produce "accurate" results for fresh serum from patients; however, further studies of this effect will be needed if we are to understand the problem. In addition, our study has shown that for the CAP survey materials and the SRM 909 serum Reference Materials, lyophilization of the material plays a significant role in the bias observed on measurement with certain enzymatic methods for cholesterol. From our studies we cannot offer an immediate solution to this problem. It would be helpful to users if manufacturers would provide information on the effect of lyophilized materials for their instruments. Such information does not address the issue that the users of instruments should have adequate materials to verify that their own instruments are accurate. Confidence in our standardization procedures will be greatly enhanced when we better understand the effect of lyophilization.

We thank Don Hochstein and Mark Heintzelman for lyophilizing our pools; Polly Ellerbe, Lorna Snapiegoski, and Edward White V for measuring cholesterol in our pools by isotope dilution/mass spectrometry; and Joan May for determining the moisture content of the lyophilized cakes.

References

CLIN. CHEM. 35/7, 1526–1531 (1989)


Nazilha F. Nuwayhid, George F. Johnson,1 and Ronald D. Feld

This is a multipoint kinetic method for simultaneously determining acetoacetate (AcAc) plus β-hydroxybutyrate and lactate plus pyruvate in a single cuvette of the Multistat III centrifugal analyzer. In the first step, AcAc and pyruvate are completely reduced, using 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30) and lactate dehydrogenase (EC 1.1.1.27) in the presence of excess NADH at pH 7.5, to β-hydroxybutyrate and lactate, respectively. After dilution, the endogenous β-hydroxybutyrate and lactate and that resulting from reduction are simultaneously oxidized by their respective dehydrogenases in the presence of excess NAD+ at pH 9.0. Adjustment of the relative enzyme concentrations allows simultaneous estimation of AcAc plus β-hydroxybutyrate and lactate plus pyruvate by analyzing multipoint absorbance data, collected during the oxidation reaction, with use of a two-component linear-regression model. Total run-to-run CVs were 6.4% and 6.1% at 5 mmol/L β-hydroxybutyrate and 5 mmol/L lactate, respectively. The method was designed to be useful for identifying the cause of an increased anion gap in serum.

Additional Keyphrases: centrifugal analyzers · multiple analyte assay by enzymic methods · high anion-gap metabolic acidosis

High anion-gap metabolic acidosis results when various anions accumulate in the blood. Accumulation of lactate (LACT) leads to the development of lactic acidosis, whereas accumulation of β-hydroxybutyrate (BOH) and acetoacetate (AcAc) leads to the development of ketoacidosis (1). Uremic acidosis in patients with acute and chronic renal failure results from the accumulation of sulfate, phosphate, and various organic acid anions (2). High anion-gap metabolic acidosis is also caused by the ingestion of toxic substances such as salicylates, methanol, ethylene glycol, and paraaldehyde (1, 2). Diagnosis of these conditions is critical in emergency medicine and requires accurate and rapid measurement of the anions involved. Determination of LACT and pyruvate (PYRU) provides information on the state of tissue oxidation, whereas measurement of BOH and AcAc aids in the diagnosis of diabetic and alcoholic ketoacidosis (3–5). Routine measurement of these analytes

1 Address correspondence to this author.
2 AcAc, acetoacetate; BOH, β-hydroxybutyrate; LACT, lactate; PYRU, pyruvate; HBDH, 3-hydroxybutyrate dehydrogenase; and MCA, Multistat centrifugal analyzer.

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in a discrete analyzer would require a single cuvette for each analyte. Measurement of lactic acid is done enzymatically, but ketone bodies are assayed by use of the semi-quantitative nitroprusside test or the enzymatic measurement of BOH and AcAc (6–8).

We recently described a kinetic method for measuring the combined concentrations of AcAc plus BOH in patients with diabetic ketoacidosis (9). We have also shown that multipoint kinetic analysis can be used for detection and quantification of an interferent that differs kinetically from the analyte (10). Here, we describe a multipoint kinetic method for the simultaneous determination of multiple analytes: AcAc plus BOH, and LACT plus PYRU. The measurement is performed in a single cuvette of a centrifugal analyzer. This could improve the cost effectiveness and efficiency of determining several analytes. Because high anion-gap metabolic acidosis may be caused by a concurrent increase in multiple anions, we used this condition as an example to demonstrate the feasibility and the potential use of the multipoint kinetic approach by measuring AcAc plus BOH and LACT plus PYRU in patients with high anion-gap metabolic acidosis. Our method would help in rapidly identifying the contribution of common metabolic anions to the measured anion gap.3

Materials and Methods

Materials

Reagents: Lyophilized 3-hydroxybutyrate dehydrogenase (HBDH, EC 1.1.1.30) from Rhodopseudomonas spheroides; β-NADH, disodium salt (grade III); β-NAD+, approximately 98% pure (grade III); lyophilized, salt-free L-lactate dehydrogenase (EC 1.1.1.27) from rabbit muscle; L(+)-lactic acid (grade L-X, crystalline), lithium salt; pyruvic acid, sodium salt; acetoacetic acid, lithium salt; DL-β-hydroxybutyric acid, sodium salt; lyophilized alanine amidotransferase (EC 2.6.1.2) from porcine heart; and L-glutamic acid, sodium salt were all from Sigma Chemical Co., St. Louis, MO 63178.

Specimens: Sera from patients with diabetic ketoacidosis and chronic renal failure, submitted to our laboratory for determination of ketone bodies or electrolytes and having an anion gap exceeding 20 mmol/L, were divided into aliquots and stored at −80 °C until assay. Also, plasma samples from patients with lactic acidosis and submitted on ice was stored at −20 °C until use. Sera from blood donors were divided into aliquots and stored at −80 °C until used. We assayed all specimens by the present method within three weeks of storage.

Reagent Preparation

Standards: Prepare stock 500 mmol/L solutions of AcAc (5.40 g/100 mL) and DL-BOH (6.30 g/100 mL), and 200 mmol/L solutions of LACT (1.92 g/100 mL) and PYRU (2.2 g/100 mL) in de-ionized water. The DL-BOH concentration is assumed to be one-half that of DL-BOH (11, 12). Prepare LACT and BOH standards (0, 2.5, 5, 10, and 15 mmol per liter) by making the appropriate dilutions of stock solutions in de-ionized water. Appropriate standards and stock solutions of AcAc and BOH and store them as described (9). Store LACT stock solutions and standards at 4 °C, and use them within one month. Prepare PYRU stock solution the day of use.

Controls: Prepare two controls by adding to 17.8 mL of pooled serum (containing, per liter, 2.0 and 0.17 mmol of LACT and AcAc plus BOH, respectively) the following volumes of the corresponding stock solutions: Control 1, 400 μL of BOH and 500 μL of PYRU; Control 2, 200 μL of AcAc and 500 μL of LACT. Adjust the total volumes to 20 mL with de-ionized water. The exogenous concentrations of AcAc, BOH, LACT, and PYRU in our controls were 5 mmol/L each. Subtract the values for the concentrations of AcAc plus BOH and LACT present in the pooled serum before control preparation from the final analytical results. Store all controls in aliquots at −80 °C and use within a month.

For interference studies, we added different concentrations of bilirubin (0, 12, 25, 50, and 100 mg/mL), hemoglobin (310, 620, 1250, 2500, 5000, and 10 000 mg/mL), and triglycerides (310, 620, 1250, 2500, 5000, and 10 000 mg/mL) to each control as described by Glick and Ryder (13), and assayed.

Prepare Tris HCl buffer (pH 9.5), phosphate buffer (pH 6.98), and NADH and NAD+ solutions as previously described except that the NAD+ solution is to be twice the concentration specified there (9). Stability and conditions of storage of these solutions were as previously described (9).

HBDH: Dissolve 100 U of lyophilized HBDH in 4000 μL of de-ionized water. Divide into aliquots and store at −20 °C until use. This is stable for at least two weeks.

LDH: Dissolve 5000 U of lyophilized lactate dehydrogenase in 400 μL of de-ionized water. Divide into aliquots and store at −20 °C until use. This is stable for at least two weeks.

ALT: Dissolve 1000 U of lyophilized alanine aminotransferase in 1000 μL of de-ionized water. Divide into aliquots and store at −20 °C until use. This is stable for at least two weeks.

Reagent 1: Mix 570 μL of pH 9.50 Tris HCl buffer (500 mmol/L), 420 μL of NAD+ (32.4 mmol/L), 420 μL of glutamate (1 mol/L), 87 μL of ALT (1 kU/mL), and 883 μL of de-ionized water just before the assay. This is enough for simultaneously determining AcAc plus BOH and LACT plus PYRU in nine samples. When only measurements of AcAc plus BOH were performed, the volumes of glutamate and ALT solutions were replaced by de-ionized water.

Reagent 2: Mix 33 μL of pH 6.98 phosphate buffer (200 mmol/L), 48 μL of NADH (40 mmol/L), 175 μL of LACT (25 kU/L), 140 μL of LDH (12.5 kU/mL), and 104 μL of de-ionized water just before the assay. Two hundred microliters of this reagent suffices for simultaneously determining AcAc plus BOH and LACT plus PYRU in 18 samples. When AcAc plus BOH and LACT plus PYRU were measured separately, only the corresponding enzyme was used. The volume of the other enzyme solution was replaced by de-ionized water.

Methods

For all measurements we used the Multistat III centrifugal analyzer (MCA) and its loader (Instrumentation Laboratory, Lexington, MA 02173).

Measurement of LACT plus PYRU: Load sample (2 μL), and reagent 2 (20 μL) into the sample (inner) well of the MCA rotor. Load reagent 1 (238 μL), and water diluent (10 μL) into the reagent well cuvette of the same rotor. Transfer the rotor to the analyzer and pre-incubate for 6 min at 30 °C, then start centrifugation to mix sample-well components with those in the reagent well. Record the increase in absorbance at 340 nm for two min after a delay of 10 s by

\[ \text{Anion gap} = [\text{Na}] - ([\text{HCO}_3] + [\text{Cl}]). \]
using water as the reference blank.

**Measurement of AcAc plus BOH**: AcAc plus BOH were measured as previously described (9), with the following modifications: 20 μL of reagent 2, 238 μL of reagent 1, high HBD concentration, and a 6-min pre-incubation. Components of the reduction reaction, including NADH, were diluted 14-fold after mixing with those of the oxidation reaction.

**Simultaneous measurement of AcAc plus BOH and LACT plus PYRU**: We measured AcAc plus BOH and LACT plus PYRU simultaneously by combining reduction reaction components (reagent 2) of the separate measurements of AcAc plus BOH and LACT plus PYRU in the inner well of the MCA rotor and those of oxidation reaction components (reagent 1) of both reactions in the reagent cuvette well. Load 2 μL of sample and 20 μL of reagent 2 into the sample well of the MCA rotor, and 238 μL of reagent 1 and 10 μL of water diluent into the reagent well of the same rotor. After a 10-s delay after the pre-incubation (6 min at 30 °C), record the increase in absorbance at 340 nm for 5 min at 10-s intervals, using water as a blank. In the reagent-well cuvette, the concentration of NADH was diluted 14-fold (optical path, 0.5 cm) and corresponded to a background absorbance of around 1.0 A for a water blank.

We used the following MCA settings: absorbance, 340 nm; temperature, 30 °C; delay time, 10 s; interval time, 10 s; number of intervals, 30; start mode, 2 (pre-incubation).

**Calculations**: We used the two-component, linear-regression (least squares) model as described by Czervionke et al. (10) to estimate the concentrations of AcAc plus BOH and LACT plus PYRU in an unknown sample. Briefly, we compared the absorbance difference of the unknown with the absorbance differences of two aqueous standards (10 mmol/L BOH and 10 mmol/L LACT) at the same time interval, for multiple intervals (at least 15). The concentrations of AcAc plus BOH and LACT plus PYRU in the unknown sample were then estimated by fitting the two-component first-order linear-regression equation to the data. The standard error of estimate (SD\textsuperscript{est} \textsubscript{err}), which is a measure of the difference between the observed (y\textsubscript{i}) and estimated (\hat{y}) absorbance, reflects the degree of fit of the model to the data. For the two-component model, the SD\textsuperscript{est} \textsubscript{err} is defined as the sum of the squared differences (y\textsubscript{i} − \hat{y})\textsuperscript{2} divided by n − 2, where n is the number of data points:

\[
SD_{\text{est}}^2 = \frac{\sum_{i=1}^{n} (y_i - \hat{y}_i)^2}{n-2}
\]

**Results and Discussion**

**Principle of the Assay**

The described method is based on the following enzyme-catalyzed reversible reactions:

1) HBDH-catalyzed reaction:

\[\text{AcAc} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{BOH} + \text{NAD}^+\]

2) LDH-catalyzed reaction:

\[\text{PYRU} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{LACT} + \text{NAD}^+\]

Under appropriate conditions and in the presence of the specific enzymes, these reactions can be made to proceed in either direction. The forward (reduction) reactions are favored by excess NADH and low pH (pH 7.0), and the backward (oxidation) reactions are favored by excess NAD\textsuperscript{+} and high pH (pH 9.5) (14–16). Our approach is to drive the reduction reactions to near completion and then measure the reduced species kinetically by the oxidation reactions. AcAc and PYRU are converted completely into BOH and LACT (first stage), and then the concentrations of total BOH and total LACT, representing AcAc plus BOH and LACT plus PYRU, respectively, are measured during their oxidation back to AcAc and PYRU (second stage).

**Design of the Multipoint Kinetic Assay**

To determine concentrations of AcAc plus BOH and LACT plus PYRU simultaneously by the multipoint kinetic method, using the two-component, linear-regression model, BOH oxidation and LACT oxidation must obey first-order kinetics (17) with sufficiently different rate constants. The following results demonstrate that both reactions are pseudo-first-order: first, the change in absorbance (ΔmA) (γ) between two time points for different time intervals is linearly related to the concentration (x) of either BOH or LACT up to 15 mmol/L. The regression equations for the 1-min interval were: γ = 12.3x − 7.8 (r = 0.9993) and γ = 5.21x + 0.01 (r = 0.9999), respectively. Second, the half-life (t\textsubscript{1/2} = ln 2/k; t\textsubscript{1/2} = half-life; k = first-order rate constant) of either reaction is constant when 2.5 to 15 mmol of either BOH or LACT liter were assayed (Table 1). For the various BOH concentrations, the average (and SD) half-life of HBDH-catalyzed reaction was 20 (1.94) s, whereas the average half-life (and SD) for the various LACT concentrations of lactate dehydrogenase-catalyzed reaction was 193 (5.07) s. By 300 s (the last absorbance measurement we made) essentially 100% of the BOH oxidation reaction and >75% of the lactate oxidation reaction would be completed. The difference in reaction rate constants is determined by the relative concentrations of enzymes used, with larger differences giving more nearly accurate estimation of the analytes. In our assay, the difference in rate constants is approximately sevenfold.

**Measurement of LACT plus PYRU**

Conditions that favor complete reduction of AcAc, as measured by the number of millimoles of NADH consumed,

| Table 1. Kinetic Characteristics of BOH and LACT Oxidation Reactions |
|----------------|-----------------|-----------------|-----------------|
| Concentration, mmol/L | Estimated (A\textsubscript{0} − A\textsubscript{f}) | Half-life, s | SD\textsubscript{est} \textsubscript{err} mA |
| BOH* | | | |
| 2.5 | 44 | 17 | 0.90 |
| 5.0 | 87 | 20 | 0.71 |
| 10.0 | 171 | 21 | 1.25 |
| 15.0 | 288 | 21 | 0.88 |
| LACT* | | | |
| 2.5 | 52 | 137 | 0.64 |
| 5.0 | 101 | 132 | 0.62 |
| 10.0 | 192 | 142 | 0.70 |
| 15.0 | 295 | 143 | 0.99 |

* BOH was assayed in the absence of LACT and (or) PYRU, and LACT was assayed in the absence of BOH and (or) AcAc with use of the simultaneous reaction as described in Materials and Methods and analyzed with a one-component exponential model: A = A\textsubscript{f} + (A\textsubscript{0} − A\textsubscript{f}) exp (−kt), where A\textsubscript{f} is the absorbance that would result if all BOH and all LACT in the sample were oxidized; A\textsubscript{0} is the initial absorbance, k is the rate constant, and t is time.
also favor complete reduction of PYRU. Substituting lactate dehydrogenase for HBDH in reagent 1, we obtained an average reduction of 107% (range, 103–110%) for 2.5–15 mmol of PYRU per liter. It was unnecessary to trap AcAc during BOH oxidation, but PYRU (the end product of LACT oxidation) must be continuously removed, to prevent the reaction from coming to equilibrium. Addition of alanine aminotransferase and glutamate to the oxidation reaction (cuvette well) results in the alanine aminotransferase-catalyzed conversion of PYRU to alanine, thus favoring LACT oxidation (16). We based the reagent conditions for LACT oxidation on the “Stat-Pack Rapid Lactate Test” kit (Behring Diagnostics, La Jolla, CA 92037). We modified these conditions by increasing the concentrations of alanine aminotransferase, lactate dehydrogenase, and glutamate by as much as sevenfold those recommended by the manufacturer. This allowed oxidation of LACT to proceed linearly in the presence of NADH (a component of the reduction stage), which is mixed in with the oxidation-reaction components. Under the modified conditions, the rate (ΔmA/2 min) (y) of the reaction was linearly related to the concentration (x) of either PYRU or LACT up to 15 mmol/L. The regression equations were: y = 7.59x + 3.03 (r = 0.998) and y = 7.29x + 2.25 (r = 0.998), respectively. The similarity of the slopes supports the conclusion that reduction of PYRU was complete during the first stage of the assay. HBDH had no effect on either the reduction or the oxidation stage of the lactate dehydrogenase-catalyzed reaction.

When different concentrations of LACT (2.5–15.0 mmol/L) and PYRU (2.5–15.0 mmol/L) were mixed together to give a final total concentration of 10 mmol/L and assayed, the average analytical recovery was 102% (range 98–107%). This indicates that the reduction of PYRU and the oxidation of LACT are independent, with no interaction. The PYRU concentration in serum is about 10% that of LACT (6), so we do not expect the PYRU in patients’ sera to contribute much to the total concentration of LACT plus PYRU. We compared LACT plus PYRU measured as described (y) with our routine laboratory method for LACT (Behring Diagnostics procedure) (x) in the sera of 16 patients with lactic acidosis and obtained the regression equation: y = 1.0x – 0.22 (r = 0.98). The means were 9.49 (range 2.5–25.20) mmol/L for LACT and 9.30 (range 1.74–24.38) mmol/L for LACT plus PYRU.

Simultaneous Measurement of AcAc plus BOH and LACT plus PYRU

**Analytical data:** Analysis of multipoint kinetic data by using different approaches has been described (10, 18). In our method, we used the two-component linear-regression approach described by Czervionke et al. (10) to estimate the concentrations of AcAc plus BOH and LACT plus PYRU. Table 1 shows a good estimate of AcAc plus BOH and LACT plus PYRU concentrations in the supplemented control sera, made by use of this approach. Table 1 also shows the SDest obtained from fitting the linear-regression model to the experimental data. At absorbance readings of 0.99 and 1.48 A the spectrophotometric noise of the MCA used, as determined by the SD for the absorbance of NADH in Tris HCl buffer (0.1 mol/L, pH 7.4 at 340 nm), was 1.0 and 1.8 mA, respectively. In our assay the initial absorbance reading is near 1.0 A, so the observed SDest is primarily due to the measured spectrophotometric noise of the MCA used and not to poor fit of the model. Using two-component linear regression, the SDest range we observed for sera from patients in diabetic ketoacidosis (n = 16), lactic acidosis (n = 20), and chronic renal failure (n = 4) was 0.30–1.25 mA. This is similar to that obtained from the analysis of supplemented control sera or aqueous standards, again demonstrating the good fit of the two-component linear-regression model to the patients’ data.

Figure 1 illustrates the fit of the data for one patient to the sequential linear-regression models. The observed absorbance difference (y) from the patient is plotted vs the estimated absorbance difference (x) of the 10 mmol/L BOH standard (Figure 1A), the 10 mmol/L LACT standard (Figure 1B), and (Figure 1C) that obtained from the two-

![Graphical illustration of linear-regression models for a diabetic patient](image_url)

**Fig. 1.** Graphical illustration of linear-regression models for a diabetic patient

The observed ΔmA represent experimental data from a patient in diabetic ketoacidosis; estimated ΔmA of BOH (A) and LACT (B) standards represent best-fit data obtained from one-component regression analysis; estimated ΔmA of the patient (C) represent the best-fit data obtained from two-component, linear-regression analysis (ΔmA = 1.41(ΔmA BOH standard) + 0.29(ΔmA LACT standard) + 2.85). ——, the regression line; ---, the patient’s data.
component linear regression of the same patient (estimated $\Delta A = 1.41(\Delta m A\ BOH\ standard) + 0.29(\Delta m A\ LACT\ standard) + 2.55$). The regression equations are: $y = 1.71x - 13.70$ ($r = 0.9918, SD_{est} = 7.00$), $y = 1.18x + 108.16$ ($r = 0.8798, SD_{est} = 25.87$), and $y = 1.00x - 0.16$ ($r = 0.9998, SD_{est} = 1.25$), respectively. Only the two-component regression model (Figure 1C, BOH and LACT) explains the observed absorbance data ($SD_{est} = 1.25$).

**Analytical Variables**

**Analytical recovery and precision:** For recovery and precision studies on sera we used controls 1 and 2. These controls were designed to contain one component of the HBDH-catalyzed reaction (AcAc or BOH) and one component of the lactate dehydrogenase-catalyzed reaction (PYRU or LACT) in such a way that one component would undergo oxidation only, whereas the other component would be first reduced, then oxidized. This was done to reveal any analytical problem at either stage (reduction or oxidation) of the assay or with any of its components. Analytical recoveries of LACT and PYRU in these controls were assessed in terms of LACT plus PYRU, and those of AcAc and BOH were assessed in terms of AcAc plus BOH (Table 2). Analytical recoveries of LACT and BOH in serum were similar to those obtained with aqueous studies. When we mixed different concentrations of aqueous BOH (2.5–10 mmol/L) with different concentrations of LACT (2.5–10 mmol/L) in various combinations and assayed by using the multipoint kinetic method, average recoveries were 98% (range 86–108%) for BOH and 97% (range 89–115%) for LACT. The recovery results in Table 2 show that reduction of AcAc and oxidation of BOH are essentially independent, with no interaction. The presence of different concentrations of BOH does not interfere with the reduction step, nor do various concentrations of AcAc affect the oxidation step. Therefore, the various concentrations of NADH remaining after the reduction step do not affect the oxidation step. A similar conclusion can be drawn from the oxidation and reduction of LACT and PYRU. The results also show that the reduction–oxidation reaction of AcAc plus BOH is essentially independent of that of LACT plus PYRU.

Within-run precision and day-to-day precision also are shown in Table 2, in terms of CV.

**Assay linearity:** With use of 10 mmol/L BOH and 10 mmol/L LACT as standards for the multipoint kinetic method, the estimated concentrations of BOH ($y$) were linear up to 15 mmol/L, the regression equation being $y = 1.04x - 0.3$ ($r = 0.998$). The estimated concentrations of LACT ($y$) were also linear up to 15 mmol/L; the regression equation: $y = 0.98x + 0.26$ ($r = 0.999$).

**Interference studies:** There was no correlation between the concentration of added bilirubin, triglycerides, or hemoglobin and the estimated concentrations of AcAc plus BOH and LACT plus PYRU. The slopes of the regression equations ranged between 0.002 and 0.005 for both controls. Also, there was no consistent pattern of change in the estimated concentrations of AcAc plus BOH and LACT plus PYRU with the increase in the concentration of bilirubin, triglyceride, or hemoglobin.

**Studies of Patients**

To evaluate the multipoint kinetic method, we estimated concentrations of AcAc plus BOH and LACT plus PYRU in 16 patients with diabetic ketoacidosis and 20 patients with lactic acidosis by the two-component, linear-regression model ($y$) and compared results with AcAc plus BOH ($x$) as previously described (9) and to LACT ($x$) as measured routinely in our laboratory. A good correlation is indicated by the regression equations: $y = 0.95x + 1.13$ ($r = 0.983, n = 16$) and $y = 0.88x + 0.94$ ($r = 0.907, n = 20$) for measurements of AcAc plus BOH and LACT plus PYRU, respectively. The respective means ($\bar{x}, \bar{y}$) were 12.58 (SD 4.66, range 2.67–17.73) mmol/L and 12.60 (SD 5.0, range 2.44–18.47) mmol/L for AcAc plus BOH in patients with diabetic ketoacidosis; and 9.01 (SD 4.90, range 4.74–23.22) mmol/L and 9.15 (SD 4.54, range 4.2–25.2) mmol/L in patients with lactic acidosis. Table 3 shows examples of results for samples from patients with diabetic ketoacidosis, lactic acidosis, and chronic renal failure, as analyzed by the multipoint kinetic method and the comparison methods. Such patients often have a high anion-gap acidosis, owing to accumulation of BOH plus AcAc, LACT, and other anions, respectively. If we assume that the average anion gap in these patients is similar to that reported by Witte et al. (19) in hospitalized patients (12 mmol/L), then adding values of AcAc plus BOH and LACT plus PYRU to the pre-existing anion gap could account for >98% of the increased anion gap only in patients with diabetic ketoacidosis and lactic acidosis but for <66% in those with chronic renal failure. This is to be expected, because the accumulated anions in patients with renal failure include anions (such as phosphate and sulfate) that are not measured by our method.

In summary, we have shown that consecutive reduction of AcAc and oxidation of BOH as well as consecutive reduction of PYRU and oxidation of LACT can be carried out simultaneously in a discrete analyzer (MCA). This

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**Table 2. Analytical Accuracy, Precision, and SD_{est} of the Multipoint Kinetic Method Assay**

<table>
<thead>
<tr>
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<th>Controls(^a,b)</th>
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<tbody>
<tr>
<td></td>
<td>#1</td>
<td>#2</td>
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<tr>
<td><strong>AcAc plus BOH</strong></td>
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<td>Within-run precision (n = 20)</td>
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<td>CV, %</td>
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<td>Within-run precision (n = 18)</td>
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<td>Total run-to-run precision</td>
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<tr>
<td>Range, %</td>
<td>93–115</td>
<td>91–120</td>
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</table>

\(^a\) Concentrations of exogenous components in the prepared controls were: control 1, 5 mmol/L PYRU + 5 mmol/L BOH; control 2, 5 mmol/L LACT + 5 mmol/L AcAc.  
\(^b\) Results shown were obtained after subtracting the endogenous AcAc plus BOH and LACT plus PYRU concentrations in the controls.
approach should be transferable to other instruments that have the capability of sequential two-reagent addition, allowing two analytes to be measured in one analytical channel with multipoint kinetic measurement. This method was designed to measure concentrations of AcAc plus BOH and LACT plus PYRU in 2-μL samples from patients with high anion-gap metabolic acidosis. Simultaneous measurement of AcAc plus BOH and LACT plus PYRU could help determine whether the anion gap can be explained by increased AcAc plus BOH or LACT plus PYRU, or both, or in part ascribable to the presence of other anions in the patient's serum.

Multipoint kinetic analysis also offers an internal analytical validation. If interferents are present in the patient's sample either as inhibitors, activators, or additional enzyme (lactate dehydrogenase or HBDH in our assay), analysis of the kinetic data would be expected to show a poor fit of the assumed model (increased SDmeas) to the actual data.

This study demonstrates the feasibility of multiple analyte assay by enzymic methods in a single cuvette. Implementation of this approach in the clinical laboratory awaits suitable instrumentation systems with appropriate software. Reaction design considerations, such as the effective half-lives of individual first-order reactions, depend on the target instrument system. Simultaneous measurement of multiple analytes is most appropriate in clinical situations in which assay of these analytes would be ordered as separate determinations. This was the genesis of our idea for an assay of total metabolic anions to help with interpretation of an increased anion gap.

References