Kinetic Serum Creatinine Assays I. The Role of Various Factors in Determining Specificity

Larry D. Bowers

We studied the specificity of kinetic methods of analysis, with emphasis on creatinine determination. The error contributed by an interferent that reacts with the analytical reagent and absorbs at the wavelength of analysis is shown to be related to the ratio of the equilibrium absorbances of the analyte and interferent, the ratio of the rate constants, the extent of the analytical reaction, and the duration of the measurement. To assess the potential diminution of interference with kinetic methods, we determined the rate constants of several \( \alpha \)-keto acids in the Jaffe reaction. Evaluation of the advantages of various measuring techniques with respect to specificity showed the main factors to be the extent of the analytical reaction and the relative value of the rate constants. Total specificity for creatine appears to be unattainable with currently used kinetic techniques.

Additional Keyphrases: Jaffé reaction • kinetic analysis • variation, source of • centrifugal analyzer

Creatinine determination has been dominated by the use of the Jaffé reaction since its introduction by Polin in 1914 (1). Although there has been some debate as to the nature of the product, Butler has presented strong evidence that the structure is a Janovsky complex (2). The proposed mechanism also agrees with the observed rate law involving creatinine, picric acid, and hydroxide ion. The reaction of picrate under the conditions used is known to be very nonspecific, occurring with any compound that possesses an active methylene group such as a keto-enol tautomer. As a result, a great deal of effort has been expended in an attempt to improve the specificity within the technique. The methods used so far include batch or column ion-exchange chromatographic adsorption onto Lloyd's reagent (3) or a cation-exchange resin (4, 5), preliminary oxidation and extraction of interferents (6, 7), and removal of noncreatinine-chromogen color by using acid-treated reaction mixtures as blanks (8, 9). Alternatives to the Jaffé reaction such as the use of 3,5-dinitrobenzoates (10), gas chromatography (11), and enzymic reactions (12, 13) have also been proposed. The most popular means of improving the specificity of the creatinine assay, however, has been the use of kinetic methods based on the Jaffé reaction.

I have investigated the role of the initial measurement time, the measurement interval, the ratio of the rate constants of the interferent and analyte, and the ratio of the equilibrium absorbances of the two species in determining the specificity of the kinetic analysis. I have also investigated the effect of measurement technique on accuracy, in an attempt to identify any increase in specificity as a consequence of method.

Materials and Methods

All rate measurements were made with GEMSAEC procedure and instrumentation (Electro-Nucleonics Inc., Fairfield, NJ 07006) with the following settings for data collection:

<table>
<thead>
<tr>
<th>IR</th>
<th>CD</th>
<th>SC</th>
<th>SA</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>2</td>
<td>10</td>
<td>1.0</td>
</tr>
<tr>
<td>RE</td>
<td>HI</td>
<td>TC</td>
<td>RM</td>
</tr>
<tr>
<td>20</td>
<td>30</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>NR</td>
<td>LO</td>
<td>AD</td>
<td>XX</td>
</tr>
<tr>
<td>20</td>
<td>0.3</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

The reaction temperature was 30 °C; wavelength, 520 nm; filter position, 430–560 nm; and reaction mode, “Auto Rate.” Data were printed out as absorbance and change in absorbance readings. Rate constants were determined in the same manner as by Shoucri and Pouliot (14). In all cases, the correlation coefficient for triplicate runs analyzed simultaneously by linear regression was at least 0.98.

The chemicals (sodium salts, except as noted) were obtained from the following sources: creatinine from Eastman Kodak (Scientific Products, Minneapolis, MN 55441); acetoacetate, lactate (lithium salt), citrate, and \( \alpha \)-ketoglutarate from Sigma Chemical Co., St. Louis, MO 63178; pyruvate and \( \beta \)-hydroxybutyrate from Boehringer Mannheim Biochemical, Indianapolis, IN 46250; and oxaloacetate from Calbiochem-Behring Corp., La Jolla, CA 92037. Solutions were prepared in de-ionized and distilled water to avoid any effect of acid on the hydroxide ion concentration and were used within 8 h of preparation.

Picric acid (J. T. Baker Co., Phillipsburg, NJ 08865), 13 g/L; sodium hydroxide, 2.5 mol/L; and de-ionized water were mixed 9/2/9 by vol to prepare the Jaffé reagent.

Results and Discussion

The kinetic approach to achieving methodological specificity is certainly not a new one. Many researchers (15–19) have discussed both the instrumentation and approaches to kinetic analysis. For two first- or pseudo-first-order reactions, each of which produces a colored product (B and C) that has some absorbance at a prescribed wavelength, the absorbance at any time \( t \) is given by

\[
A_t = \epsilon_B[B_0]_0[1-\exp(-k_BT)] + \epsilon_C[C_0]_0[1-\exp(-k_CT)]
\]

where \( \epsilon_B \) and \( \epsilon_C \) are the molar absorptivities for the interferent B and analyte C, respectively, \( b \) is the optical path length, \( [B]_0 \) and \( [C]_0 \) are the initial concentrations of B and C, and \( k_B \) and \( k_C \) are the first-order rate constants. Because the absorbance at any time is a function of the product of the path length, the molar absorptivity, and the initial concentration, we can represent this absorption product as \( A_{-B} \) and \( A_{-C} \) for compounds B and C, respectively. The molar absorptivities are determined by the products of (e.g.) the Jaffé reaction. It should be apparent from equation 1 that the second determining factor in the production of an absorbance at any time is the rate constants, \( k_B \) and \( k_C \). These are again determined by the choice of reaction (e.g., Jaffé) and the reaction temperature. The change in absorbance, \( \Delta A \), from time \( t_1 \) to time \( t_2 \) can be written as

\[
\Delta A = A_{t_2} - A_{t_1} = A_{-B}[\exp(-k_BT) - \exp(-k_BT_2)] + A_{-C}[\exp(-k_CT) - \exp(-k_CT_2)]
\]

\[
= A_{-B}[\exp(-k_BT_1) - \exp(-k_BT_2)] + A_{-C}[\exp(-k_CT_1) - \exp(-k_CT_2)]
\]

\[
+ A_{-C}[\exp(-k_CT_1)][1-\exp(-k_CT_2)]
\]

\[
(2)
\]
where $\Delta t$, the measurement interval, is the difference between $t_2$ and $t_1$. We can now write an equation for the relative error, $\theta$, in the observed absorbance change.

$$\theta = \left[ \frac{A_{\infty, C}}{A_{\infty, B}} \cdot \exp(-k_{Ct_1}) \cdot 1 - \exp(-k_{C\Delta t}) \right]^{-1} \left[ \frac{A_{\infty, B}}{A_{\infty, C}} \cdot \exp(-k_{Bt_1}) \cdot 1 - \exp(-k_{B\Delta t}) \right]$$

(3)

Rearranging,

$$\theta = \left[ \frac{A_{\infty, C}}{A_{\infty, B}} \cdot \exp(-(k_C - k_B)t_1) \cdot 1 - \exp(-k_{C\Delta t}) \right]^{-1} \left[ \frac{A_{\infty, B}}{A_{\infty, C}} \cdot \exp(-(k_B - k_C)t_1) \cdot 1 - \exp(-k_{B\Delta t}) \right]$$

(4)

If $r$ is the ratio of the rate constants, $k_B/k_C$, further simplification yields

$$\theta = \left[ \frac{A_{\infty, C}}{A_{\infty, B}} \cdot \exp(-(1 - r)k_{Ct_1}) \cdot 1 - \exp(-k_{C\Delta t}) \right]^{-1} \left[ \frac{A_{\infty, B}}{A_{\infty, C}} \cdot \exp(-(1 - r)k_{Bt_1}) \cdot 1 - \exp(-k_{B\Delta t}) \right]$$

(5)

The error in the absorbance change measurement is now a function of the measurement interval and of three dimensionless quantities: the ratio of the equilibrium absorbances, the ratio of the rate constants, and the extent of the reaction of analytical interest at the initial absorbance measurement. I emphasize that $\theta$ is the relative error in absorbance; to estimate the relative error in analyte concentration, the results must be multiplied by a concentration conversion factor ($\Delta A/k_{CB}$).

Now consider the ratio of the rate constants for several cases of analytical interest. If the rate of the interfering reaction is slow compared with that of analytical reaction, $r < 1$ and one would expect to make the measurement soon after initiation of the reaction; i.e., $k_C t_1$ is small. Equation 5 would indicate increasing precision with this approach. In the case of creatinine, however, the rate constant for acetacetate has been shown to be larger than that for the analyte, i.e., $r > 1$. This would require a greater extent of reaction for the analyte before the contribution of the interference to the absorbance change can be eliminated. Equation 5 predicts a decrease in error under these circumstances. The intermediate case presents considerable difficulties in the use of kinetic analysis with one- or two-point measurements. We will restrict our discussion to cases where $r$ is greater than or equal to 1.

Because one is attempting to improve the specificity of the Jaffe reaction by using kinetic methodology, a plot of error as a function of extent of reaction should yield useful information about several of the parameters of interest. Figure 1 illustrates the effect of the ratio of the rate constants. If there is an interferent whose rate constant is larger than that observed for the analytical species, one should ideally wait until the interfering reaction reaches equilibrium before measuring the absorbance change. The length of time one can wait for component B to reach completion, however, is limited, because at least 10% of component C must remain to assure that a significant rate of reaction is observed (17). This requires that the reaction of interest proceed for three half-lives or less, e.g., $k_C t_1 \leq 1.983$. If $r$ is 1.5, the interfering reaction contributes about 50% of the absorbance change. When $r$ is 3 or greater, the error in the measurement is less than 5% under the conditions stipulated. Under the more reasonable limit of one half-life elapsed from initiation for the reaction of analytical interest, an $r$ value of less than 7 yields more than 5% error when $A_{\infty, C}/A_{\infty, B} = 1$. Figure 2 shows the effect of varying the ratio of absorbances for an $r$ value of 3.0. When the absorbance product of the analyte is 10-fold greater than the absorbance product of the interferent, the three-half-life extent of reaction criterion can be met with only 5% error, even when the $r$ value is 1.5. Note that as the initial concentration of B increases, $A_{\infty, B}$ increases and, as shown in Figure 2, the error at any extent of conversion increases.

Figure 3 illustrates the effect of the measurement interval on the error in analysis. The larger the interval, the greater the sensitivity of the assay. Thus, when $\Delta t$ is very large, there is, in effect, an equilibrium analysis. At the opposite extreme, a pure derivative method uses a measurement interval of essentially zero. It has been implied that the derivative method is more specific than two-point kinetic methods because the interference B is measured to a greater absolute degree with a larger $\Delta t$ (20). As shown in Figure 3, however, the main determinant in achieving specificity is the initial measurement time. One way of explaining this result is to consider that although the absolute amount of absorbance due to B increases with increasing $\Delta t$, the amount in each additional increment...
of time relative to the absorbance change due to the analyte C in that increment decreases if B is a faster reacting species. Thus one would expect that specificity, as well as precision due to the larger absorbance change, would improve with increasing ΔA. From Figure 3 this appears to be true, within reason. Obviously, at equilibrium one loses all specificity, and equation 5 reduces to \( (A_{r=C}/A_{r=B}) = 1 \).

One final conclusion regarding the role of the measurement technique in achieving specificity can be gleaned from Figure 4. Figure 4A illustrates an absorbance vs time curve for several analyte–interferent mixtures. By choosing \( t \) and Δ\( t \), the observed absorbance change can be obtained. For kinetic specificity to occur, the curves for the mixture of materials must be parallel to the curve for the pure analyte. This can be shown to be true after about 30 s for curve \( c \). Figure 4B illustrates the rate of change of absorbance for the curves in Figure 4A, obtained by taking the derivative of equation 1. The y-value at any time \( t \) is proportional to the concentration of analyte. In this case, the curves for the mixture and pure analyte must be coincident to remove any contribution of the interference. Note that curve \( c \) converges with curve \( a \) at about 30 s, the same time as the occurrence of parallelism in the absorbance–time illustration. In neither case can the interference with a rate constant twice the rate constant of the analyte (curve \( b \)) be eliminated from quantitation with the species of interest. Thus the specificity, which is determined by the reaction kinetics, is not changed by method.

To ascertain whether creatinine could be accurately determined in the presence of metabolites such as pyruvate, I tested a series of common metabolites. Citrate, \( \beta \)-hydroxybutyrate, and lactate, up to 50 mmol/L, showed no reactivity with alkaline picrate. These results were anticipated because the presence of hydroxy groups instead of ketone functions would result in a relatively unreactive methylene group. Acetoacetate reacted so rapidly that its reaction was essentially complete after 10 s, the first time observable with the GEMSAEC. This would require a rate constant of at least 0.5 s\(^{-1}\). The apparent first-order rate constants for all species that showed a significant reaction rate were as follows: creatinine, 0.0022 s\(^{-1}\); oxaloacetate, 0.0027 s\(^{-1}\); \( \alpha \)-ketoglutarate, 0.0046 s\(^{-1}\); and pyruvate, 0.0049 s\(^{-1}\). This would imply that the determination of any of the latter species in the presence of the others would be impossible, if single- or two-point kinetic or equilibrium methodology were used. Accurate determination of creatinine in the presence of acetoacetate should be feasible (\( r = 227 \)) with any kinetic measurement.

The present discussion applies only to the application of single- and two-point kinetic methods (such as those currently in use in the determination of serum creatinine) to kinetic situations where the rate constant of the interferent is greater than that of the analyte. Extensive discussions of multicomponent kinetic methods and their associated errors, particularly when \( r < 1 \), have been presented elsewhere (17) and are beyond the scope of this paper. An approach to discriminating between compounds with similar rate constants has been reported, in which multipoint regression methods were used (21). Landis and Pardue (22) were able to quantify mixtures of conjugated and unconjugated bilirubin by using kinetic measurements of their reaction with \( p \)-diazobenzenesulfonic acid, despite the fact that \( r = 3.4 \). However, their data were acquired during at least three reaction half-lives to improve precision, a procedure that would improve the precision of two-point methods as well. Thus, methods have been described that can potentially resolve problems such as those observed here and in other situations where the ratio of the rate constants is small. Unfortunately, these methods are not used in the clinical laboratory.

In summary, kinetic methods of serum creatinine analysis rely on a constant rate of reaction between creatinine and alkaline picrate to achieve a more specific and therefore more accurate analysis. Shoucri and Pouliot (14) have reported the variation in rate constants for serum creatinine and the attendant difficulties of obtaining accurate results. I have, on a theoretical basis, evaluated the effect of the measurement interval and the ratio of the rate constants, the initial concentrations of analyte and interferents, and their molar absorptivities with respect to the error in absorbance observed due to an interfering reaction. The most important parameters, despite the attention given to various measuring techniques, are the fractional conversion of the analyte of interest.

**Fig. 4.** Continuous monitoring (A) and derivative monitoring (B) of reaction observed for two co-reacting species

\( a \), the analyte of interest; \( b \), an equimolar mixture of the analyte and a species whose rate constant is twice that of the analyte; \( c \), an equimolar mixture of the analyte and a species whose rate constant is 20 times that of the analyte. In any case \( A_{r=a}/A_{r=b} = 1 \).
and the ratio of the rate constants. Moreover, a number of keto-acids have rate constants that preclude obtaining total specificity with currently used single- and two-point kinetic methods.

References