Table 4. Effects of Hemoglobin, Bilirubin, and Turbidity

<table>
<thead>
<tr>
<th>Hemoglobin added, g/L</th>
<th>Magnesium measured, mg/L</th>
<th>Bilirubin added</th>
<th>Magnesium measured, mg/L</th>
<th>Intralipid* added</th>
<th>Magnesium measured, mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>17.1</td>
<td>5</td>
<td>16.4</td>
<td>0</td>
<td>17.5</td>
</tr>
<tr>
<td>0.7</td>
<td>17.3</td>
<td>58</td>
<td>16.3</td>
<td>800</td>
<td>17.8</td>
</tr>
<tr>
<td>1.7</td>
<td>17.1</td>
<td>111</td>
<td>16.5</td>
<td>1600</td>
<td>17.4</td>
</tr>
<tr>
<td>3.5</td>
<td>17.6</td>
<td>163</td>
<td>16.3</td>
<td>2400</td>
<td>17.6</td>
</tr>
<tr>
<td>5.4</td>
<td>17.6</td>
<td>210</td>
<td>16.3</td>
<td>3200</td>
<td>17.6</td>
</tr>
<tr>
<td>8.9</td>
<td>17.8</td>
<td>283</td>
<td>16.4</td>
<td>4000</td>
<td>17.5</td>
</tr>
</tbody>
</table>

*Appearance of the specimens ranged from clear to milk-like (4000 mg/L).

Use of EDTA to enable a sample blank absorbance measurement has been reported previously for calcium methods (5, 8–10). We are not aware of any previous reports discussing the similar use of EDTA for the assay of magnesium. Our approach should be adaptable to other instruments capable of adding two reagents and making the necessary absorbance measurements.

References


Creatine Kinase Isoenzymes in Human Cerebrospinal Fluid and Brain
Wayne L. Chandler,1 Kathleen J. Clayson,1 W. T. Longstreth, Jr.,2 and James S. Fine1

Extracts of normal brains obtained at autopsy and cerebrospinal fluid (CSF) from patients with global brain ischemia were analyzed for creatine kinase (CK; EC 2.7.3.2) isoenzymes. We used both qualitative and quantitative assays (electrophoresis and immunoinhibition). Brain extracts contained CK-BB isoenzyme and mitochondrial CK. In 54 CSF samples free of blood contamination and with total activities ranging from 7 to 100 U/L (mean 202 U/L), virtually all of the CK activity was due to CK-BB, and none to CK-MM or CK-MB. We conclude that brain contains CK-BB and mitochondrial CK, but lacks CK-MM and CK-MB. After cardiac arrest, CK-BB is released into the CSF. Any CK-MM in the CSF is probably from blood contamination, in which case immunoinhibition with anti-CK-M antibodies accurately quantifies CK-BB.

Additional Keyphrases: electrophoresis, cellulose acetate · immunoinhibition · mitochondrial CK · brain ischemia · cardiac arrest

Acute neurological injury releases creatine kinase (CK; EC 2.7.3.2) into the cerebrospinal fluid (CSF) (1).2 In humans after cardiac arrest, the greater the concentration of CK in the CSF, the more extensive the brain damage and the worse the prognosis for recovery of neurological function (2–5). Despite the potential utility of CSF-CK as an index of brain damage after cardiac arrest, uncertainty remains as to which isoenzymes of CK are present in brain (6); which isoenzymes leak into the CSF following brain damage (7); and which method is best for determining CSF-CK (8). In this study we address these three problems.

Materials and Methods
Brain tissue. Four samples of normal human cerebral cortex and one sample of putamen were obtained at autopsy from five different brains, each within 24 h of death. Specimens were stripped of meninges and superficial vessels, then rinsed with saline. Homogenates of 250 mg of tissue per milliliter of buffer were extracted with either Tris (50 mmol/L, pH 7.4 at 25 °C), containing N-acetylcysteine (50 mmol/L) and EDTA (0.5 mmol/L), or ammonium acetate.

1 Department of Laboratory Medicine and 2 Division of Neurology, Department of Medicine, University of Washington School of Medicine, Seattle, WA 98195.
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3 Nonstandard abbreviations: CK, creatine kinase; CSF, cerebrospinal fluid: DTT, dithiothreitol.
(100 mmol/L, pH 9.5 at 25 °C) (6, 8). After stirring for 30 min, we centrifuged the homogenate (15,000 × g, 30 min) and stored the clear supernate at −70 °C until assaying for CK content.

**CSF specimens.** CSF was obtained by lumbar puncture from patients who were comatose after cardiac arrest. Samples were placed on ice immediately after collection, transported to the laboratory, and stored at 4 °C. Before assay, we treated the CSF samples with dithiothreitol (1 mg/0.1 mL of CSF) for at least 5 min to reactivate reversibly oxidized CK. We determined total CK activity by a modification of the method of Rosalki (9, 10), using a Cobas Bio centrifugal analyzer (Roche Analytical Instruments, Nutley, NJ 07110) at 30 °C. The final CK reaction mixture contains, per liter, 10 mmol of DTT (CK reactivator), 15 mmol of Mg²⁺, and 25 mmol of sodium fluoride (adenylate kinase inhibitor). For samples with CK activity less than 20 U/L we increased the sample volume fourfold, from 10 to 40 μL (8). Interference by uninhibited adenylate kinase was controlled by including blanks in which buffer was substituted for creatine phosphate. Only samples with total CSF-CK activities exceeding the reference range of 0–5 U/L, after addition of DTT, were included in the study.

**Isoenzyme determinations.** CK isoenzymes were determined qualitatively by cellulose acetate electrophoresis with membranes and Tris-barbital buffer (pH 8.6) from Helena Laboratories, Beaumont, TX 77704. We analyzed the isoenzyme pattern by the fluorescence method of Somer and Konttinen (11) with CK reagents from Sclavo Inc., Wayne, NJ 07470. We determined CK-MM by immunoinhibition with a "Cardiozyme Plus CK-MB" kit (EM Science, Gibbstown, NJ 08027), which contains anti-CK-M antibodies incorporated in an optimized CK reaction mixture. We performed this assay with programming parameters described by Wu and Bowers (12) for a Cobas Bio centrifugal analyzer, also at 30 °C.

We used anti-CK-M antibodies to ascertain the ability of immunoinhibition to quantitatively differentiate CK-BB (brain damage) from CK-MM (blood contamination). To samples of normal pooled CSF with no detectable CK activity we added brain homogenate (greater than 99% CK-BB) to produce activity concentrations of 15, 27, and 112 U/L. Serum samples with greater than 99% CK-MM activity were obtained from the general patient population. A known volume of serum (0.01, 0.03, 0.1, 0.3, or 1.0 mL) with total CK activity of 160 U/L was added to a 1-mL aliquot of CK-augmented CSF to simulate contamination with blood. We measured the total CK activity in each sample before and after immunoinhibition with anti-CK-M antibodies, and compared the results with predicted values.

**Results**

**Brain extracts.** The ammonium acetate and Tris buffers extracted essentially the same total CK activity from samples of cortex, but the ammonium acetate extracts of putamen had considerably less total CK activity (Table 1). Two distinct CK isoenzymes were found in all the brain samples tested: CK-BB and a non-CK-MM, cathodally migrating CK, probably mitochondrial CK (13). CK-MM and CK-MB were not detected in any of the samples by electrophoresis or immunoinhibition.

**CSF-CK isoenzymes.** We analyzed for CK isoenzymes 55 CSF samples from 49 patients who were comatose after cardiac arrest; 21 of the 55 samples were assayed for total CK before and after addition of DTT. Mean total CK activity increased from 107 to 247 U/L after the reactivating agent was added; therefore, we reactivated all subsequent samples with DTT before determining total CK. We also measured adenylate kinase activity for 17 samples, and found that it ranged from 0 to 4 U/L (mean 1 U/L). In 16 of the 17 samples, uninhibited adenylate kinase activity was less than 1% of the total CK activity, but in one sample it was 7% of the total CK.

To increase precision for CSF samples with less than 20 U/L total CK activity, we increased the sample volume fourfold, from 10 to 40 μL (8). For CK activity of 5 U/L, the within-run CV was 40% for 10-μL samples vs 4% for 40-μL samples. Although Urdal and Strømme recommended increasing sample volume 10-fold (8), we did not improve precision by using sample volumes larger than 40 μL. Furthermore, their recommendation of adding EDTA (3 mmol/L) to CSF (8) did not increase the CK activity in either the 10- or 40-μL samples.

Total CK activity for 54 CSF samples not contaminated with blood ranged from 7 to 2010 U/L (mean 202 U/L). On electrophoresis, 46 samples showed CK-BB only; eight samples were more than 95% CK-BB and demonstrated small CK bands migrating in the zone between CK-MB and albumin. These small CK bands may result from a change in the electrophoretic mobility of CK-BB. A similar band has been described for CK-BB incubated at 37 °C in serum and may also occur in CSF (14–17). Occasionally, we noted a little diffuse fluorescence slightly anodal to the CK-MM region on samples with CK activities greater than 350 U/L. Electrophoresis of a specimen grossly contaminated with blood showed primarily CK-MM with only faint CK-BB.

For four of the CSF samples we used immunoinhibition with anti-M antibodies. In three samples that had only CK-BB on electrophoresis, anti-M antibodies had no effect on total CK activity; in the single sample with gross blood contamination, which showed primarily CK-MM on electrophoresis, 95% of the CK activity was inhibited by anti-M antibodies.

**Simulated CSF contamination.** We measured total CK activity for each combination of CSF, serum, and brain homogenate (see above) and compared the results with the predicted values. A least-squares analysis of predicted vs measured total CK values showed excellent correlation (r = 0.998), with a slope of 0.99 and an intercept of 1.9 U/L. We again measured CK for each sample after addition of anti-CK-M antibodies and compared these values with the predicted CK-BB activity (assuming all residual activity was due to to CK-BB). Analysis of the resulting CK-BB concentrations resulted in r = 0.999, slope = 0.99, and intercept = 2.1 U/L. Measured CK-BB activity tended to be slightly greater than predicted but within the error limits of the method.
Discussion

Different methods used to decide which CK isoenzymes are present in brain have yielded various results. Brain specimens obtained at surgery contain only CK-BB, whereas autopsy specimens contain both CK-BB and mitochondrial CK (13, 18, 19). Difficulty in releasing mitochondrial CK from intact mitochondria may explain its absence in surgical specimens. Lindsey and Diamond (6) found CK-MM in appreciable amounts in brain, and suggested that it was the predominant isoenzyme in regions such as basal ganglia. We were unable to replicate their results. In this study, regardless of the source of the sample (cerebral cortex or putamen), the method of extraction (Tris or ammonium acetate), and the method of determination (electrophoresis or immunoinhibition), we found no CK-MM and CK-MB in brain. Instead, we detected various amounts of CK-BB and mitochondrial CK in all brain extracts.

For CSF our findings agree with those of most other investigators (3–5): CK-BB is the major isoenzyme in CSF samples free of blood contamination. The absence of CK-MM in CSF is a further indication that this isoenzyme is not present in substantial quantities in brain. Meberg et al. found small amounts of CK-MM in CSF from infants with neonatal hypoxia (7), but total CSF-CK activity was low in most of their samples. Accurate analysis of CK isoenzyme by electrophoresis alone is difficult on samples with less than 20 U/L total CK activity (8). Fluorescent artifacts, uninhibited adenylate kinase, unsuspected blood contamination, or some combination of these factors makes interpretation of electrophoresis results difficult in the CK-MM region.

The optimum method for analyzing CSF CK isoenzymes has not been determined. Our experience with this study and our review of the literature lead us to the following recommendations:

- Immediately after obtaining CSF, place the specimen on ice and store at 4°C. Before determining total CK, add a reactivating agent such as DTT (or equivalent) to the CSF sample and incubate for at least 5 min.
- Optimize the current method for total CK to increase precision at low CK activities. This involves increasing the assay sample volume and adjusting the concentrations of magnesium and EDTA in the CK reaction mixture to prevent interference from calcium (8). If total CSF-CK is less than or equal to the upper reference limit, no further work is needed.
- If electrophoresis or immunoinhibition shows no CK-MM, then total CSF-CK is equivalent to CSF-CK-BB.
- In CSF samples with known or suspected blood contamination, use immunoinhibition with anti-CK-M antibodies and blanks for adenylate kinase to control potential interference and to determine CSF-CK-BB.
- CK activity can be lost through either reversible oxidation or irreversible temperature-dependent inactivation (16, 20). Reactivating agents in the CK reaction mixture are not sufficient; we found that CSF-CK activity more than doubled after addition of DTT directly to the CSF sample, even though the CK reagents also contained DTT. Cooling the CSF to 4°C effectively slows irreversible inactivation such that the sample may be stored for 24 to 48 h without important loss of CK activity (3, 17).

Finally, immunoinhibition with anti-CK-M antibodies is excellent for differentiating CK-BB from CK-MM in CSF contaminated with blood. Even when CSF-CK-MM activity was 10-fold greater than CSF-CK-BB activity, the immunoinhibition method estimated CK-BB to within 3 U/L of the predicted value.

References