from other tissues besides myocardium, and that it also comes from two different cellular components: mitochondria and cytoplasm (21, 22). Mercer, on the basis of its column-chromatographic behavior on Sephadex G-200 and immobilization studies, suggests that the atypical isoenzyme is a CK-linked macromolecular complex (immunoglobulin G) with properties similar to macromolecular complexes described for amylase and lactate dehydrogenase (23). Either a conformational isomerism or a combination with carrier proteins might explain this finding. Others (6) postulate interaction of the enzyme with a serum beta-lipoprotein. According to Wevers et al. (24), CK3 is not an homogeneous dimer, and different components can be observed in different pathological states. These interpretations might justify our findings on the basis of molecular heterogeneity of the enzyme, but of course no pathophysiological explanation can be put forward at present.

To conclude, I believe that the proposed method represents a suitable tool to show the three principal CK isoenzymes for diagnostic purpose and to clearly demonstrate an unusual isoenzyme. It is in any case a reliable confirmatory method.

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References