pyruvate, in contrast to the original rate fluorometric assay of Keitt (6), which was sensitive to above-normal erythrocyte pyruvate concentrations causing interference when evaluating rejuvenated blood (unpublished data, M. E. Ledford). In the original procedure of Keitt (2, 6) perchloric acid extracts were neutralized by titration with KHC\textsubscript{2}O\textsubscript{3} to pH 7 with a pH meter, the volume of KHC\textsubscript{2}O\textsubscript{3} being recorded for calculation of the dilution factor. This method was slow, requiring 15 to 20 min per sample. To shorten sample preparation time, this step was modified by establishing a fixed ratio between the volume of KHC\textsubscript{2}O\textsubscript{3} stock solution needed to “neutralize” the supernatant fluid of a sample precipitated from the stock perchloric acid solution. Therefore, this ratio was used in future samples, eliminating the need for constant verification of pH after adding aliquots of KHC\textsubscript{2}O\textsubscript{3} to attain the desired pH. After the samples were treated with the KHC\textsubscript{2}O\textsubscript{3}, they were vortex-mixed and left in an ice bath for about 1 h with occasional vigorous mixing to release CO\textsubscript{2}, then capped and frozen until assayed. Mixing several times during the hour is important, to obtain a stable “neutral” pH before freezing the samples. Control samples, adjusted in pH increments between pH 5 and 8, were stored at °20 C and were stable for at least six months. This procedure shortened sample preparation time by 75%.

The data indicate that the spectrophotometric assay is adequate for routine screening of blood to determine the approximate 2,3-DPG content, although a small proportional error is present in the system. However, fluorometry gave a more accurate, precise assay of 2,3-DPG. The limiting factor in obtaining good results with the fluorometric assay is the need for skilled use of the calibrated micropipets necessary for sample addition in the enzymatic analysis step.

We appreciate the assistance of SP5 Victor Cheong in performing the spectrophotometric assay, and to Dr. Carl C. Peck for assistance with data analysis. Requests for reprints should be addressed: Commander, ATTN: Medical Research Librarian, Letterman Army Institute of Research, Presidio of San Francisco, Calif. 94129. The opinions or assertions contained herein are those of the authors, and are not to be construed as reflecting the views of the Department of the Army.

References
2. Keitt, A. S., Reduced nicotinamide adenine dinucleotide-linked analysis of 2,3-

Mary Edith Ledford
Gerald L. Moore
Thomas A. Bensinger

Blood Research Division
Department of Surgery
Letterman Army Institute of Research
Presidio of San Francisco, Calif.
94129

Use of Dihydrofolate Reductase from Bovine Liver, Rather Than from L. casei, for Determining Methotrexate

To the Editor:

Finley and Williams recently reported the use of bovine liver dihydrofolate reductase (EC 1.5.1.3.) for assay of methotrexate with a centrifugal analyzer (I). The assay conditions were essentially identical to those of Falk et al. (2), who used the bacterial Lactobacillus casei enzyme.

I currently use the bovine liver enzyme for methotrexate analysis under significantly different conditions. Ov-

erdyp (3) found that the pH optimum was near 5.3 in 1 mol/liter citrate buffer, but decided to use pH 5.9, where the reaction was linear for more than 5 min. Rowe and Russel (4) have done extensive studies on the bovine liver enzyme and found maxima at pH 4.3 (acetate buffer), 6.2 (phosphate buffer), and 6.4 (sodium citrate buffers). The pH profile changes, with significant activation, on adding urea or KCl, with maxima at pH 5.5 (4 mol/liter urea) and 6.0 (0.6 mol/liter KCl).

There is a greater than sixfold increase of activity at pH 6 (0.1 mol/liter phosphate buffer with 0.6 mol/liter KCl) as compared to a pH 7.5 tri(hydroxymethyl)methylamino-HCl buffer (0.15 mol/liter).

I analyze for methotrexate by using a thermostat Beckman 25 pipet cell assembly at 30 °C connected to a Monroe computer, which measures the absorbance change at 340 nm for 60 s after a 20-s delay. The reaction buffer includes, per liter, 0.05 mol of D-mercaptoethanol, 0.1 mol of phosphate buffer, pH 6.0, 0.6 mol of KCl, 1 × 10\textsuperscript{-4} mol of NADPH, and 17 U of bovine liver enzyme. The dihydrofolate substrate, kept on ice, is phosphate buffer (2 mmol/liter, pH 6.0) containing D-mercaptoethanol. The assay is performed by adding 1.0 ml of reaction buffer to a 12 × 75 mm tube containing 50 µl of plasma. To start the reaction, 30 µl of substrate is added. Under these conditions, the following observations have been made:

1. With aqueous standards, sometimes increased activity has been observed, as compared to protein-based ones, even at a 21.6-fold dilution of plasma. This may represent the presence of an unknown inhibitor in normal plasma.
2. The enzyme is relatively unstable (as compared to the bacterial enzyme), exhibiting decreased activity after 1 h.

An invitation to membership in the

AMERICAN ASSOCIATION FOR CLINICAL CHEMISTRY

is extended to all readers of Clinical Chemistry

Dues: $60.00 per year

(includes subscription to Clinical Chemistry)

For application forms and information, write:

American Association for Clinical Chemistry
1725 K Street, NW
Washington, D. C. 20006

This necessitates performing the assay on the standards, controls and unknowns within 30 min of each other.

3. There appears to be no advantage to using ethylenediaminetetraacetate in the reaction buffer for either stabilizing or activating the enzyme.

It would appear that, except for cost, the bacterial L. casei enzyme offers the advantages of no protein dependence and increased stability, calibration curves being good for two days.

References


Richard J. Brooks

Laboratory
John Muir Memorial Hospital
1601 Ygnacio Valley Rd.
Walnut Creek, Calif. 94598

Evaluation of the BMC Column-Chromatographic Method for Creatine Kinase Isoenzyme MB

To the Editor:
The major growth industry of identification of creatine kinase (EC 2.7.3.2) MB isoenzyme utilizes principally electrophoretic and column-chromatographic methods, with immunological methods recently gaining ground. Electrophoretic methods have been criticized (1) because of the demands they make on time and technological expertise. Others have claimed (2) that it is the most reliable technique for identification of CK-MB. The costs of any separation technique are seldom mentioned, yet this has probably inhibited many laboratories from providing this additional tool for the diagnosis of myocardial infarction. A rapid and inexpensive column-chromatographic method has considerable appeal. No column method is perfect, but an awareness of the limitations of any method is a prerequisite and not a contraindication to the use of that method. If near perfection were demanded of any test procedure before it is introduced, then perhaps we would only now be able to make an informed guess as to when we might be able to introduce analyses for sodium and potassium into clinical medicine.

We have evaluated a simple column-chromatographic method (BMC Canada Limited, Montreal, P.Q.) for separating the creatine kinase MB isoenzyme from serum. For comparison we used the same samples in isolating the MB isoenzyme by the modified Mercer procedure (3) by agarose electrophoresis (4).

The BMC column-chromatography kit consists of 20 polypropylene columns and three bottles: bottle 1, DEAE-Sephadex A-50 (200 ml/g liter) and imidazole buffer (100 mmol/liter, pH 6.7); bottle 2, MM buffer concentrate, dilute with 200 ml of distilled water to give imidazole buffer (100 mmol/liter, pH 6.7); bottle 3, MB buffer concentrate, dilute with 100 ml of distilled water to give imidazole buffer (100 mmol/liter, pH 6.7) and 150 mmol/liter MgCl2. All the solutions are said to be stable for one year at 4 °C. Each column has a plastic sieve to retain the Sephadex, and the columns may be re-used if they are rinsed several times with distilled water after the Sephadex is carefully removed at the end of the analysis.

Since we completed the evaluation we have been informed that there are now two bottles included in each kit containing, after reconstitution, 3 ml of lyophilized CK-MB control sera.

The column is set on the reagent tube and 2.0 ml of the Sephadex A-50 and 0.5 ml of serum are pipetted carefully. When the solution has passed through the sieve, 5 ml of MM buffer is added twice to the column. The manufacturer recommends discarding these eluates. For the purpose of this evaluation we retained eluate 1 (11-fold dilution) and eluate 2 (10-fold dilution).

To elute the CK-MB fraction, one sets the column on a new reagent tube and successively adds three 1-ml portions of MB buffer (MB, sixfold dilution). The activities of the fractions were measured with an ABA-100 analyzer, with use of a 100-µl sample of each eluate. We determined the enzyme activities of the eluates of the BMC and Mercer columns with use of the BMC CK-NAC reagent and measured the activity of each serum specimen with the use of both CK-NAC and Worthington CK reagents, following the ABA protocol. The results with the Worthington reagents were used to express the percentage of total activity represented by the MB isoenzyme as separated with the electrophoretic procedure. The analyses were done with the ABA-100 analyzer, incubation time 5 min. A 100-µl sample of eluate was manually pipetted into the cuvet and then incorporated into the reaction mixture, with use of a 1.51 syringe plate and 380/340 nm filters. The MB fraction (sixfold dilution) has a calibration factor of 389, eluate 1 (11-fold dilution) 713, and eluate 2 (10-fold dilution) 608.

As a result of measuring MM eluates 1 and 2, we were satisfied by the manufacturer's claim that there is no significant carryover of MM into the MB fraction. Discarding these eluates ensures that the time to constitute the column and obtain the MB fraction does not exceed 15 min. Several columns can readily be handled at the same time by one operator without causing confusion.

Regression of the results obtained with the BMC column against those with the modified Mercer column show a good correlation (r = 0.985, n = 23, slope = 0.679, y-intercept = 0.439). The MB fraction (modified Mercer column) ranged from 0.7 to 16.8% of total CK activity. For the BMC columns the MB range was from 0.8 to 12.2% of CK activity. The electrophoretic procedures are only semiquantitative, and it is hard to compare them with the numerical data provided by column chromatography. Substantially higher percentages for MB activities were obtained by electrophoresis, and in one sample there was a significantly positive MB of 16.3% by electrophoresis, whereas the same sample gave only 2.9 and 2.9% MB by column chromatography. We cannot say from this study if this represents a false-positive MB by electrophoresis. None of the positive results by column was negative by electrophoresis.

The serum activities with the BMC-NAC and Worthington reagents also exhibited a good correlation (r = 0.997, n = 23, slope = 1.234, y-intercept = 65.54). Creatine kinase activities ranged from 97 to 4400 U/liter with the Worthington reagents, and from 124 to 5420 U/liter with the BMC-NAC reagents.

We applied the same sample to four of the BMC columns, to assess the variability from column to column. The sample was followed by 10 ml of MM buffer, the eluates being collected as recommended by the manufacturer (21-fold dilution). A third MM eluate (twofold dilution) was obtained by using 1 ml of the MM buffer as an additional check for carryover of MM. The results, tabulated below, are insufficient for statistical analysis but indicate a reasonable reproducibility from column to column and again show that the MM fraction is removed by the recommended procedure.

<table>
<thead>
<tr>
<th>Fraction 1</th>
<th>Fraction 2</th>
<th>MB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity, U/liter</td>
<td>Activity, U/liter</td>
<td>Activity, U/liter</td>
</tr>
<tr>
<td>A</td>
<td>350</td>
<td>49</td>
</tr>
</tbody>
</table>

The CK-BB isoenzyme could be eluted with the MB fractions, giving rise to the