Decreased Storage Stability of Creatine Kinase in a Cardiac Reperfusion Solution

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Creatine kinase (CK; EC 2.7.3.2) has been used as an indicator of myocardial cellular damage. In this study we used a Krebs–Henseleit (KH) solution to reperfuse isolated rat hearts after 24 h of cold preservation and collected the KH reperfusionate for assay of CK to assess cellular damage. We wanted to determine the stability of CK in the KH solution at different cold-storage temperatures and albumin concentrations. CK activity (mean ± SEM) after one week of refrigeration (5 °C) was 93% ± 1% of control values, whereas CK activity in nitrogen-frozen (−200 °C) samples was only 1.6% ± 1% of control values, and that in samples frozen at moderately low temperatures (−10 °C) was 63% ± 1% of control values. To enhance stability, we added albumin to several concentrations (49, 25, 12, and 6 g/L) to reperfusion collections in which CK had been previously determined. Specimens were frozen (−10 °C), then re-analyzed for CK weekly for three weeks. CK activity was maintained (100% ± 5%) only in samples containing 25 g/L or more albumin. These data suggest that refrigeration (5 °C) for one week maintains normal CK activity in KH solution; however, if prolonged storage is necessary, a stabilizer such as albumin (≥25 g/L) will maintain analyte stability in frozen storage (−10 °C) for at least three weeks.

Additional Keyphrases: variation, source of · sample handling · enzyme activity · organ preservation · albumin · rat heart tissue

The maintenance of optimal conditions for cardiac function during periods of ischemia and of optimal storage conditions during prolonged preservation for heart transplantation is critical for successful recovery of heart function. Preservation experiments designed to establish the specific conditions required for optimal cardiac function involve the use of biochemical markers to assess myocardial damage. The measurement of creatine kinase (CK; EC 2.7.3.2) in plasma is routinely used as an index of heart damage (1–6) and thus seems an appropriate biochemical marker for the assessment of ischemic and post-ischemic myocardial damage in preservation experiments.

During studies involving 24-h heart preservation, even with left ventricular function impaired and visible tissue discoloration, we found that CK activity in samples stored frozen (−10 °C) did not always correspond to the severity of cellular damage. The specimens assayed immediately after collection displayed higher CK activity than did frozen samples, regardless of the experimental conditions. This suggested that a loss of CK activity had occurred, either during frozen storage or when the samples were thawed. Like any enzyme, CK may denature and lose activity if stored inappropriately (7); however, the storage conditions used in our laboratory were similar to those used in hospital laboratories where serum samples are routinely frozen and re-assayed. Serum or plasma samples, however, contain from 60 to 80 g of protein per liter (8), whereas the buffer solutions used during heart-preservation experiments contain very little (0.1 g/L) or no protein.

These observations prompted a study to determine the stability of total CK (about 85% CK-MM and 15% CK-MB) in the heart-preservation solution and to determine whether the addition of protein would enhance the stability of total CK during storage.

Materials and Methods

Hearts from 46 normotensive, 250- to 350-g Wistar Kyoto (WKY) rats were isolated and perfused by the Langendorff technique (9). Rats were anesthetized intraperitoneally with Brevital (Lilly, Indianapolis, IN), 75 mg/kg of body weight, and their hearts were removed and rinsed in cold Krebs–Henseleit buffer (per liter, 119 mmol of NaCl, 4.7 mmol of KCl, 2.5 mmol of CaCl₂, 1.2 mmol of MgSO₄, 1.2 mmol of KH₂PO₄, 25 mmol of NaHCO₃, 14 mmol of glucose, and 0.1 g of albumin). We trimmed off the atria, then attached the hearts by an aortic cannula to a hydrostatic perfusion apparatus and retrograde-perfused the hearts at 10.6 kPa (80 mmHg), 37 °C, with Krebs–Henseleit solution plus albumin (10), bubbling the solution with O₂/CO₂ (95/5, by vol). After perfusion we removed the hearts, with cannula attached, from the hydrostatic apparatus and connected them to a pulsatile roller pump (Minipuls 2; Gilson, Middleton, WI) for perfusion at a rate of 2 mL/min with a recirculating cardioplegic solution (per liter, 119 mmol of NaCl, 15 mmol of KCl, 0.8 mmol of CaCl₂, 5.2 mmol of MgCl₂, 1.2 mmol of KH₂PO₄, 25 mmol of NaHCO₃, 11 mmol of glucose, 68 mmol of mannitol, 0.12 mmol of corticosterone, 0.14 mmol of pyruvic acid, 10.4 mmol of ATP, 24 units of insulin, and 0.1 g of albumin), again bubbling the solution with O₂/CO₂ (95/5, by vol). The hearts and pump were transferred to a cold chamber (5 °C) overnight. Twenty-four hours later, we re-attached the cannulated hearts to the hydrostatic apparatus and reperfused with the warm (37 °C) Krebs–Henseleit buffer containing mannitol, 68 mmol/L, and produced a hyperosmolar solution (330 mosmol/L) to enhance water removal from tissue (11).

Collections of the reperfusion fluid were analyzed immediately for CK with reagents from Sigma Diagnostics (St. Louis, MO) and a Multistat III Analyzer (Instrumentation Laboratory, Lexington, MA) and then divided into three aliquots. The first aliquot was refrigerated (5 °C), the second was frozen in liquid nitrogen (−200 °C), and the third was frozen at −10 °C. After storage for one week, all specimens were re-analyzed for CK and the percentage of the original activity that was recovered was calculated.

One of the major differences between the Krebs–Henseleit buffer and serum or plasma is the low protein content in the former (0.1 g/L). Therefore, in the second part of the experiment, we determined whether the addition of albumin to the Krebs–Henseleit buffer would stabilize the CK during freezing. Samples of reperfusion fluid from 24-h
heart-preservation experiments (n = 46) were collected as previously described, aliquoted in sufficient numbers so that each specimen was thawed only once, supplemented with various albumin concentrations (final concentration 49, 25, 12, and 6 g/L), and analyzed for CK, weekly for three weeks. All experiments were performed within the National Institutes of Health guidelines, "The Principles of Laboratory Animal Care," with procedures approved under the University Animal Research Committee guidelines.

Results were analyzed for significance by one-way ANOVA, Pearson correlation coefficients, and Student's t-test and expressed as mean values ± SEM. Significance was assumed if P < 0.05.

Results

The CK, measured after storage at 5, -10, and -200 °C, was 93% ± 1%, 63% ± 1%, and 1.6% ± 1% of controls, respectively (n = 46). The two types of frozen specimens (-10 and -200 °C) showed significantly less CK activity (P < 0.001) than did the refrigerated (5 °C) specimens. The CK assay reagent insert (12) states that serum is stable for 24 h at room temperature (18-26 °C), or for seven days when refrigerated (2-6 °C) or frozen. Clearly the frozen stability of CK activity in serum or plasma is not the same as in Krebs-Henseleit buffer.

Figure 1 shows the change in the percentage of CK activity in frozen specimens (-10 °C) over time in the presence of various concentrations of added albumin. There was no significant decrease with time in the 49 or 25 g/L albumin concentration samples; however, in the presence of lower concentrations of albumin—12, 6, and 0.1 g/L—there was a significant decrease (P < 0.001, P < 0.01, and P < 0.001, respectively) from the initial CK values. In the samples with albumin <25 g/L, CK activity decreased by 20% during the first seven days; however, in the next week, the samples in the lowest albumin concentrations (0.1 g/L) showed a very rapid decrease (51%), the decrease for the other albumin concentrations (12 and 6 g/L) being less severe (9%). Figure 2 shows a significant correlation (r = 0.669, P < 0.001) between various albumin concentrations and the percentage of CK activity remaining after one week of frozen (-10 °C) storage.

Discussion

Enzymes are relatively fragile molecules. They may undergo denaturation, with subsequent loss of catalytic activity (7), as the result of many factors, including extremes of pH, excess thermal energy, or the freezing and thawing processes associated with sample storage and reconstitution in clinical and research laboratories. It is common practice in clinical laboratories to freeze plasma or serum samples for batch-profile assays (e.g., for determining enzyme activities for CK, lactate dehydrogenase, and others) to maximize cost effectiveness and assay precision. Enzyme activity in such samples is not adversely affected by storage conditions. However, the results from this study indicate that fast or slow freezing of Krebs-Henseleit buffer samples collected during isolated heart reperfusion adversely affects the stability of CK. Because refrigerated samples of reperfusion buffer retained acceptable CK activity, we conclude that the freezing and thawing process inactivates the enzyme.

The addition of albumin has been used to stabilize enzymes in solution (13, 14). Similarly, we have found that CK activity in the Krebs-Henseleit aqueous sample was retained after frozen storage for as long as three weeks if the albumin concentration in the sample exceeded 25 g/L. Presumably, the albumin prevents enzyme denaturation by providing buffering during the initial stages of freezing or thawing. Also, albumin may contribute trace-metal impurities, which are known to stabilize some enzymes (15).

We conclude that addition of albumin (≥25 g/L) to Krebs-Henseleit reperfusion buffer sample before frozen storage is required to stabilize CK activity in samples that are not to be assayed immediately. Albumin is not necessary to maintain CK stability in samples stored refrigerated (5 °C) for up to one week. Presumably, albumin will also provide a stabilizing environment for enzymes in other aqueous media; however, experimental verification of this is required for each such situation.

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**Fig. 1.** Effect of albumin on percent creatine kinase activity in Krebs-Henseleit buffer stored at -10°C for three weeks
Albumin concentrations were 49 (C), 25 (■), 12 (△), 6 (▲), or 0.1 (□) g/L. Analysis of variance of all groups for the three-week period was significant (P < 0.001). Variances for the lower albumin concentration groups were also significant: P < 0.001 (△, □) and P < 0.01 (▲). Means ± SEM are shown (for 0.1 g/L albumin group, n = 46; for the heart samples and for all other groups, n = 8)

**Fig. 2.** Correlation between percent creatine kinase activity retained and albumin concentration after one week of frozen storage (-10 °C): y = 6.56x + 70 (r = 0.669, P < 0.001)

n = 8 hearts, but multiple samples of each were assayed
Accuracy and Precision of Methods for Theophylline Measurement in Physicians' Offices
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We tested the accuracy and precision of five theophylline methods intended for use in physicians' offices. The Syntex AccuLevel, Ames Seralyzer, and 3M Diagnostics TheoFAST methods were less reproducible (CVs 6.3% to 9.2%) than the Abbott Vision and Kodak DT-60 (CVs 2.2% to 3.3%). Caffeine interfered with the Vision, Seralyzer, and AccuLevel methods, and theobromine interfered with the Vision, Seralyzer, and TheoFAST methods. Only the DT-60 method was free from interference from any of the 24 compounds tested. Results by all methods correlated well with those by the HPLC comparison method (Clin Chem 1981;27:1931–3) and by the Abbott TDx method for assay of 100 serum (or, when appropriate, paired whole-blood) samples. The frequency of sample results differing from the comparison method by >2.0 mg/L was as follows: TDx, 11%; Vision (serum), 12%; Vision (whole blood), 16%; DT-60, 14%; AccuLevel, 18%; Seralyzer, 25%; and TheoFAST, 31%. The Kodak DT-60 method was the most nearly accurate and precise among these physician's office methods. Some physician's office methods for theophylline analysis are not adequate to guide dosage adjustments.

Additional Keyphrases: intermethod comparison • physician's office testing

Theophylline is a potent smooth-muscle relaxant especially affecting bronchial smooth muscle. The drug is used as a bronchodilator to treat bronchial asthma and to control reversible bronchoconstriction in obstructive pulmonary disease and neonatal apnea.

Theophylline has a narrow therapeutic index. Toxicity, caused by transient stimulation of the central nervous system, is expressed as nausea, vomiting, headache, diarrhea, irritability, and insomnia. Severe toxicity is characterized by seizures and cardiac arrhythmias. The elimination kinetics of the drug are influenced by age, smoking, and liver function, and it is common to encounter patients for whom prompt measurement of serum theophylline is essential.

To facilitate theophylline measurements in physicians' offices, clinics, and other locations remote from centralized laboratories, several systems for theophylline quantification in nonlaboratory settings have become available. These systems are designed and marketed for use by minimally trained personnel.

Our goal in this study was to examine the accuracy and precision of these devices for quantifying theophylline in serum or whole blood.

Materials and Methods

Pooled serum and whole blood. Pools were prepared by combining serum or whole blood (with EDTA anticoagulant) collected from healthy, drug-free donors. All serum-based materials were mixed well, filtered, and stored at −20°C in 1.5-mL capped polypropylene tubes until use. Whole-blood specimens were analyzed within 1 h after collection.

Standards. Separate portions of serum pool were supplemented with an aqueous theophylline stock standard (1.00 g/L) prepared from reference standard theophylline (U.S. Pharmacopeia Convention, Rockville, MD 20852).

Linearity studies. Portions of the serum and whole-blood pools were supplemented with the 1.00 g/L theophylline