Evaluation of a Kinetic Method for Simultaneous Determination of Conjugated and Unconjugated Bilirubin

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This paper describes a fast kinetic method for the simultaneous determination of unconjugated and conjugated bilirubin in the same reaction solution. A stopped-flow mixing system with a stabilized photometer and small computer is used to mix sample and reagent rapidly and to record 250 data points during a 700-ms reaction time, and a regression program is used to resolve these kinetic data into the concentrations of unconjugated and conjugated bilirubin. Data are reported for synthetic single and two-component samples and for serum samples. Kinetic results for synthetic mixtures and serum samples are compared with results obtained by a conventional two-step procedure. Regression equations show good linearity between kinetically determined absorbance changes and concentration, good agreement between taken and found values for total, unconjugated, and conjugated bilirubin for synthetic samples in human serum albumin, and a good correlation between kinetic and equilibrium results for these species in sera. Regression slopes for kinetic vs. equilibrium assay results for total, unconjugated, and conjugated bilirubins in sera were 1.01 ± 0.05, 1.04 ± 0.03, and 0.91 ± 0.04, respectively, with intercepts of 6.8, 3.8, and 3.8 μmol/liter, and standard errors of estimate of 28, 14, and 20 μmol/liter. These data reflect uncertainties in both the kinetic and equilibrium methods.

Additional Keyphrases:icterus, disease, "direct" and "indirect" assays for bilirubin

Reliable determination of both unconjugated bilirubin (UCB) and conjugated bilirubin (CB) in body fluids represents one of the difficult analytical problems in clinical laboratories. Commonly used "direct" and "indirect" procedures take advantage of the different solubilities and reaction rates of UCB and CB with p-diazobenzenesulfonic acid in aqueous and methanolic solutions (1). Unconjugated bilirubin is determined by difference from total bilirubins (TB) and CB, determined under vastly different conditions.

Here we describe a kinetic method that permits simultaneous determination of both UCB and CB in a single step in the same reaction mixture. The method makes use of the facts that at certain pH values, the apparent first-order rate constants for UCB and CB reacting with p-diazobenzenesulfonic acid differ significantly (2), and the different rate constants permit the two species to be resolved quantitatively by use of kinetic data (3). Samples containing bilirubin are mixed with the reagent in a stopped-flow system, and 250 values of absorbance at 530 nm are recorded during a period of about 700 ms. UCB and CB concentrations are evaluated from these data with regression programs described later.

Results are reported for single- and two-component synthetic samples of UCB and CB and for several sera containing both components. Kinetic results are compared with equilibrium results obtained with conventional procedures that use caffeine (4). Results for 50 synthetic samples containing, per liter, 0–400 μmol of UCB, 0–250 μmol of CB, and 0–450 μmol of total bilirubins processed by the kinetic method gave regression equations for found vs. added with slopes of 1.01, 0.99, and 1.05 and intercepts of 7.15, 4.26, and 7.8 μmol/liter for TB, UCB, and CB, respectively. Application of the equilibrium method to the same samples gave regression slopes of 0.97, 0.88, and 0.78 and intercepts of 5.5, 31.2 and 0.5 μmol/liter for TB, UCB, and CB, respectively. These data suggest potential performance advantages for the kinetic method in comparison with the conventional two step-equilibrium method.

General Considerations

Figure 1a shows absorbance vs. time data for UCB and CB, recorded under the same conditions and on the same time scale. Clearly, the UCB reaction is significantly faster than the CB reaction. It is this difference in reaction rates that is used for the simultaneous kinetic determination of UCB and CB in the same reaction mixture. Figure 1b is a plot of absorbance vs. time for a solution containing both species. The data rate and time scale on the plot were changed part way through the experiment, so that adequate time resolution is achieved for both reactions without collecting an excessive number of data points for the slower reaction. Although UCB has its major effect during the early part of the response curve and CB has its major effect during the latter part of the curve, the data in Figure 1a show that both species are contributing throughout the reaction time. The mathematical procedure used in this work takes account of the simultaneous contributions. The
rationale for the kinetic method is discussed briefly here and the mathematical procedure is discussed in more detail in the Appendix.

**Reaction Model**

Earlier work has shown that both UCB and CB react with p-diazobenzenesulfonic acid in two steps, with the first step involving the production of azobilirubin (AZB) and hydroxypropyromethene carbinoi (HPC), and the second step involving conversion of HPC to azobilirubin (5). In our early work with this system, we observed that the second step involving conversion of HPC to AZB is catalyzed by sulfanilic acid, and that if the reaction takes place in the absence of sulfanilic acid, then the second step is slow as compared to the first (2). Under this condition and in the presence of an excess of p-diazobenzenesulfonic acid, both UCB and CB reactions follow pseudo-first-order kinetics. We also observed that in the pH range between 6 and 8 there are significant differences in the apparent first-order constants for UCB and CB. We also confirmed that the azobilirubin products from UCB and CB under these conditions have virtually identical absorption spectra, with maxima at 530 nm. These observations lead to a simplified reaction model involving two parallel pseudo-first-order reactions that can be represented as follows

\[ \text{UCB} \xrightarrow{k_u} \text{AZB} \quad (1a) \]

and

\[ \text{CB} \xrightarrow{k_c} \text{AZB} \quad (1b) \]

where \( k_u \) and \( k_c \) represent apparent first-order rate constants for UCB and CB.

**Mathematical Model**

For reactions involving either UCB or CB, the time-dependent concentration of azobilirubin will be given by

\[ [\text{AZB}]_t = C_u^0 (1 - e^{-k_u t}) \quad (2a) \]

or

\[ [\text{AZB}]_t = C_c^0 (1 - e^{-k_c t}) \quad (2b) \]

where \( C_u^0 \) and \( C_c^0 \) represent initial concentrations of the species. For a reaction involving both UCB and CB, the product concentration will be additive, and the time-dependent concentration of azobilirubin will be the sum of reactions \(2a \) and \(2b \), or

\[ [\text{AZB}]_t = C_u^0 (1 - e^{-k_u t}) + C_c^0 (1 - e^{-k_c t}) \quad (3a) \]

If it is assumed that molar absorptivities at 530 nm for AZB produced from UCB and CB are the same, then equation \(3a \) can be rewritten as

\[ A_t = c_b [C_u^0 (1 - e^{-k_u t}) + C_c^0 (1 - e^{-k_c t})] + A_b \quad (3b) \]

where \( A_t \) is the time dependent absorbance, \( c \) is the molar absorptivity of AZB, \( b \) is the path length, and \( A_b \) is a blank absorbance from any source(s). If all UCB and CB were converted to AZB, then they would produce absorbances equivalent to \( A_{w,u} = c_b C_u^0 \) and \( A_{w,c} = c_b C_c^0 \), assuming \( c_u = c_c \), so that equation \(3b \) can be rewritten in terms of these equivalent absorbances as

\[ A_t = A_{w,u} (1 - e^{-k_u t}) + A_{w,c} (1 - e^{-k_c t}) + A_b \quad (3c) \]

where \( A_{w,u} \) and \( A_{w,c} \) represent absorbances that would result if all UCB and all CB present in a sample were converted to AZB.

If \( k_u \) and \( k_c \) were known, then absorbances measured at three points in time for a reaction would give three equations in three unknowns \( (A_{w,u}, A_{w,c}, \text{and } A_b) \) that could be solved for the absorbances, and then \( C_u^0 \) and \( C_c^0 \) could be computed or evaluated from a calibration plot. In practice, such a three-point procedure would result in large uncertainties, and we have used a multipoint procedure (6) involving 250 data points. The data are collected on-line with a small computer, and a least-squares program is used to determine values \( A_{w,u}, A_{w,c}, \text{and } A_b \) that give the best fit of absorbances computed with equation \(3c \) to the experimental values. Concentrations of UCB and CB are determined from a calibration plot. A more complete description of the mathematical procedure is presented in an appendix to this paper.

**Materials and Methods**

**Instrumentation and Software**

All kinetic experiments were performed on a computer-controlled stopped-flow system described earlier (7, 8) and equilibrium absorbance measurements were made on a commercial spectrophotometer (Spectronic 100; Bausch and Lomb, Inc., Rochester, N.Y. 14625). Both spectrometers used cells with 10-mm light paths.

The computer software used in this work consisted of simultaneous kinetic analysis programs developed by Ridder and Margerum (3) added as subroutines to the program of Mieling et al. (7) for the computerized stopped-flow system.
An important feature of the kinetic analysis program is the ability to change data-acquisition rates during the course of an experiment. Data rates are adjusted automatically by the program (see Fig. 1b) to obtain suitable number of data points during four half-lives of each first-order reaction. Rate constants needed to compute the time per half-life are obtained from preliminary runs on UCB and CB under the analysis conditions. The program also makes use of the estimated mixing time (5 ms) to establish zero reaction time. The program evaluates 'best fit' values of $A_b$, $A_{b,m}$, and $A_{b,c}$ (see equation 3c) to compute absorbance changes resulting from UCB and CB, and these data are used to read UCB and CB concentrations from a calibration plot.

The program also controls the reagent handling and mixing functions to perform analyses automatically. Samples can be processed at a rate of 60 per hour with the system.

Reagents

All reagents were prepared in doubly distilled water (Megapure Still; Corning Glass Works, Corning, N.Y. 14830) and reagent-grade chemicals were used whenever possible.

**p-Diazobenzensulfonyl acid.** The diazonium salt was prepared and standardized as described earlier (2) and a 1.00 mmol/liter solution in 1.2 mol/liter sodium perchlorate was used in the analysis procedure. The sodium perchlorate is included to adjust the ionic strength to that of the sample mixture described below to ensure efficient mixing.

**Buffer–caffeine–benzoate.** This solution was prepared by dissolving 37.5 g of anhydrous caffeine (Sigma Chemical Co., St. Louis, Mo. 63178), 56.3 g of sodium benzoate (Sigma Chemical Co.), 0.58 g of the disodium ethylenediaminetetraacetate (Fisher Scientific Co., Fairlawn, N.J. 07410), 1.44 g of NaH$_2$PO$_4$.H$_2$O, and 37.86 g of Na$_2$HPO$_4$ (both from Mallinckrodt, Inc., St. Louis, Mo. 63147) in water and diluting to 1 liter. The resulting solution was filtered and was stable for at least one year.

**Unconjugated bilirubin standards.** Standard solutions of bilirubin were prepared by the method of Doumas et al. (9) from a commercial preparation (lot no. 5256; Harleco, Gibbstown, N.J. 08027) certified by the College of American Pathologists. Standards in the range from 17.0 to 428 μmol/liter were prepared from a 428 μmol/liter stock solution in 40 g/liter human serum albumin by dilution with a 40 g/liter solution of human serum albumin (Fraction V, lot no. A2358; Sigma Chemical Co.). Standards were kept frozen at −20 °C in the dark until needed.

**Conjugated bilirubin.** Conjugated bilirubin was isolated as described earlier (10) and dissolved in a 40 g/liter human serum albumin solution. The resulting solutions were standardized against UCB standards with the kinetic procedure described here. Solutions containing normal concentrations of 17.1 to 428 μmol/liter CB in 40 g/liter human serum albumin solution were prepared daily because CB is oxidized to biliverdin when stored for several hours at room temperature.

**Synthetic samples.** Synthetic single- and two-component samples were prepared by diluting appropriate amounts of UCB and CB stock solutions with 40 g/liter human serum albumin solution.

**Procedures**

**Kinetic method.** One part of bilirubin samples was mixed with four parts of the buffer–caffeine–benzoate solution and the resulting solution was mixed with an equal amount of the p-diazobenzensulfonyl acid reagent in the stopped-flow instrument. This procedure corresponds to reaction conditions involving pH 7.54, 77.3 mmol/liter caffeine, 156 mmol/liter benzoate, 480 μmol/liter p-diazobenzensulfonyl acid, and an ionic strength of 1.2 controlled by sodium perchlorate in the diazo reagent and buffer components in the buffer–caffeine–benzoate solution. The reaction temperature was 25.0 °C. Absorbances were measured on line with a small computer. A total of 250 absorbance values were recorded for each sample, with 161 points being collected at 1.5-ms intervals during the first 242 ms of the reaction and with 89 points being collected at 5.0-ms intervals between 242 and 687 ms of the reaction time.

Concentrations of the bilirubin species were determined from these data as discussed earlier.

**Equilibrium method.** A modified (11) Jendrassik–Gróf method was used to obtain data for comparison purposes. The method was similar to that described in Tietz (4) except that the volume of water was increased from 150 μl to 200 μl for TB and the color-development time for CB was extended to 10 min.

**Results and Discussion**

The results presented here are intended to explain the rationale for experimental conditions chosen for the analytical procedure and to illustrate performance characteristics for single- and two-component mixtures of the bilirubin species in synthetic samples and for both species in sera.

**Analytical conditions.** Earlier work (2) has shown that the bilirubin reactions follow pseudo-first-order conditions for a wide range of conditions. The most critical factors in the selection of conditions were to adjust the reaction rates so the time scales would be in a range easily handled by the stopped-flow instrument, to obtain the largest possible ratio between the rate constants for UCB and CB, and to minimize effects of the second step in the UCB reaction. Conditions described above correspond to rate constants of 11.3 s⁻¹ and 3.3 s⁻¹ for UCB and CB, respectively. These rate constants correspond to half-lives of 61 ms and 210 ms, respectively, for the two reactions, which are ideally suited to the stopped-flow instrumentation.

Previously reported pH studies (2) show that rate constants for UCB and CB are significantly different in the pH range of 6 to 8, and any pH in this range gives a ratio of the rate constants that is useful for analytical purposes. The final choice of pH was dictated by the second step in the reactions involving conversion of hydroxypropyromethene carbinol to azobilirubin.

Although both bilirubin species react in two steps, it is the second step in the UCB reaction that is of most concern, because the hydroxypropyromethene carbinol produced by the faster UCB reaction could interfere with measurements involving the slower CB reaction. Accordingly, our attention was focused on the second step in the UCB reaction. Figure 2 shows the effects of pH on both the first-order rate constant for the second step in the UCB reaction and the total absorbance produced in the reaction. The influence from this reaction is minimized for pH values below 6 and above 7.5. Data presented earlier show that the absorbance change produced by UCB is dependent upon pH below 6 and insensitive to pH above 6. To minimize the dependence upon pH, to maximize the sensitivity for UCB, and to minimize the effect of the second step in the UCB reaction, we chose a pH of 7.54 for these analyses. At this pH the observed rate constant for the interfering reaction is about 0.1 s⁻¹, compared to values of 11.3 and 3.3 s⁻¹ for UCB and CB. The primary effect of the second step in the UCB reaction will be to give an erroneously high reaction velocity during the latter part of the reaction when data related mainly to the CB reaction are being collected. While it is beyond the scope of this paper to present a detailed analysis of the effect of this second step of the UCB reaction on the total analysis, it is important to identify the nature of the problem for some selected situations. To do this,
we shall consider initial concentration ratios of CB to UCB of 10:1, 1:1, and 1:10, and we shall consider just one point in time, namely 0.23 s when the UCB reaction is about 50% complete and the CB reaction is about 50% complete. For these conditions, the ratio of the reaction velocity for CB to that for the second step in the UCB reaction would be about 200:1, 20:1, and 2:1, respectively, for the 10:1, 1:1, and 1:10 concentration ratios mentioned above. While it is difficult to project actual errors from these data, it is easy to see that there will not be much problem when CB concentration is greater than UCB concentration and that significant problems should be expected when CB concentration is much smaller than that for UCB. We re-emphasize that the data presented above are those for only one of 250 total data points the regression subroutine uses to evaluate concentrations; accordingly, it would be improper to draw simplistic conclusions concerning actual errors from these data.

Analytical Data

The kinetic method was evaluated for single-component samples of UCB and CB, for synthetic mixtures of these species, and for serum samples. The mixture and serum samples were also analyzed by an equilibrium method (4) for comparison purposes. As the comparisons are made below, it should be kept in mind that the equilibrium procedure for CB (4) involves a reaction at low pH without any caffeine present and the use of a calibration plot that was established with UCB at high pH with caffeine present so that any matrix effects that produced different behavior under these different conditions could cause systematic errors. All uncertainties are reported at the one standard deviation unit level (±1 SD). All UCB and CB concentrations (C₀ and C₀, respectively) are expressed as micromoles per liter.

Calibration plots. Standards containing UCB in concentrations ranging from 17 to 430 μmol/liter were run by the kinetic and equilibrium methods. During the kinetic runs we also measured the absorbance, Aₘ, after the reaction had gone to more than 99.6% completion. These data permit a comparison between ΔA values computed from kinetic data and measured values of ΔA. Regression equations for two types of determinations are

\[ Aₘ = (2.20 ± 0.02) \times 10^{-3} C₀₀ + 0.011 \]
\[ ± 0.05; \text{SEE} = 0.007, r = 0.9999 \]

and

\[ ΔA_{kin} = (2.19 ± 0.02) \times 10^{-3} C₀₀ - 0.010 \]
\[ ± 0.004; \text{SEE} = 0.006, r = 0.9999 \]

where SEE is the standard error of estimate and r is the correlation coefficient. It is clear that the two procedures give virtually identical results. The regression equation for absorbance (Aₘ) obtained with the conventional equilibrium method (4) vs. concentration is

\[ Aₘ = (3.51 ± 0.01) \times 10^{-3} C₀₀ + 0.003 ± 0.002; \text{SEE} = 0.03, r = 0.9999 \]

The statistical parameters are similar for the three approaches except that the standard error of estimate (SEE) shows a significantly larger scatter about the least-squares line for the equilibrium method than for the kinetic method. The slopes of these plots are not expected to be the same, because AZB is monitored directly in the kinetic method and a product modified by the addition of tartrate is measured in the equilibrium method.

Slopes of the data on Aₘ and ΔA_{kin} vs. C₀ are related to the molar absorptivities of AZB. Taking account of the 10-fold dilution of the sample, and the effective path length of about 0.8 cm, the molar absorptivity for the AZB product is estimated to be 2.8 × 10⁴ liter mol⁻¹ cm⁻¹. This value applies for 1 mol of UCB producing 1 mol of AZB and would correspond to a value of 5.6 × 10⁴ liter mol⁻¹ cm⁻¹ for both steps in the bilirubin-p-diazobenzenesulfonic acid reaction going to completion.

Single-component samples. Studies of single-component samples were conducted, both to confirm the validity of the calibration procedures and to determine if there is any dependence of the apparent first-order rate constant on concentration for either UCB or CB. Concentrations included in this study ranged from 8.6 to 462 μmol/liter for UCB and from 26 to 256 μmol/liter for CB. For nine UCB samples, the regression equation of concentration found (C₀₀) vs. that taken (C₀₀) was

\[ C₀₀ = (0.996 ± 0.007) C₀₀ + 1.3 ± 1.6; \text{SEE} = 3.1, r = 0.9999 \]

and the regression equation for nine CB samples was

\[ C₀₀ = (0.987 ± 0.003) C₀₀ + 1.7 ± 3.8; \text{SEE} = 5.4, r = 0.998 \]

Plotted curves and these regression data demonstrate excellent linearity for both bilirubin species. Joint confidence limits show that the slopes and intercepts are not statistically different from unity and zero at the 95% confidence level. Standard errors of estimate (SEE) show a somewhat larger scatter about the line (5.4 vs. 3.1 μmol/liter) for CB than for UCB. These slightly less ideal characteristics from CB appear to be related to one errant point (192.2 vs. 205 μmol/liter). When this point is excluded from the regression the resulting equation is

\[ C₀₀ = (1.012 ± 0.008) C₀₀ + 0.09 \]
\[ ± 1.1; \text{SEE} = 1.6, r = 0.9998 \]

and, although we are not aware of any reason why this sample should have been different than the others, this equation is judged to be more representative of the method than that in which the errant point was included.

For the kinetic method to be valid, it is necessary that the rate constants, kₙ and kₚ, not be dependent upon concentration. Regression equations for rate constants (determined by a nonlinear regression program) vs. concentration on the samples described above were

\[ kₙ = (-3.6 ± 5.6) \times 10^{-4} C₀₀ + 11.3 ± 0.14; \text{SEE} = 0.26; r = 0.24 \]

and

\[ kₚ = (4.6 ± 6.7) \times 10^{-4} C₀₀ + 3.3 ± 0.01; \text{SEE} = 0.014; r = 0.93 \]
In each case, the intercepts represent the "best fit" values of the apparent rate constants. The slopes are not statistically different from zero at the 95% confidence level, suggesting little or no dependence of rate constants upon concentration.

These data were judged to confirm the potential of the method for simultaneous determinations of UCB and CB.

Synthetic mixtures. Prepared samples containing, per liter, 10–450 μmol of TB, 0–400 μmol of UCB, and 0–250 μmol of CB, with concentration ratios (C_S/C_0) of CB to UCB between 10/1 and 1/10 were run by the kinetic and equilibrium methods, and results are summarized in Figures 3–5 and in the first six data lines of Table 1. Slopes and intercepts that deviate badly from unity and zero are noted in the table and represent equilibrium values for UCB and CB. In both cases, slopes are well below unity and in the case of UCB, the intercept is at 31.5 μmol/liter. The intercept for the kinetically determined CB is outside the 95% confidence limit. The scatter about the regression line (as measured by the SEE) is about the same for the equilibrium and kinetic methods (15 vs. 12 μmol/liter), while the scatter for UCB is somewhat worse by the equilibrium method (22 vs. 13 μmol/liter) and the scatter for CB is somewhat worse for the kinetic method (14 vs. 6 μmol/liter). These observations can be rationalized on the basis that in the equilibrium method, UCB is determined by difference from experimental values for TB and CB and the uncertainties in both TB and CB would be expected to be propagated to UCB, while in the kinetic method, results for CB are influenced by the second step in the UCB reaction. In the case of the equilibrium UCB data, it may appear surprising that a near-unity slope for TB and a slope less than unity for CB result in a slope less than unity for UCB because low values for CB should lead to high values for UCB. It was pointed out earlier that slopes and intercepts are negatively correlated (12), and it is the group of UCB values above the least-squares line in the range from 20 to 180 μmol/liter that leads to the large positive intercept (31.2), "elevates" the least-squares line at low concentrations, and causes the slope to be less than unity.

These data indicate that the kinetic method will give slightly high values for total bilirubin, somewhat higher values for conjugated bilirubin, and slightly low values for unconjugated bilirubin. The equilibrium method gives high results for TB at low concentrations, significantly lower results for CB at all concentrations, high results for UCB up to 250 μmol/liter, and low results for UCB above 250 μmol/liter. Of the results presented here, the direct CB method exhibits the least scatter about the least-squares line, the indirect UCB method the greatest.

Serum samples. Results for 56 serum samples run by the two methods are summarized in Figures 6a–c and in the last three data lines of Table 1. Slopes and intercepts for TB and UCB and the intercepts for TB are not significantly different from unity and zero at the 95% confidence level, but the 95% confidence limit of the slope for CB does not include unity. It is not surprising that the slope for CB is slightly different from unity, but it is surprising that it is less than unity. The
Table 1. Regression Data for Bilirubin in Synthetic and Serum Samples

<table>
<thead>
<tr>
<th>Method</th>
<th>Components</th>
<th>Slopes ± SD</th>
<th>Intercepts ± SD</th>
<th>SEE</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>µmol/liter</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TB</td>
<td>0.97 ± 0.02</td>
<td>5.5 ± 5.1</td>
<td>15</td>
<td>0.99</td>
</tr>
<tr>
<td>Kinetic</td>
<td>TB</td>
<td>1.01 ± 0.02</td>
<td>7.2 ± 3.6</td>
<td>12</td>
<td>0.99</td>
</tr>
<tr>
<td>Equil.</td>
<td>UCB</td>
<td>0.88 ± 0.03</td>
<td>31.2 ± 5.5*</td>
<td>22</td>
<td>0.97</td>
</tr>
<tr>
<td>Kinetic</td>
<td>UCB</td>
<td>0.99 ± 0.02</td>
<td>-4.3 ± 2.7</td>
<td>13</td>
<td>0.99</td>
</tr>
<tr>
<td>Equil.</td>
<td>CB</td>
<td>0.78 ± 0.02*</td>
<td>0.5 ± 1.6</td>
<td>6</td>
<td>0.99</td>
</tr>
<tr>
<td>Kinetic</td>
<td>CB</td>
<td>1.05 ± 0.03</td>
<td>7.8 ± 3.2</td>
<td>14</td>
<td>0.98</td>
</tr>
</tbody>
</table>

Synthetic samples

Serum samples (n = 56) (kinetic vs. equilibrium)

|        | TB         | 1.01 ± 0.05 | 6.6 ± 8.1       | 28  | 0.95 |
|        | UCB        | 1.04 ± 0.03 | -2.7 ± 2.7      | 14  | 0.98 |
|        | CB         | 0.91 ± 0.04 | 3.8 ± 4        | 20  | 0.96 |

* Significantly different from unity and zero at 95% confidence level.

Data and analysis were performed without caffeine to free the bilirubin from HSA. Possibly all the CB in the synthetic samples did not react completely with p-diazobenzenesulfonic acid, but we can not confirm this and additional work is needed to identify the cause of the discrepancy.

The scatter of data about the least-squares line is greater for these serum data than for the data on synthetic samples. This is not surprising because uncertainty in both the kinetic and equilibrium methods are propagated into these comparisons, while the taken vs. found data in Figures 3-5 involve uncertainties from only one analytical method.

Summary

Data presented in this paper show that the kinetic method offers potential for the determination of unconjugated, conjugated, and total bilirubin in sera. In contrast to conventional equilibrium methods, it permits all data needed for the analysis to be collected rapidly on a single reaction mixture in which both UCB and CB are subjected to the same reaction conditions and it provides dynamic compensation for both sample and reagent blanks. On the other hand, the kinetic method probably is more sensitive to variations in experimental variables such as temperature than is the equilibrium method. The two methods offer interesting contrasts in terms of their performances for UCB and CB. While the kinetic method is most reliable for UCB and least reliable for CB because of the effect of the second step of the UCB reaction, it is probable that the equilibrium method could be most reliable for CB and least reliable for UCB, because UCB is determined by difference and any uncertainty in the CB result will be reflected in the UCB result.

There is evidence that there are more than two bilirubin species in human sera (13-15). If there were other species present in significant concentrations, and if they reacted with rate constants very different from those of the two species assumed to be in the samples, then they could influence the kinetics as well as the equilibrium results. Aside from monitoring kinetic data for unusual variations in statistical parameters associated with the regression method, we made no attempt to detect or account for such species.

It is reasoned that the kinetic method would be more attractive in the immediate future if the reactions were slow enough to be handled with conventional instrumentation. The variables that might be manipulated to slow the reaction are caffeine concentration and pH. Unfortunately, the caffeine concentration used in this work is near its upper solubility limit. It is possible that a pH below 5 could result in reactions slow enough to be monitored with conventional mixing systems and fast enough to give reasonable analysis times, but
rewritten as
\[ \dot{A}_t = \dot{A}_{w,u} (1 - e^{-k_{t} t}) + \dot{A}_{w,c} (1 - e^{-k_{c} t}) + \dot{A}_b \]  
(A-1a)
Because \( k_t \) and \( k_c \) will have been determined experimentally and treated as constants, the \( (1 - e^{-k_{t} t}) \) terms will be fixed at each point in time and it will greatly simplify notation to define \( k_{t,t} = (1 - e^{-k_{t} t}) \) and \( k_{c,t} = (1 - e^{-k_{c} t}) \), in which case equation A-1a can be written as
\[ \dot{A}_t = k_{t,t} \dot{A}_{w,u} + k_{c,t} \dot{A}_{w,c} + \dot{A}_b \]  
(A-1b)
If estimated values of the parameters in equation A-1b were used to compute an estimated value of \( \dot{A}_t \) at several points in time, then error (\( \mu_t \)) in the computation at each point in time would be the difference between the computed and experimental values
\[ \mu_t = \dot{A}_t - A_t \]  
(A-2a)
This error equation is made explicit in the parameters of interest by substituting for \( \dot{A}_t \) from equation A-1b to yield
\[ \mu_t = k_{t,t} \dot{A}_{w,u} + k_{c,t} \dot{A}_{w,c} + \dot{A}_b - A_t \]  
(A-2b)
The criterion for the best fit is that the sum of the squares of the error terms
\[ \mu^2 = \sum (k_{t,t} \dot{A}_{w,u} + k_{c,t} \dot{A}_{w,c} + \dot{A}_b - A_t)^2 \]  
(A-3)
must be a minimum. The mathematical procedure for minimizing such a function is to equate the first derivative to zero. When partial derivatives of equation A-3 with respect to \( \dot{A}_{w,u} \), \( \dot{A}_{w,c} \), and \( \dot{A}_b \) are equated to zero and rearranged, the following equations result
\[ \sum k_{t,t} A_t = \sum (k_{t,t}^2 \dot{A}_{w,u} + k_{c,t} \dot{A}_{w,c} + k_{t,t} \dot{A}_b) \]  
(A-4a)
and
\[ \sum k_{c,t} A_t = \sum (k_{c,t}^2 \dot{A}_{w,u} + k_{c,t} \dot{A}_{w,c} + k_{c,t} \dot{A}_b) \]  
(A-4b)
and
\[ \sum A_t = \sum (k_{t,t} \dot{A}_{w,u} + k_{c,t} \dot{A}_{w,c} + \dot{A}_b) \]  
(A-4c)
Although these equations may appear complex at first glance, they are simple functions of \( k_t \), \( k_c \), \( t \), and \( A_t \) that are determined experimentally and they represent three equations that can be solved for the three unknowns \( \dot{A}_{w,u} \), \( \dot{A}_{w,c} \), and \( \dot{A}_b \) that give the “best fit” to the experimental data. When these values are obtained, then the changes in absorbance due to UCB and CB are computed as
\[ \Delta A_u = \dot{A}_{w,u} - \dot{A}_b \]  
(A-5a)
and
\[ \Delta A_c = \dot{A}_{w,c} - \dot{A}_b \]  
(A-5b)
and Beers law or a calibration plot is used to evaluate \( C_0 \) and \( C_{0,c} \) from \( \Delta A_u \) and \( \Delta A_c \), respectively.

This investigation was supported by Research Grant No. GM 13326-11 from the NIH, USPHS.

References