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DIRECT ASSAY FOR CREATINE KINASE BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A direct assay for creatine kinase (CK) activity was developed based on the separation and quantitation of adenosine triphosphate (ATP) by high-performance liquid chromatography. The total incubation time is 13 min and the elution time for ATP is 16 min. Using lyophilized CK as the sample, a sensitivity in the range of 8 U/l (units/liter) was obtained. The method presented also has clinical significance in that CK levels in serum can easily be determined with minimal sample preparation. Using serum samples from a healthy patient and a heart attack victim, activities of 26.6 U/l and 609.0 U/l, respectively, were obtained. Because of the direct measurement of ATP, this method eliminates the coupled reactions encountered in the common spectrophotometric and colorimetric methods of analysis resulting in a simpler and inexpensive assay.

INTRODUCTION

The assay of creatine kinase (ATP: creatine N-phosphotransferase; EC 2.7.3.2), hereafter referred to as CK, for the diagnosis of myocardial infarction and progressive muscular dystrophy has already been reported [1,2]. The activity of the BB isoenzyme of CK has also been shown to be of clinical importance in the presence of various neoplastic diseases [3—5].

The usual method for the assay of CK is basically the method of Oliver [6] which utilizes the more thermodynamically favored reverse reaction. It is known that the reverse CK catalyzed reaction proceeds approximately 6 times faster than the forward reaction. The entire enzymatically coupled assay is shown as follows:

$$\text{ADP} + \text{phosphocreatine} \xrightleftharpoons{\text{CK}} \text{ATP} + \text{creatine} \quad (1)$$

$$\text{ATP} + \text{D-glucose} \xrightleftharpoons{\text{HK}} \text{ADP} + \text{D-glucose-6-phosphate} \quad (2)$$

$$\text{D-glucose-6-phosphate} + \text{NAD}^+ \xrightleftharpoons{\text{G-6-PD}} \text{6-phosphogluconate} + \text{NADH} \quad (3)$$

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where ATP is adenosine triphosphate, ADP is adenosine diphosphate, HK is hexokinase, G-6-PD is glucose-6-phosphate dehydrogenase, and NAD^+ and NADH are the oxidized and reduced forms, respectively, of nicotinamide adenine dinucleotide. The increase in NADH is measured spectrophotometrically at 340 nm.

A problem that arises with this type of scheme is that the activity of CK is somewhat dependent on the coupled reactions chosen for the assay. The equilibrium constants for reactions 1, 2, and 3 have been reported [7] as 1.4×10^8, 1.55×10^2, and 6.0×10^{-7}, respectively. Since the assay utilizes the more favorable reverse reaction in the initial step, reaction 3, due to its low equilibrium constant, may not always reflect accurately the rate of formation of ATP in reaction 1.

The lack of agreement between optimal assay conditions has also been reported in the literature. Morin [8] has examined several sets of assay conditions in addition to the presentation of his own optimum conditions. The omission of reactions 2 and 3 in the coupled system would eliminate several of the differences in reactant concentrations by completely eliminating the reactants.

The presence of adenylate kinase, which catalyzes the conversion of two ADP molecules to 1 molecule of ATP and 1 molecule of AMP (adenosine monophosphate) presents a problem in real serum samples. Walter [9] has shown that when AMP is used to inhibit this competing enzyme, it also inhibits reaction 3 of the CK coupled system. Elimination of reaction 3 would thus enable the use of AMP as an inhibitor of adenylate kinase without affecting the assay for CK.

High-performance liquid chromatography (HPLC) has been utilized for the assay of several enzymes [10—12]. To facilitate the assay of CK, we have used HPLC for the rapid separation and quantitation of ATP produced in reaction 1. The amount of ATP can be directly related to the activity of CK. Besides eliminating the necessity of a coupled enzyme system, many of the problems stated previously can be avoided.

EXPERIMENTAL

Apparatus

An Altex Model 110A high-pressure pump equipped with an Altex Model 153 UV detector, constant wavelength (254 nm), (Altex, Berkeley, CA, U.S.A.) was used for the direct assay method. The injector used was a Rheodyne Model 7125 (Rheodyne, Berkeley, CA, U.S.A.). A 5 cm × 4.1 mm I.D. precolumn and 15 cm × 4.1 mm I.D. working column were used for the separations. The columns were packed with 10-μm RP-18 LiChrosorb (E. Merck, Darmstadt, G.F.R.) using a Micromeritics Model 705 column packer (Micromeritics, Norcross, GA, U.S.A.) and the Altex pump. Peaks were recorded by an Ommiscribe Model B-5000 recorder (Houston Instrument, Austin, TX, U.S.A.). The spectrophotometric assay was performed using a Cary Model 14 spectrophotometer (Cary Instrument, Monrovia, CA, U.S.A.) equipped with a Lauda K-2/R thermostated water bath (Brinkman Instrument Inc., Westbury, NY, U.S.A.).

Chemicals

All water used was triply distilled. Tetrabutylammonium hydrogen sulfate was supplied by either Sigma (St. Louis, MO, U.S.A.) or Aldrich (Milwaukee, WI, U.S.A.). Trizma Base [tris(hydroxymethyl)aminomethane], ATP (sodium salt), ADP (sodium salt or di-monocyclohexyl ammonium salt), AMP, phosphocreatine (disodium salt), β-NAD^+, α-D(±)glucose, monothioglycerol, CK (lyophilized from rabbit muscle), HK (crystalline suspension in 3.2 *M* ammonium sulfate), G-6-PD (lyophilized from *Leuconostoc mesenteroides*), and control serum (type 1-A, normal) were all obtained from Sigma. Potassium dihydrogen phosphate was obtained from Fisher Scientific Co. (Fair Lawn, NJ, U.S.A.) and NaOH was obtained from Matheson, Coleman, and Bell (Norwood, OH, U.S.A.). Methanol distilled in glass was obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Acetic acid was obtained from Scientific Products (McGaw Park, IL, U.S.A.), magnesium acetate from J.T. Baker (Phillipsburg, NJ, U.S.A.), and $KF \cdot 2H_2O$ from Mallinckrodt (St. Louis, MO, U.S.A.). All substrates and enzymes were stored refrigerated at 0—5°C or frozen in a desiccator as recommended. All chemicals were reagent grade or the highest purity available. Real serum samples, normal and elevated, were obtained from McCullough-Hyde Memorial Hospital (Oxford, OH, U.S.A.) and refrigerated at 0—5°C until use.

Procedures

CK timed reaction. All solutions were made or diluted with 0.1 *M* Tris buffer adjusted to pH 6.8 with acetic acid. A 0.5-ml aliquot of a reactant solution containing 6 m*M*/l ADP, 6 m*M*/l AMP, and 60 m*M*/l magnesium acetate was placed in a 75-mm glass test-tube. To this, 0.5 ml of a reactant solution containing 90 m*M*/l phosphocreatine and 18 m*M*/l $KF \cdot 2H_2O$ was added. Inhibition of adenylate kinase was performed by the addition of 2 m*M*/l AMP and 6 m*M*/l $KF \cdot 2H_2O$ (reaction concentrations) as recommended by Meiattini et al. [13]. In using the fluoride concentrations recommended by Morin [8], problems with the precipitation of MgF_2 occurred. The test-tube was then pre-incubated for 3 min at 30°C in a water bath equipped with a Polyscience Model 73 thermostat (Polyscience, Niles, IL, U.S.A.). A 0.5-ml aliquot of a pre-incubated (30°C) stock solution of 0.04 μg/ml CK solution containing 1.2 m*M*/l monothioglycerol was added to the test-tube. Reactions proceeded for 10, 20, and

TABLE I

REACTION CONDITIONS FOR THE HPLC AND THE SPECTROPHOTOMETRIC ASSAY OF CK

Reactant	Concentration (m*M*/l)	Reagents added additionally in the spectrophotometric assay	Concentration
ADP	2.0	NAD^+	2 m*M*/l
AMP	2.0	D-Glucose	15 m*M*/l
Magnesium acetate	20.0	HK	3000 NAD units/l
Phosphocreatine	30.0	G-6-PD	2500 NAD units/l
$KF \cdot 2H_2O$	6.0		

30 min and were terminated by placing the test-tubes in boiling water for approximately 75 sec. Final reaction concentrations are listed in Table I. Upon cooling, a 10-μl sample of the reaction mixture was injected into the chromatographic system. A solution of 0.40 μg/ml CK and 12 m*M*/l monothioglycerol was reacted in this manner.

Varying CK concentrations in control serum. All solutions were made or diluted with 0.1 *M* Tris buffer adjusted to pH 6.8 with acetic acid. A 0.5-ml aliquot of a reaction solution containing 10 m*M*/l ADP, 10 m*M*/l AMP, and 100 m*M*/l magnesium acetate was mixed with 0.5 ml of a reactant solution containing 150 m*M*/l phosphocreatine and 30 m*M*/l $KF \cdot 2H_2O$. Final reaction conditions are also listed in Table I. This ADP—phosphocreatine mixture was then pre-incubated at 30°C for 3 min. In a 75-mm glass test-tube, a 0.5-ml aliquot of a

TABLE II

ENZYME AND ACTIVATOR CONCENTRATIONS FOR THE HPLC ASSAY OF CK IN CONTROL SERUM

CK (μg/ml)	Monothioglycerol (m*M*/l)
0.08	2.4
0.20	6.0
0.40	12.0
0.60	18.0
0.80	24.0

CK—monothioglycerol solution of varying concentration (Table II) was added to 1.0 ml of control serum and pre-incubated at 30°C for 3 min. At time 0 min, the ADP—phosphocreatine mixture was added to the CK—serum mixture. The reaction proceeded for 10 min and was terminated by placing the test-tube in boiling water for approximately 75 sec. The test-tube was centrifuged at about 400 *g* for approximately 5 min on a semi-micro bench-top centrifuge to separate the coagulated protein from the liquid. A 10-μl volume of the decanted supernatant was then injected into the chromatographic system.

Real serum assay by HPLC. The procedure for real serum samples was essentially identical to that of the procedure for the CK time study. The CK solutions were replaced with the serum samples and only a reaction time of 10 min was used. For the normal serum, 0.5 ml of serum with 60 m*M*/l monothioglycerol (20 m*M*/l reaction concentration) was pre-incubated for 3 min at 30°C. To this, the ADP—phosphocreatine mixture was added and allowed to react for 10 min. The reaction was then terminated and treated in the same manner as the CK in the control serum procedure. For the elevated CK in serum, 0.5 ml of serum was diluted to 2.0 ml with 0.1 *M* Tris buffer (pH 6.8). The diluted serum solution also contained 60 m*M*/l (20 m*M*/l reaction concentration) of monothioglycerol. A 0.5-ml aliquot of this solution was then used as in the above procedure.

CK assay spectrophotometric method. For the spectrophotometric assay of CK, the method of Bowers and Kelley [14] was utilized with several minor adjustments. The blank used was 0.1 *M* Tris buffer (pH 6.8) with acetic acid. All solutions were made or diluted with the 0.1 *M* Tris buffer. The concentrations of the substrates, cofactors, activators, and inhibitors used in the HPLC assay are very similar to those recommended by Bowers and Kelley and were kept the same for the spectrophotometric method. Final reaction concentrations for glucose, NAD, HK, and G-6-PD are given in Table I. Serum volumes of 0.2 or 0.25 ml were used for each assay.

Calculations

Peak areas were calculated using a HP 3000 minicomputer equipped with a HP 7221A plotter (Hewlett Packard, Palo Alto, CA, U.S.A.). The activity in units/l were calculated from the area of the ATP peak and an ATP calibration curve. A constant injection volume of 10 μl was used to eliminate any necessary conversion factors. Activity was calculated by the following equation:

$$\text{Units/l} = \frac{[\text{PA} - \text{YI}] \times \text{TV}}{\text{SC} \times \text{T} \times \text{SV}}$$

where

PA = peak area of ATP produced (cm^2)
YI = *Y* intercept of the ATP calibration curve (cm^2)
TV = total reaction volume (μl)
SC = slope of the ATP calibration curve [$cm^2/(\mu M/\mu l)$]
T = reaction time (min)
SV = volume of sample (l)

The YI and SC terms must correspond to the same detector range as the PA term.

Activity in units/l for the spectrophotometric method were calculated in the typical manner [15].

RESULTS

In selecting a suitable mobile phase for the separation of AMP, ADP, and ATP, the method of Hoffman and Liao [16] was considered. Resolution using this method was good; with a slight adjustment in pH and composition percentage, a slightly better separation of the components was achieved. The mobile phase consisted of an 88% mixture of 0.1 *M* KH_2PO_4, 0.025 *M* butylammonium hydrogen sulfate, and 12% methanol. To this, enough 0.75 *N* NaOH was added to adjust the pH to 6.8. All three components could be resolved in 16 min as shown in the chromatograms.

For the assay proposed, the chromatographic unit must exhibit a linear response to the product being quantitated, ATP. Table III lists a series of various ATP concentrations that were injected into the system and detected at 0.04 absorbance units full scale (a.u.f.s.). Each concentration was injected three times. The correlation coefficient shows that linearity of the system to ATP

TABLE III

INSTRUMENT RESPONSE FOR ATP AT 0.04 a.u.f.s.

Slope = 5.96 × 10^3; intercept = 0.11; correlation coefficient = 0.9980.

ATP concentration × 10^{-4} (M)	Peak area (cm^2)	R.S.D. (%)
0.31	0.30 ± 0.05	16.67
1.24	0.84 ± 0.06	7.14
2.48	1.65 ± 0.18	10.91
3.73	2.22 ± 0.06	2.70
4.97	3.13 ± 0.21	6.71

was excellent. A similar response curve was obtained for ATP at 0.08 a.u.f.s. Six different concentrations of ATP ranging from 4.97×10^{-4} M to 0.62×10^{-4} M were injected into the system. Each concentration was injected three times with relative standard deviations of peak areas ranging from 1.19% at higher concentrations to 16.15% at lower concentrations. The response curve

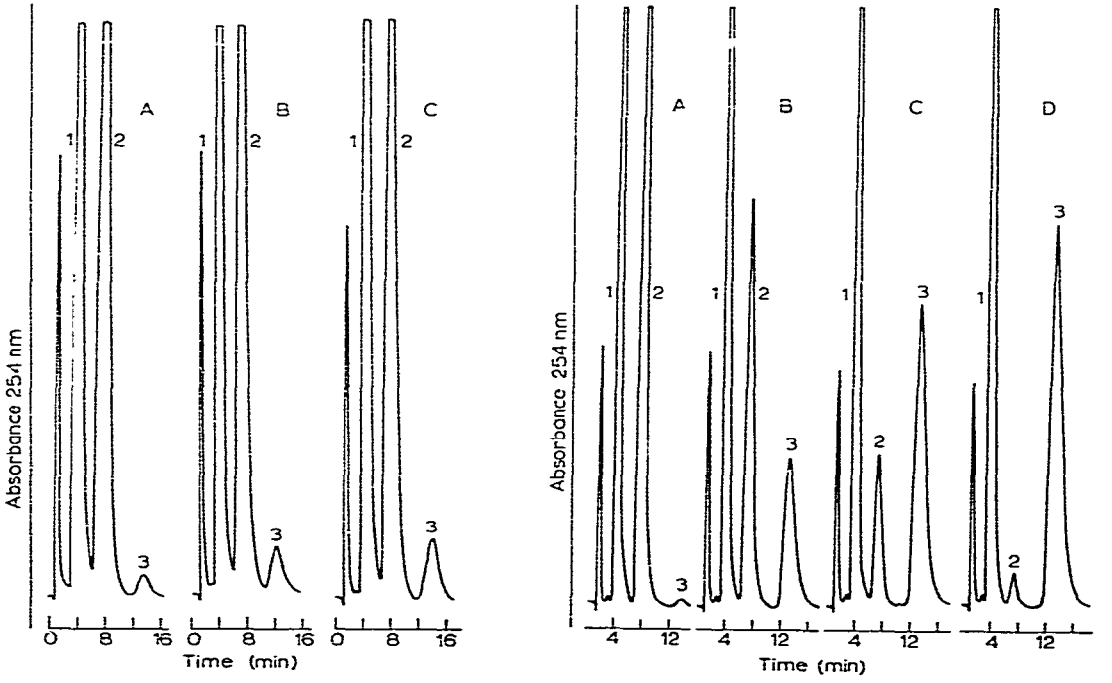

Fig. 1. Separation of AMP (1), ADP (2), and ATP (3) for reaction times of 10 min (A), 20 min (B), and 30 min (C) using 0.04 μg/ml CK. Chromatographic conditions: column, LiChrosorb C_{18}; eluent, see text; flow-rate, 2.0 ml/min; temperature, ambient; detection, UV 254 nm, 0.04 a.u.f.s.; injection volume, 10 μl.

Fig. 2. Separation of AMP (1), ADP (2), and ATP (3) for reaction times of 0 min (A), 10 min (B), 20 min (C), and 30 min (D) using 0.40 μg/ml CK. Chromatographic conditions: same as in Fig. 1 except detection range is 0.08 a.u.f.s. Chromatograms B, C, and D show the reduction of substrate ADP in addition to the leveling of the product ATP as time proceeds.

TABLE IV

LINEARITY OF CK ACTIVITY WITH RESPECT TO REACTION TIME FOR 0.04 μg/ml CK

Trial	Time (min)	Peak area (cm^2)	R.S.D. (%)	Slope	Intercept	Correlation coefficient
1	10	0.35 ± 0.02	5.7	0.046	−0.10	0.9999
	20	0.82 ± 0.11	13.4			
	30	1.27 ± 0.02	1.6			
2	10	0.34 ± 0.03	8.8	0.046	−0.13	0.9980
	20	0.75 ± 0.05	6.7			
	30	1.25 ± 0.06	4.8			
3	10	0.47 ± 0.07	14.9	0.042	−0.03	0.9519
	20	0.66 ± 0.06	9.1			
	30	1.32 ± 0.07	5.3			

for this range had a slope of 3.025×10^3, an intercept of 0.105, and a correlation coefficient of 0.996.

Once the linearity of the system to ATP was determined, the linearity of the reaction to time was investigated. Figs. 1 and 2 show the increase in the ATP peak area as time proceeds using CK concentrations of 0.04 μg/ml and 0.40 μg/ml. A visual inspection of the ATP peaks obtained using the CK concentration of 0.40 μg/ml (Fig. 2) is sufficient to demonstrate the leveling off the reaction between 20 and 30 min. Not only is the increase in the ATP indicative of this, but also the peak corresponding to ADP shows that the level of this substrate is insufficient to sustain the reaction at the initial rate.

Peak areas of ATP generated using a CK concentration of 0.04 μg/ml were quantitated and are listed in Table IV along with the corresponding reaction times. Slopes, intercepts, and correlation coefficients for each set of reaction times were calculated and are also listed in Table IV. As can be seen, linearity was achieved up to 30 min at this concentration of CK. All injections were performed in triplicate. For subsequent reactions, a reaction time of 10 min was adopted in that it seemed to be sufficiently long for low CK concentrations to be detected but not excessively long for high CK concentrations so that linearity was maintained. In addition to this, 10 min is not an excessive reaction time for the analysis of clinical samples.

Linearity of the assay with respect to CK concentration was determined. ATP peak areas obtained using the concentrations of CK in control serum in Table II were quantitated and used to calculate the respective units/l of CK. A plot of units/l of CK versus μg/ml of CK is shown in Fig. 3. Relative standard deviations for nine determinations are on the order of 3—10%. A slope of 671.3 was obtained with an intercept of −7.50.

Fig. 4 illustrates the difference in serum CK levels between a healthy patient and a heart attack victim. It is important to note that 0.5 ml of undiluted serum was used for the assay of the normal sample whereas 0.5 ml of a 2-ml

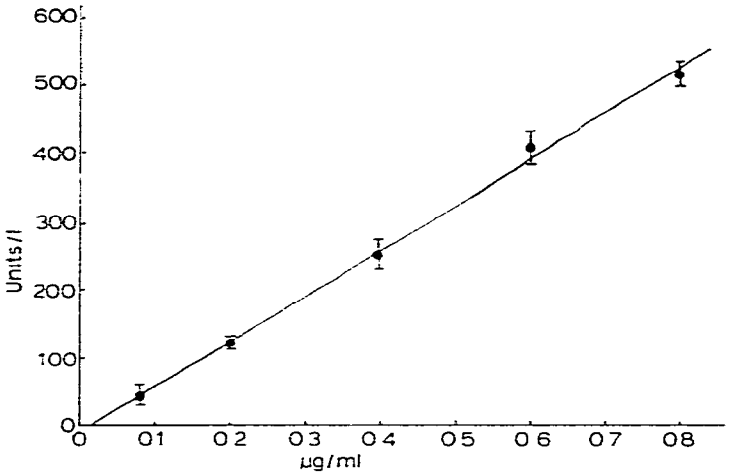

Fig. 3. Units/l of CK in control serum measured by the HPLC assay as a function of the CK concentrations listed in Table II. Conditions used are given in Table I. Each point represents the mean of nine determinations. Error bars indicate the standard deviation. Correlation coefficient = 0.9986.

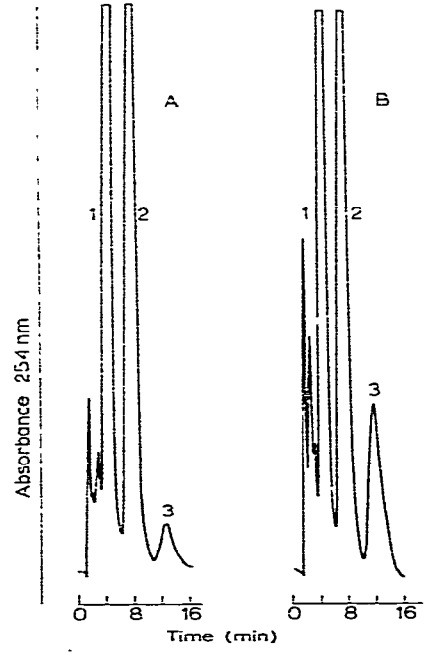

Fig. 4. Separation of AMP (1), ADP (2), and ATP (3) for CK assay of serum from a healthy patient (A) and a heart attack victim (B). Due to dilution of the elevated serum, the ATP peak area for (B) should be magnified 4 times when comparing to the ATP peak of (A). Chromatographic conditions are the same as those in Fig. 1.

solution containing 0.5 ml of serum was used for the assay of the elevated serum. Therefore, in order to give a proper perspective, the ATP peak in the elevated sample should be magnified four times due to the difference in sample volumes.

CK activities for real serum samples and standards determined using both the

TABLE V

COMPARISON OF THE HPLC AND THE SPECTROPHOTOMETRIC METHOD FOR THE ASSAY OF CK

CK source	HPLC method			Spectrophotometric method		
	Activity (U/l)	n^*	R.S.D. (%)	Activity (U/l)	n^*	R.S.D. (%)
0.04 μg/ml lyophilized CK	12.4 ± 4.5	3	36.3	10.0	1	—
0.40 μg/ml lyophilized CK	304.2 ± 26.2	4	8.6	87.3	1	—
Normal serum	26.6 ± 1.5	3	5.6	36.0 ± 1.0	3	2.8
Elevated serum	609.0 ± 28.3	2	4.6	256.0	1	—

*n = number of trials.

HPLC and spectrophotometric methods are shown in Table V. When serum samples were assayed by both methods, the normal sample produced similar values for the different assay procedures. However, when the elevated serum sample was assayed by both methods, a large difference in values was obtained. Possibly the serum matrix itself was a contributing factor to the difference in activities observed. To determine this, the ATP peaks corresponding to a reaction time of 10 min in the HPLC time studies were quantitated and converted to units/l. Both enzyme concentrations of 0.04 μg/ml CK and 0.40 μg/ml CK were used. These two solutions were then assayed by the spectrophotometric method. The activities obtained for the 0.04 μg/ml CK solution were similar for the two methods; however, the values obtained for the 0.40 μg/ml CK solution from the two methods differed considerably. This result, similar to that found for the serum samples, indicates there is some inhibitory factor in the coupled system and that the serum matrix was not a problem. Apparently, at high CK activities, the unfavorable equilibrium constant for the G-6-PD catalyzed reaction in the spectrophotometric method is a limiting factor. In addition, AMP inhibition of G-6-PD could also play a role. At low activities of CK, the G-6-PD catalyzed reaction is not a limitation and close CK activities for the two methods can be obtained.

DISCUSSION

The linearity of the system has been established for ATP over a concentration range of approximately one order of magnitude. Although the range is not extremely wide, it has proven to be sufficient for the purpose of this assay. For samples containing high levels of CK activity, simple dilutions will enable the ATP peak produced to be within this range. Although ATP concentrations above the reported upper limit were not tested, linearity would be expected to be maintained. At a.u.f.s. settings lower than 0.04, accurate quantitation of the ATP peak was difficult due to the inability of obtaining a stable baseline with the given mobile phase.

In performing this assay, the concern is mainly to determine whether or not

the CK levels in the serum are normal, borderline, or elevated. Bowers and Kelley [14] have reported the following ranges for CK activities in serum: normal 0—100 units/l, borderline 100—200 units/l, and elevated > 200 units/l. In using these ranges, the values obtained spectrophotometrically have shown that we have a serum sample with normal CK levels and also a serum sample with elevated CK levels. Before utilizing the HPLC procedure as a routine clinical assay, many more serum samples would need to be run to establish the normal, borderline, and elevated ranges for CK in serum. However, for these two serum samples, the HPLC method could clearly distinguish which is from a healthy patient and which is from a heart attack victim.

The direct assay for creatine kinase described offers several advantages over the usual enzymatically coupled spectrophotometric assays. The main advantage is that the measurement of the product is only dependent upon the rate of the CK reaction and not some function of a coupled reaction system. For the spectrophotometric method using NAD^+ as a substrate, G-6-PD must come from *L. mesenteroides*. This enzyme preparation is quite expensive, and the elimination of this enzyme would reduce analysis cost considerably. Analysis time is comparable; total analysis time, including incubation, is approximately 30 min. The separation is free from serum interferences and utilization of the substrate ADP could also be monitored if desired. Application of this assay for CK to a variety of other types of samples such as cerebrospinal fluid should be straightforward.

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