An Enzymic, Reaction-Rate Assay for Serum Creatinine with a Centrifugal Analyzer

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We describe a procedure for specific, rapid, kinetic determination of creatinine, in which a manual coupled-enzyme micro-scale assay is adapted to a centrifugal analyzer. The creatinine reaction is ultimately linked to NADH utilization, which is measured by the absorbance change at 340 nm. This procedure requires 15 µL of serum and the standard curve is linear to a creatinine concentration of 200 mg/L. A four-point kinetic algorithm allows the dynamic range of the assay to be extended without sacrificing sensitivity, and makes a separate serum blank unnecessary. The within-run precision (CV) for samples with a creatinine concentration of 11 and 52 mg/L was 5.6 and 2.4%, respectively; day-to-day CV for a creatinine concentration of 11 mg/L was 7.7% (n = 21). We compared this procedure with a kinetic Jaffé procedure, with excellent agreement (r = 0.996; y = 0.986 x + 2.4 mg/L). Bilirubin, non-esterified fatty acids, and ketone bodies do not affect creatinine determinations by this method; thus the method is especially useful for monitoring the renal function of diabetics.

Additional Keyphrases: pediatric chemistry - algorithm for assays involving coupled first-order reactions

Many methods have been presented for determining creatinine in biological samples. The most widely used analytical procedures are based on the Jaffé alkaline picrate reaction. Many biological and pharmacological compounds interfere with the Jaffé procedure (1–4). Its shortcomings as a kinetic assay (5, 6) have recently been reviewed (7, 8). These problems have motivated many investigators to devise alternative methods for creatinine determination: specific adsorption (9), chromatography (10, 11), and coupled enzymic assays (12, 13). Chromatographic techniques and adsorption procedures add specificity to creatinine determinations but remain, at best, semi-manual methods with less-than-acceptable analysis time. Here we report our adaption of the Boehringer Mannheim Diagnostics coupled enzymic creatinine assay (Creatinine Enzymatic UV-Method) to the Instrumentation Laboratory centrifugal analyzer (Multistat III). This coupled-enzyme assay for creatinine (Figure 1) involves creatinine amidohydrolase (EC 3.5.2.10), creatine kinase (EC 2.7.3.2), pyruvate kinase (EC 2.7.1.40), and lactate dehydrogenase (EC 1.1.1.27). The change in absorbance at 340 nm reflects the progress of the reaction. The manual enzymic method is relatively specific but still has the problem of long sample analysis time inherent in most manual analytical procedures.

The approach that we have taken to this creatinine assay can be applied to all centrifugal analyzers that have a design such that both double reagent addition and data collection at early time points are possible, thus allowing a kinetic creatinine determination. In addition we have applied a data-reduction algorithm to the assay that negates the need for sample blanks for each determination and extends linearity to 200 mg/L.

Materials

Reagents. NADH in pre-weighed vials was from Sigma Chemical Co., St. Louis, MO 63178 (disodium salt; grade III). Creatinine was from the United States National Bureau of Standards, as SRM No. 914 (purity: 99.8%). "Acetest" reagent tablets for ketone body determination were from Ames Co., Elkhart, IN 46515.

All other reagents were supplied in the kit from Boehringer Mannheim Diagnostics, Indianapolis, IN 46250. Vial 1 in this kit contains glycine (100 mmol/L), potassium phosphate (75 mmol/L, pH 8.0), and detergent. Vial 2 contains NADH (8 mmol/L), ATP (44 mmol/L), and phosphoenolpyruvate (15 mmol/L). Vial 3 contains creatine kinase (>500 kU/L), pyruvate kinase (>200 kU/L), lactate dehydrogenase (>500 kU/L), and magnesium chloride (100 mmol/L). Vial 4 contains creatinine amidohydrolase (>500 kU/L). Vial 5 contains pyruvate (2 mmol/L).

Creatinine standards. Standards—10, 25, 50, 100, 150, and 200 mg/L—were prepared the same day they were to be used by diluting, with distilled water, a stock creatinine solution (1.000 g/L in 50 mmol/L HCl). Stock solutions were prepared freshly every two weeks.

Instrumentation. We used the Astra 8, a discrete analyzer (Beckman Instrument Inc., Fullerton, CA 92634) and the Multistat III (Instrumentation Laboratory, Lexington, MA 02173). Multistat III settings were as follows: temperature, 30°C; absorbance λ, 340 nm; delay interval, 5 s; data interval, 45 s; and number of data points, 12.

Methods

Reagent dilution. The contents of vials 2 and 3 were each diluted with 2 mL of distilled water; and the contents of vials 1, 4, and 5 were used undiluted. These reagents were adapted for use with the Multistat III loader as follows: 60 µL of reagent 1 (50 µL from vial 4 diluted with 2.0 mL from vial 1); 75 µL of reagent 2 (100 µL from vial 2, 200 µL from vial 3, 1.5 mg of NADH, and 2.5 mL from vial 1); and a 15-µL sample were pipetted into each transfer disk used for centrifugal analysis.

Ketone body determination. For semi-quantitative ketone body determinations we used Acetest reagent tablets. The intensity of the color formed after serum was placed on a tablet was compared to a color-comparison strip enclosed with...
the tablets. Acetest is specific for the detection of acetoacetic acid and acetone. It is about 10-fold more sensitive to acetoacetic acid than to acetone and does not react with β-hydroxybutyric acid. Results were categorized as negative, small, moderate, or large. The "small" color block corresponds to about 200 mg of acetoacetic acid per liter, the "moderate" color block to 300–400 mg/L, and the "large" color block to about 800–1000 mg/L.

Data acquisition and reduction. The data-reduction algorithm is based on the assumption that the assay proceeds as a series of first-order reactions superimposed on a zero-order background rate. This model predicts that four absorbance data points can be used to calculate standard and unknown creatinine values: 

\[(A_1 - A_2) - (A_3 - A_4) = \Delta A,\]

where \((t_2 - t_1) = (t_4 - t_3)\). Furthermore this model allows two standard curves to be generated from one set of results. Examination of late time points \((t_{1L} \text{ through } t_{1A} = 45, 225, 270, \text{ and } 450 s, \text{ respectively})\) facilitates accurate determination of creatinine values that are <50 mg/L; early time-point analysis \((t_{E1} \text{ through } t_{E4} = 45, 135, 180, 270 s, \text{ respectively})\) extends the linearity of the determination to 200 mg/L. We constructed standard curves from data on a water blank and two creatinine standards (50 and 200 mg/L). Unknowns with \(\Delta A\) calculated to be less than that for the 50 mg/L standard were read off the two-point (water blank, 50 mg/L) standard curve; those with a greater calculated \(\Delta A\) were read from a second standard curve (linear regression line prepared by using the water blank and the 50 and 200 mg/L standards).

Results

Data acquisition. Figure 2 illustrates a plot of raw absorbance data vs time. The creatinine concentration in Figure 2 is 50 mg/L with the four early time points \((t_{E1} \text{ through } t_{E4})\) and four late time points \((t_{1L} \text{ through } t_{1A})\) indicated (note that \(t_2 - t_1 = t_4 - t_3\)). Linearity. We analyzed a series of creatinine standards run in the same rotor and plotted creatinine concentration \((x)\) vs \(\Delta mA\) (\(y\), as defined in Methods), using early and late data points as discussed above. Early time points yielded the regression equation \(y = 0.30x + 5.4\ mA\); the later time points are characterized by the regression equation \(y = 1.08x + 2.5\ mA\). In both cases the correlation coefficient \((r)\) equaled 0.999. The curve is linear to 200 mg/L (Figure 3).

![Fig. 2. Absorbance as a function of time for a 50 mg/L creatinine standard, with the four early \((t_{E1} \text{ through } t_{E4})\) and four late time points \((t_{1L} \text{ through } t_{1A})\) indicated.](image)

![Fig. 3. Standard curves for the present method.](image)

Precision. We assessed within-run precision by examining two specimens of pooled sera containing 11 and 52 mg of creatinine per liter \((n = 13)\). The CVs were 5.6% and 2.4%, respectively. Day-to-day precision was determined by the use of a pooled specimen of serum containing 11 mg of creatinine per liter, assayed on 21 consecutive days. The CV was 7.1% (Table 1).

Method comparison. We compared results by the present method with those by the Astra 8 technique. The results for 55 determinations with creatinine concentrations ranging from 4 to 200 mg/L gave the following equation for least-squares regression: \(y = 0.966x + 2.4\ mg/L\ (r = 0.996; \text{ mean values for } x \text{ and } y, 78.3 \text{ and } 77.5, \text{ respectively}).

Interference: studies on patients. We determined creatinine values for sera containing different concentrations of ketone bodies with the present method and the Beckman kinetic Jaffe procedure (Astra 8). Results with the Astra 8 for five diabetic patients in ketoacidosis were consistently higher \((31, 30, 27, 22, 18\ mg/L)\) for sera than were values obtained with the present method \((16, 14, 12, 12, \text{ and } 10\ mg/L)\). All these samples contained large amounts of ketone bodies \((>800\ mg/L)\) according to Acetest reagent-tablet results. After these patients had been treated with insulin for less than 24 h, sera were again sampled and creatinine determined. This time the Astra 8 measurements were lower, approaching the original enzymatic determination \((±2\ mg/L)\) in all cases. Furthermore this decline in apparent creatinine, as measured by the Astra 8, was accompanied by a decline in serum ketone bodies to <200 mg/L. We also examined icteric samples, using both methods. Astra 8 \((x)\) determinations on three samples containing greatly supranormal concentrations of unconjugated bilirubin \((285, 330, \text{ and } 442\ mg/L)\) resulted in lower values than those obtained with the Multistat III \((y)\). The values obtained by the present method were 22, 12, and 15 mg/L, respectively, differing by 6, 4, and 6 mg/L from the Astra 8 method. We examined 15 additional samples with concentrations of unconjugated bilirubin between 42 and 285 mg/L and conjugated bilirubin between 61 and 402 mg/L, with

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<tr>
<th>Creatinine, mg/L</th>
<th>Within-run ((n = 13))</th>
<th>Day-to-day ((n = 21))</th>
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<td>Mean</td>
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<td>SD</td>
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<td>0.8</td>
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<td>CV, %</td>
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Table 1. Precision of Creatinine Determination with the Automated Coupled-Enzyme Assay

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comparable results ($\bar{x} = 18$ mg of creatinine per liter; $\bar{y} = 20$ mg/L).

We investigated the negative interference of non-esterified fatty acids with the Astra 8 creatinine procedure, using three concentrations of them added to pooled serum with a creatinine concentration of 18 mg/L. At 0.5, 1.9, and 3.2 mmol/L, the Astra 8 creatinine values were 17, 15, and <0 mg/L, respectively. Creatinine determinations by the present method were 19 ± 1 mg/L at all three non-esterified fatty acid concentrations.

**Discussion**

The reaction scheme pictured in Figure 1 can be modeled as a series of pseudo-first-order reactions, if the reaction intermediates creatine, ADP, and pyruvate reach and maintain low concentrations with respect to their Michaelis constants. Other reactants in the process must either be in excess or continually regenerated. The appearance of NADH with time can then be described as a series of time-dependent exponential terms

$$C(t) = CR(0) \sum_{i=1}^{n} a_i e^{-k_i t}$$

where $C(t)$ and CR(0) are the concentrations of NADH at time $t$ and of creatinine at zero time, respectively. A zero-order background rate is observed and ascribed to endogenous activities in the serum, such as alkaline phosphatase (13). This background rate can vary substantially from sample to sample. The following model can then be postulated to explain the data on absorbance vs time shown in Figure 1:

$$A_t = \Delta A_{CR} f(t) + A_0 + bt$$

where:

- $A_t$ = absorbance of reactant NADH at time $t$
- $\Delta A_{CR}$ = absorbance change of NADH due to creatinine
- $f(t)$ = a sum of exponential terms
- $A_0$ = initial absorbance at $t = 0$
- $b$ = zero-order background rate constant

With this model the $\Delta A$ calculation described in Methods can be shown to be directly proportional to creatinine concentration.

$$(A_1 - A_2) - (A_3 - A_4) = \Delta A_{CR} \left[ f(t_1) - f(t_2) - f(t_3) + f(t_4) \right] + b(t_2 - t_1 - (t_4 - t_3))$$

Given $(t_2 - t_1) = (t_4 - t_3)$, the zero-order term, $b(t_2 - t_1) - (t_4 - t_3)] = 0$; and $[f(t_1) - f(t_2) - f(t_3) + f(t_4)] = a$ constant $(k)$, then $(A_1 - A_2) - (A_3 - A_4) = k\Delta A_{CR}$.

Thus the model then predicts that the calculated $\Delta A$ is proportional to creatinine concentration for any values of $t_1$ through $t_4$ if $(t_2 - t_1) = (t_4 - t_3)$. The data in Figure 3 are experimentally consistent with this prediction, showing a linear increase in $\Delta A$ with added creatinine. This model describes the absorbance data only if the NADH concentration does not at any time become the rate-limiting reactant. Samples were routinely run with minimal delay between the loader and the analyzer. Depending on the background rate, a significant amount of NADH could be consumed during any long delay between rotor loading and analysis. By varying the choice of time intervals to be measured, greatly different creatinine concentrations can be determined with acceptable sensitivity and without time-consuming dilution steps.

Consequently, low creatinine concentrations are determined at longer time intervals to maximize $\Delta A$ and sensitivity, while high creatinine concentrations are determined at shorter time intervals to ensure that the NADH concentration does not become significantly depleted. The two-standard-curve plot presented in Figure 3 demonstrates the sensitivity and increased linearity of this approach. Although this data-reduction method is somewhat complex, it is necessary to obtain linearity as well as sensitivity. The previously reported failure to implement this assay on a centrifugal analyzer is most probably a result of the authors' data-reduction approach as well as the inability of their instrumentation to perform double reagent addition (14).

In general, we have shown that results by our present method compare very well with those by the Beckman kinetic Jaffé procedure (Astra 8). The latter method, however, does give clinically misleading results as compared with the enzymic method under certain conditions. Interference by ketone bodies and non-esterified fatty acids with the kinetic Jaffé reaction has been well documented (3, 4, 15). The present method is unaffected by ketone bodies or non-esterified fatty acids, making it a more nearly accurate method for monitoring renal function in diabetic patients. In addition, we have demonstrated that sera containing high concentrations of unconjugated bilirubin can falsely lower results by the kinetic Jaffé method, but the present method is unaffected. This is consistent with data reported by others using specific creatinine methods (2, 3).

The creatinine method described here offers significant advantages over both popular Jaffé procedures and the manual procedure. Sensitivity and dynamic range are comparable between this method and automated kinetic Jaffé procedures, whereas the dynamic ranges in all cases (13, 16, 17)—and the sensitivity in one report (14)—of existing manual procedures are significantly less acceptable. Although the accuracy and precision of the manual method has been well documented (13, 16, 17), the present method significantly decreases both sample-analysis time and reagent consumption. The present method yields a technique with analysis time comparable to that of many routine laboratory procedures: water blank, 50 and 200 mg/L standards, low and high controls, and 14 patient samples require 16 min of instrument time (8 min loading time plus 8 min analysis time). The automation of this technique has also lowered reagent consumption, increasing to at least 700 the number of tests that can be done with an 80-test kit. In addition, the proposed data-reduction algorithm negates the need for separate sample blanks. A further advantage of the present method for pediatric samples is the reduction of sample volume from 200 to 15 μL.

In summary, the present method makes the coupled-enzyme creatinine assay feasible for use in the clinical laboratory in terms of analysis time and reagent expense. This method also displays sensitivity not achievable by current Jaffé analytical procedures without prior sample adsorption. Our data-reduction algorithm can be used with any assay system that experimentally appears to follow a series of coupled first-order reactions. The capability of having multiple standard curves, by sampling at early and late times, can dramatically improve the dynamic range of an assay. The centrifugal analyzers as a class of discrete analyzers are especially suited to this approach, because all reactions are started simultaneously and are exposed to the same temperature conditions.

**References**


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Detergent Activation of the Binding Protein in the Folate Radioassay

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A minor cow's whey protein associated with β-lactoglobulin is used as binding protein in the competitive radioassay for serum and erythrocyte folate. Seeking to optimize the assay, we tested the performance of binder solutions of increasing purity. The folate binding protein was isolated from cow's whey by means of CM-Sepharose CL-6B cation-exchange chromatography, and further purified on a methotrexate-AH-Sepharose 4B affinity matrix. In contrast to β-lactoglobulin, the purified protein did not bind folate unless the detergents cetrimethylammonium (10 mmol/L) or Triton X-100 (1 g/L) were present. Such detergent activation was not needed in the presence of serum. There seems to be a striking analogy between these phenomena and the well-known reactivation of certain purified membrane-derived enzymes by surfactants (lipids/detergents).

Materials and Methods

The following chemicals and reagents were used: [3',5', 7,9-3H]folic acid, potassium salt, with a specific activity of 20–40 kCi/mol (Amersham International Limited, Amersham, U.K.), Dextran T40 (Pharmacia, Uppsala, Sweden), activated charcoal (untreated powder, 250–350 mesh, cat. no. C-5260, Sigma Chemical Co., St. Louis, MO 63178), cetrimethylammonium bromide (British Drug House, Poole, Dorset, U.K.), Triton X-100, sodium ascorbate, thioethylene glycol (2-mercaptopethanol), and β-lactoglobulin (Sigma).

Methods for isolating and purifying the folate binding protein from cow's milk whey were recently reported in detail by us (7). In brief, folate binder obtained after cation-exchange chromatography of whey on a column of CM-Sepharose CL-6B (Pharmacia) was further purified by affinity chromatography on a methotrexate-AH-Sepharose 4B gel (Pharmacia). The binding capacities of all binder solutions, including β-lactoglobulin, were determined by equilibrium dialysis against [3H]folate (3).

Binding experiments were performed under assay-like conditions in glass or polystyrene test tubes, each containing 1.0 mL of Tris buffer (170 mmol/L, pH 7.4). The [3H]folate concentration of the assay buffer was 5 mmol/L, and the protein concentration was adjusted to a binding capacity of 2.5 mmol/L. Incubations were at 20, 7, or 37 °C for 1 h, after which the distribution between bound and free [3H]folate had reached a steady state. Free and protein-bound [3H]folate were separated by adding 100 μL of dextran-coated charcoal to the test tubes, vortex-mixing, and, after standing 5 min, centrifuging (1000 × g, 10 min). Aliquots (400 μL) of the supernatants were pipetted into vials containing 10 mL of scintillation fluid (“Lumagel”; Lumac B.V., The Netherlands), and the radioactivity was counted as previously described (3). Folate binding was calculated after subtracting blank values obtained from tubes assayed without added folate binder.

Results

To study the binding of folate, we used three types of binder preparations: β-lactoglobulin; folate binder isolated from cow's milk whey after cation-exchange chromatography; and

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