Improved Separation of Creatine Kinase Isoenzymes by Use of DEAE-Sepharose CL-6B

Richard J. L. Bondar, Douglas G. Shevchik, Mei-Yung Hsu, and Margaret M. Kohler

We describe here a simple, rapid chromatographic procedure for quantitatively separating serum creatine kinase isoenzymes (EC 2.7.3.2), with diethylaminoethyl (DEAE)-Sepharose CL-6B as the anion-exchanger. We established the column bed height and the elution parameters by use of a simplex procedure. DEAE–Sepharose CL-6B equilibrated in tris(hydroxymethyl)aminomethane (50 mmol/L, pH 7.5, and containing 30 mmol of NaCl per liter) is packed into a miniature polystyrene column with bed dimensions of 0.7 × 1.5 cm. The DEAE–Sepharose column system was evaluated and compared with a DEAE–Sephadex A-50 column system. The results indicate that the Sepharose column yields MM, MB, and BB isoenzymes uniquely, without cross contamination. Coefficients of variation (CV’s) for 10 replicate column assays were 2.8, 5.9, and 15.2% for 569 U of MM per liter, 82.3 U of MB per liter, and 9.0 U of BB per liter, respectively. The serum sample was enriched with baboon heart extract. Day-to-day reproducibility for a serum control assayed on 10 days yielded CV’s of 4.8, 9.9, and 40.3% for 391, 45.3 and 1.9 U of isoenzymes MM, MB, and BB per liter, respectively. A reference interval for each isoenzyme was derived from data on 81 men and 63 women.

Additional Keyphrases: ion-exchange chromatography • enzyme activity • reference intervals • simplex analysis • heart disease

Determination of CK-MB isoenzyme in serum is quite specific and sensitive in the diagnosis of acute myocardial infarction (1–4). Several column-chromatographic procedures have been described for separation of the CK isoenzymes for routine clinical use (5–10). Morin (11) evaluated several ion-exchange procedures and showed that factors such as ion-exchanger capacity, column dimension, pH, salt concentration, and flow rate could affect resolution and reliability. Recently, Desjardins et al. (12) reported that the binding-site saturation of DEAE-cellulose1 at different pH values will affect the elution pattern of the MB isoenzyme.

We describe here a new column procedure with DEAE–Sepharose CL-6B as the anion-exchanger for CK isoenzyme separation. Once separated, all three isoenzymes can quickly be quantitated.

Materials and Methods

Tissue and Serum Preparation

Human brain and baboon heart were kept frozen (−20 °C) until used. One gram of tissue was cut into small pieces and homogenized at 4 °C in 4 mL of a solution containing, per liter, 1 mmol of disodium ethylenediaminetetraacetate, 10 mmol of KCl, and 1 mmol of dithioerythritol at pH 7.8. After the homogenate was centrifuged at 15 000 rpm for 20 min at 4 °C in a Sorvall Superspeed RC-2B centrifuge (DuPont Co. Instrument Products, Biomedical Division, Newtown, CT 06470), the supernatant fluid was collected and used. The CK-MB fraction from baboon heart was further purified by chromatography on a DEAE–Sepharose column and used to enrich serum pools. The column control is a lyophilized material prepared from extracts of human blood and baboon heart. Human sera were obtained from a local hospital and kept at −20 °C until used.

Column Chromatography

DEAE–Sepharose CL-6B columns. DEAE–Sepharose CL-6B (Pharmacia Fine Chemicals, Division of Pharmacia Inc., Piscataway, NJ 08854) gel suspension is re-equilibrated several times with Tris buffer [50 mmol/L tris(hydroxymethyl)aminomethane, pH 7.5] containing 30 mmol of NaCl per liter before packing to a height of 1.5 cm in a polystyrene column with an inside diameter of 0.7 cm. Before a sample is applied, the column is washed with 6 mL of MM-eluting buffer (see below) and drained completely. Then 0.25 mL of serum sample or control is applied to the top of the column and allowed to enter the resin bed. The three CK isoenzymes are then eluted as follows: elute MM isoenzyme with 3 mL of Tris buffer containing 30 mmol of NaCl per liter; elute MB with 4 mL of this buffer containing 145 mmol of NaCl per liter; elute BB with 2 mL of Tris buffer containing 300 mmol of NaCl per liter.

DEAE–Sephadex A-50 columns. DEAE–Sephadex A-50 (Pharmacia) was prepared by swelling 10 g in 1 L Tris buffer containing 30 mmol of NaCl per liter, overnight at room temperature. The fines were removed by decantation and the resin was re-equilibrated with fresh buffer several times before packing into a polystyrene column (0.7 × 3.0 cm). After washing twice with 5-mL portions of MM-eluting buffer and applying a sample as described for the Sepharose column, the CK isoenzymes were eluted as follows: elute MM isoenzyme with 5 mL of Tris buffer containing 30 mmol of NaCl per liter; elute MB with 3 mL of Tris buffer containing 205 mmol/L NaCl; elute BB with 5 mL of Tris buffer containing 300 mmol of NaCl per liter. This procedure has been available from Worthington Diagnostics, Freehold, NJ 07728, as cat. no. 27638.

Roche column method. Roche columns and buffers (“CPK-CS,” cat. no. 43092; Roche Diagnostics, Division of Hoffman–La Roche Inc., Nutley, NJ 07110) were used ac-

1 Nonstandard abbreviations used: CK, creatine kinase (ATP: creatine phosphotransferase, EC 2.7.3.2); MM, skeletal-muscle CK isoenzyme; MB, cardiac-muscle CK isoenzyme; BB, brain CK isoenzyme; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane.
According to the manufacturer's directions. One milliliter of serum sample was applied to each column, and MM, MB, and BB were eluted with two 4.0-mL applications of Roche buffers A, B, and C, respectively.

Assay of CK Activity

CK activity in serum and in the column fractions was assayed by the method of Oliver (13), as modified by Rosalki (14), with a Model 2400 (Gilford Instruments, Oberlin, OH 44074) or a Model PM6 (Carl Zeiss, Inc., New York, NY 10018) spectrophotometer and "Statzyme CK-I" reagent (cat. no. 27251; Worthington Diagnostics). For the assay of total CK activity, the reagent was reconstituted according to the manufacturer's directions: 3 mL of reconstituted reagent was mixed with 0.1 mL of the serum sample and assayed at 30 °C. For the assay of the CK isoenzymes, the reagent was reconstituted to one-half the volume recommended for total CK activity, i.e., 8 mL for the 16-mL vial. Then 1.5 mL of the concentrated reagent was mixed with 1.5 mL of the column fraction and assayed at 30 °C. Activity was calculated from the rate of production of NADH, as measured by the absorbance change at 340 nm.

Electrophoresis

Corning/ACI agarose electrophoresis system (Corning Medical Diagnostics, Corning Glass Works, Medfield, MA 02052) was used to identify the CK isoenzymes and to study the purity of the column fractions. The agarose film was equilibrated for 1 h with a solution of (per liter) 20 mmol of Tris and 150 mmol of glycine, pH 8.3-8.4, then drained for 10 min before use. The column fractions were concentrated 90- to 100-fold by use of the Minicon-B15 concentrator (Amicon Corp., Lexington, MA 02173) before electrophoresis. A 2- to 4-mL aliquot of the concentrated fraction was applied to the agarose film and electrophoresed in the Tris-glycine buffer for 30 min at 90 V at room temperature. A 16-mL reagent vial of Worthington's "Statzyme CP" (cat. no. 27763) was reconstituted with 8 mL of de-ionized water, and 1 mL of the solution was spread on the agarose surface. We incubated the agarose film at 40 °C for 30 min and identified the isoenzyme bands under an ultraviolet light.

Results

To optimize the isoenzyme elution from the Sepharose column, we applied a simplex strategy (15, 16) to determine the resin bed height, elution volume, and NaCl concentration in the MB eluting buffer. The volumes of serum sample (0.25 mL), MM-eluting buffer (3 mL), and BB-eluting buffer (2 mL) were kept constant during the optimization study. Table 1 lists the 18 vertices tested. The step sizes (increments) were 20 mmol/L for NaCl concentration in the MB buffer, 3 mL for MB-eluting buffer elution volume, and 5 mm for resin bed height. The performance at each vertex was evaluated with two serum pools: one enriched only with MB extract (baboon heart preparation), the other only with the BB extract (human brain preparation). The performance criteria were the ability to separate the isoenzymes individually and the total time required for the separation (less than 50 min was desired). Any appreciable amount of CK activity in the BB fraction with use of the MB-enriched serum was initially assumed to be MB- to-BB carryover, and any appreciable amount of CK activity in the MB fraction with use of the BB-enriched serum was assumed to be BB- to-MB carryover. The column fractions were not checked by electrophoresis during this phase. Table 2 shows the results of the first four vertices. Vertex 3 was eliminated because of the high BB- to-MB carryover. This elimination generated vertex 5 (see Table 1). Vertex 3 was eliminated for the same reason, generating vertex 6 (see Table 1).

A new set of serum pools was made because the first set was depleted. Vertices 1 and 4 through 18 were run with the new pools (Table 3). Any new vertices generated after vertex 18 are duplicates of ones already run, thus ending the simplex. Vertices 4 and 13 were judged to be the best, showing the least apparent MB-to-BB and BB-to-MB carryover. Because both vertices yield similar conditions, the final system (described in Column Chromatography, above) is an average.

The final Sepharose column system was evaluated and compared with the Sephadex column system with several samples. Figures 1 through 3 show chromatograms (CK activity of every 0.5-mL column fraction vs the fraction number) of both Sepharose and Sephadex columns, and indicate better resolution of the isoenzyme peaks with the Sepharose column than with the Sephadex column. The two column systems were also evaluated by comparing for each the isoenzyme activities (Table 4) in the three eluted fractions (see Materials and Methods) and by electrophoresis (Figure 4). Assays were made on duplicate columns, and each isoenzyme fraction was pooled and concentrated 80- to 100-fold. Then 4 mL of the concentrated fraction was applied to the agarose film for electrophoresis. We evaluated several serum pools that had been enriched with different concentrations of MB and BB. Sample 1 was enriched only with partly purified MB, sample 2 only with human brain extract (BB). Samples 3 and 4 were

| Table 1. Simplex Vertices for Optimization of the MB Buffer and Resin Bed |
|------------------|----------------|----------------|----------------|
| Vertex no. | NaCl concn, mmol/L | Buffer vol, mL | Bed height, mm |
| 1 | 135 | 3.0 | 10 |
| 2 | 155 | 3.0 | 10 |
| 3 | 145 | 4.5 | 10 |
| 4 | 145 | 3.5 | 14 |
| 5 | 130 | 4.5 | 12 |
| 6 | 130 | 3.0 | 14 |
| 7 | 135 | 4.3 | 17 |
| 8 | 140 | 5.2 | 15 |
| 9 | 145 | 4.5 | 10 |
| 10 | 135 | 4.0 | 9 |
| 11 | 130 | 3.5 | 13 |
| 12 | 135 | 3.7 | 17 |
| 13 | 145 | 4.3 | 16 |
| 14 | 145 | 4.5 | 11 |
| 15 | 150 | 3.7 | 15 |
| 16 | 155 | 3.2 | 19 |
| 17 | 135 | 3.6 | 18 |
| 18 | 130 | 4.4 | 13 |

| Table 2. Relative Percentage of Isoenzymes Obtained from the First Four Vertices of the Simplex Optimization |
|------------------|----------------|----------------|----------------|
| Vertex no. | MB-enriched pool | BB-enriched pool | % of isoenzyme in |
| 1 | 29.2 | 74.2 | 3.4 |
| 2* | 27.2 | 70.7 | 2.1 |
| 3* | 24.5 | 72.7 | 2.4 |
| 4 | 22.5 | 74.7 | 2.7 |

a Subsequently eliminated (see text).
enriched with all three isoenzymes. The results for samples 1 and 2 (Table 4 and Figure 4) indicate that the fractions eluted from the Sepharose column show no cross contamination, but those from the Sephadex column do. Note the lack of significant difference in isoenzyme activity between the Sepharose and Sephadex columns for samples 3 and 4; but there is cross contamination in the Sephadex column, as demonstrated by electrophoresis. Samples 3 and 4 were also run with the Roche column procedure: Figure 5 shows carryover of MB into the MM and BB fractions as well as BB into the MB fraction by electrophoresis.

Table 5 shows a comparison of CK isoenzyme separations with Sepharose, Sephadex, and the Roche columns. We studied human sera with normal and above-normal CK concentrations, and serum samples enriched with different quantities of baboon heart extract and human brain extract. In general the Roche method shows low MM and MB values...
Table 4. Sepharose and Sephadex Columns Compared for Serum Samples * Enriched with Different Amounts of MB and BB

<table>
<thead>
<tr>
<th>Isoenzyme</th>
<th>Sample 1 (CK = 145 U/L)</th>
<th>Sample 2 (CK = 95 U/L)</th>
<th>Sample 3 (CK = 773 U/L)</th>
<th>Sample 4 (CK = 1270 U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sepharose</td>
<td>Sephadex</td>
<td>Sepharose</td>
<td>Sephadex</td>
</tr>
<tr>
<td>MM, U/L</td>
<td>35.5</td>
<td>30.4</td>
<td>20.9</td>
<td>23.6</td>
</tr>
<tr>
<td>MB, U/L</td>
<td>116</td>
<td>79.2</td>
<td>4.6</td>
<td>21.3</td>
</tr>
<tr>
<td>BB, U/L</td>
<td>4.1</td>
<td>27.1</td>
<td>69.4</td>
<td>48.3</td>
</tr>
</tbody>
</table>

| Total activity, U/L | 156 | 137 | 94.9 | 93.2 | 852 | 831 | 1150 | 1160 |
| % recovery          | 107 | 94.3 | 99.7 | 98.1 | 110 | 108 | 90.3 | 91.3 |

*Samples 1–4 are four different pools of normal human serum enriched with (1) partly purified MB extract; (2) human brain extract; (3 and 4) baboon heart and human brain extracts.

Fig. 4. Results of electrophoresis of column fractions, for samples indicated in Table 4

Positions 1 and 8 are whole-serum samples; MM, MB, and BB fractions from the Sepharose column are in positions 2, 3, and 4, respectively. MM, MB, and BB fractions from the Sephadex columns are in positions 5, 6, and 7, respectively.

by activity, but by percentage the distributions agree with those by the Sepharose method. The apparent proportion of BB activity is greater with the Sephadex column for sera with above-normal CK activity, indicating carryover of MB into the BB fraction. The Sephadex column, however, is able to detect above-normal MB, showing that the method is none-theless diagnostically valid.

To investigate the linear range of the Sepharose column, we used different sample sizes and plotted sample size vs activity in the fractions (Figure 6). The results indicate that the column performs as expected for 0.1- to 0.5-mL samples containing 1200 U/L. Electrophoresis of the concentrated column fractions, however, indicates slight MB contamination in the MM fraction with the 0.5-mL sample.

Within-day precision of the column separation was determined on three samples (normal, above-normal, and an enriched human serum pool), each assayed on 10 columns. Day-to-day precision was determined over a period of 10 days.

Fig. 5. Results of electrophoresis of fractions from Roche column, for samples 3 and 4 from Table 4

Positions 1 and 5 are whole-serum samples; positions 2, 3, and 4 are MM, MB, and BB fractions, respectively. CK isoenzyme activities (U/L) and percent recoveries are: MM 546, MB 150, BB 82.3, and 101% recovery for sample 3; MM 764, MB 193, BB 116, and 84.8% recovery for sample 4.
Table 5. CK Isoenzyme Separation: Comparison of Sepharose, Sephadex, and Roche Columns

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total CK</th>
<th>Sepharose column</th>
<th>Sephadex column</th>
<th>Roche column</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td>MM</td>
<td>MB</td>
<td>BB</td>
</tr>
<tr>
<td><strong>Activity, U/L</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>5.0</td>
<td>5.3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>22.4</td>
<td>20.9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>42.4</td>
<td>33.4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>192</td>
<td>176</td>
<td>10.3</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>234</td>
<td>205</td>
<td>10.3</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>312</td>
<td>270</td>
<td>18.0</td>
<td>2.6</td>
</tr>
<tr>
<td>7</td>
<td>374</td>
<td>351</td>
<td>18.0</td>
<td>5.2</td>
</tr>
<tr>
<td>8</td>
<td>728</td>
<td>595</td>
<td>175</td>
<td>82.3</td>
</tr>
<tr>
<td>9</td>
<td>1250</td>
<td>997</td>
<td>165</td>
<td>16.7</td>
</tr>
<tr>
<td>10</td>
<td>1270</td>
<td>809</td>
<td>234</td>
<td>104</td>
</tr>
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</table>

Table 6. Precision Data

<table>
<thead>
<tr>
<th>MM</th>
<th>MB</th>
<th>BB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within-day (10 runs)</td>
<td></td>
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<tr>
<td>Normal serum</td>
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<tr>
<td>Mean, U/L</td>
<td>24.6</td>
<td>0</td>
</tr>
<tr>
<td>SD, U/L</td>
<td>2.3</td>
<td>---</td>
</tr>
<tr>
<td>CV, %</td>
<td>9.5</td>
<td>---</td>
</tr>
<tr>
<td>Above-normal serum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean, U/L</td>
<td>148</td>
<td>4.6</td>
</tr>
<tr>
<td>SD, U/L</td>
<td>3.6</td>
<td>1.6</td>
</tr>
<tr>
<td>CV, %</td>
<td>2.4</td>
<td>35.2</td>
</tr>
<tr>
<td>Serum enriched with baboon heart extract</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean, U/L</td>
<td>569</td>
<td>82.3</td>
</tr>
<tr>
<td>SD, U/L</td>
<td>15.9</td>
<td>4.8</td>
</tr>
<tr>
<td>CV, %</td>
<td>2.8</td>
<td>5.9</td>
</tr>
<tr>
<td>Day-to-day (10 days)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean, U/L</td>
<td>391</td>
<td>45.3</td>
</tr>
<tr>
<td>SD, U/L</td>
<td>17.9</td>
<td>4.5</td>
</tr>
<tr>
<td>CV, %</td>
<td>4.6</td>
<td>9.9</td>
</tr>
</tbody>
</table>

by separating and assaying the isoenzymes in a serum control. The results are shown in Table 6.

We estimated the reference interval (Table 7) for each of the isoenzymes from data on 81 men and 63 women from the general population who were asymptomatic and normal as evidenced by results of multiphasic screening. The expected MB reference intervals are 0–2.7 and 0–3.4 U/L for men and women, respectively.

Discussion

The objective of this study was to develop an improved, simple, rapid, and quantitative column procedure for the separation of CK isoenzymes. We chose DEAE–Sepharose CL-6B because it has a high binding capacity and an extremely stable bed volume. No appreciable shrinkage of the resin was observed during the eluting process as the salt concentration was increased. Consequently, the three isoenzyme fractions were better resolved, even with a very short column. DEAE–Sepharose CL-6B is a macroporous, bead-formed ion exchanger derived from cross-linked agarose gel and supplied in a suspension. The gel can be easily resuspended and equilibrated in any suitable buffer.

Under the conditions described above, MM is not bound to the Sepharose gel but is eluted in the equilibration buffer.
Because cross-contamination is most frequently observed between the MB and BB fractions, we adopted the simplex strategy to determine the optimum bed height, NaCl concentration, and elution volume of the MB buffer. The resulting column system shows essentially complete separation of the three isoenzymes. Although baboon heart extract was used in the optimization study, MB from both human and baboon heart have been shown to have the same elution profile on DEAE-Sephadex column chromatography and to migrate the same on electrophoresis (8). Furthermore, Figure 3 demonstrates distinct separation of the three isoenzymes in a human serum sample to which no extracts from non-human sources were added. Consequently, we believe that the conditions presented here are adequate for separation of CK isoenzymes of human origin.

The slight apparent carryover of BB into MB for sample 3 on the Sepharose column (Figure 4) is an artifact, because the fluorescent spot in the BB region of the MB fraction appeared before CK-substrate application. As can be seen, sample 4 contains greater concentrations of both MB and BB than sample 3, yet shows no carryover when assayed under similar conditions. This indicates that the amounts of MB and BB in the samples did not exceed the column capacity.

The proportion of each isoenzyme activity in the eluate fractions from the Sepharose column compares very well with that from the Roche column. The time required to elute all three isoenzymes is about 45 min for either Sepharose or Sephadex columns. The Sephadex column described here shows some cross-contamination between MB and BB, but it is still able to detect increased MB in the above-normal serum samples. In no case would the ability to detect increased MB in serum be compromised. Values for BB, on the other hand, would be estimated as falsely high.

Recently, much attention has been directed to the study of diseases that result in detectable BB in the serum: renal failure (17), nervous system atrophies and neuromuscular disorders (18), oest-cell carcinoma, metastatic gastric adenocarcinoma (19), stage D carcinoma of the prostate (20, 21), and hypertonia (22). Reportedly (23-25), a fluorescent artifact resembling BB interferes with electrophoretic measurement of BB. Appropriate control experiments (such as examination of naturally fluorescent bands before CK-staining, incubation of electrophoresis strips with reagent not containing creatine phosphate, addition of brain extract to samples, or other appropriate reagent blanks) should be performed to verify the suspected presence of the BB band. The current immunochemical methods (26-29) for detecting CK isoenzymes do not distinguish MB from BB. The improved column procedure we describe allows quantitative estimation of the enzyme and will help establish the diagnostic importance of the enzyme of CK.

We thank Dr. Richard Easterday (Pharmacal Fine Chemicals, Piscataway, NJ) for invaluable discussions and Kirsta Voigtlander for extremely competent technical assistance.

References

Table 7. CK isoenzyme Reference Intervals

<table>
<thead>
<tr>
<th>Isoenzyme activity, U/L (30 °C)</th>
<th>MM</th>
<th>MB</th>
<th>BB</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Men (ages: 17–79 years)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>43.3 (14.9)</td>
<td>1.32 (0.70)</td>
<td>0.15 (0.20)</td>
</tr>
<tr>
<td>Reference interval</td>
<td>13.5–73.1</td>
<td>0–2.7</td>
<td>0–0.55</td>
</tr>
<tr>
<td><strong>Women (ages: 14–85 years)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>32.4 (9.7)</td>
<td>1.40 (0.99)</td>
<td>0.22 (0.31)</td>
</tr>
<tr>
<td>Reference interval</td>
<td>13.2–51.8</td>
<td>0–3.4</td>
<td>0–0.83</td>
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</table>


