A Systems Analysis of GEMSAEC Precision Used as a Kinetic Enzyme Analyzer

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Use of the GEMSAEC as a kinetic enzyme analyzer has been one of the most promising applications for this instrument. Several important variables must be defined if a practical system and components are to be developed for use in the clinical laboratory. The effect and inter-relationship of four of these are defined and analyzed to determine their impact on the precision of GEMSAEC: starting absorbance of the sample-reagent mixture, rate of change of absorbance, length of time for which the linear kinetic reaction progresses, and the uncertainty of the analog-to-digital conversion combined with electronic noise. A set of curves is generated from equations derived to demonstrate the coefficient of variation based on these variables. The influence of these variables, as compared to the variations of sample and reagent addition and of temperature control, is also briefly reviewed. The performance of the ENI GEMSAEC against these errors is summarized. The techniques described also define a convenient and practical way to evaluate the limits of precision that can be expected from analyzers for kinetic analysis based on the rate of the reaction rather than on a specific chemical determination.

Additional Keyphrases spectrophotometric error analyses • digitization effects on spectrophotometric instruments • the effect of chemical and instrument parameters on precision of rate reactions • Rotoloader • instrumental variables • precision limits for fast analyzers

The advent of high-speed multiple analyzers of the GEMSAEC type makes necessary a more rigorous study of the factors involved in the precision of kinetic measurements. This analysis, used as the basis for the design of the (ENI)4 GEMSAEC, resulted in a simplified graphical method for the selection of parameters to optimize precision, in terms of coefficient of variation, in the laboratory. It quantitatively demonstrates the effects on kinetic chemistry of absorbance, rate, the time allowed for linear reaction, and instrument quality.

Basically, the GEMSAEC, invented at Oak Ridge National Laboratories by Anderson (1), consists of a flat rotor containing multiple cuvets, a light-source, a photo detector, and a readout system. Samples and reagents are metered into a transfer disc, which is rotated, to move the fluids centrifugally into the multiple-cuvet rotor. The sample and reagent are mixed by drawing air through siphons connected to the cuvets and causing the air to bubble through the sample reagent mixture.

The cuvets are washed and dried by flushing them with water and then air.

Transmittance of the samples is measured as the stationary light beam scans all the cuvets during rotation. Transmittance can be converted to absorbance by analog and digital means or by computer conversion exclusively.

The Electro-Nucleonics system, Figure 1, which has been designed and produced for commercial use, is a modular version of GEMSAEC, the "Rotoloader," the Analyzer, the Control Cabinet, and the Digital Computer with Teletypewriter.

The Rotoloader automates the addition of sample and reagent. The analyzer houses the spinning rotor, a monochromator, and the photosensor. The control cabinet provides controls for timing reactions as well as controls to automate mixing, washing, and drying in the analyzer. The PDP-8 computer and Teletypewriter with 4K memory is used to accumulate and process data and to display the final results.

The digital computer is used to select and operate on the linear portion of the kinetic reactions, thus providing a system that makes multiple kinetic enzyme analysis both rapid and precise.
Definitions

The development of GEMSAEC by ENI for the kinetic mode of operation required optimization of critical system variables and components for the mechanization described.

The variables are those related to optical, electronic (analog and digital computer), mechanical, chemical, and laboratory operational requirements.

The following mathematical analyses were used to define the directions to take to obtain optimal performance of a GEMSAEC for the level of precision desired.

The first variables considered are: \( R_c \), the exact rate of enzyme activity in absorbance/minute; \( T \), the time during which linear reaction is calculated, in minutes; \( \Delta t \), the uncertainty of the measurement of time, in minutes; \( \Delta A \), the exact absorbance at the beginning of the rate measurement, in absorbance units; \( \Delta f \), the exact absorbance at the end of the rate measurement, in absorbance units; \( \Delta A \), the uncertainty of absorbance measurement, in absorbance units; \( \Delta A + \Delta \), the uncertainty of the absorbance measurement at the beginning and end of the reaction, respectively, in absorbance units.

These variables combine to establish the limits of precision for GEMSAEC and will be termed, \( E_n \), the coefficient of variation due to instrument uncertainty.

Subsequently, the variables of temperature and fluid addition will be considered: \( E_t \), the coefficient of variation due to temperature; \( E_i \), the coefficient of variation due to sample and reagent additions.

These two variables will be combined and termed \( E_i \), the coefficient of variation due to the combination of temperature and fluid additions.

The effects of all variations will be considered \( E_i \), the coefficient of variation due to the combined effects of instrument uncertainty, temperature variations, and variations in fluid volume.

Electronic drift is not a factor on the GEMSAEC, since the system virtually operates as a double beam spectrophotometer, by referencing each sample to the blank located in Cuvet #1 of the rotor, every 133 milliseconds, the time for one revolution of the rotor.

The uncertainty, \( \Delta A \), is the smallest increment of absorbance that can be measured and is different at different values of absorbance. This difference can be due to two causes: (a) computer conversion of \%T to absorbance; and (b) electro-optical noise.

The effect of computer conversion of \%T is the same as seen from transmittance-absorbance charts used in the past (Table 1).

<table>
<thead>
<tr>
<th>( %T )</th>
<th>Absorbance</th>
<th>( \Delta A ) (minimum change in absorbance)</th>
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</thead>
<tbody>
<tr>
<td>1.0</td>
<td>2.0</td>
<td>0.097</td>
</tr>
<tr>
<td>1.25</td>
<td>1.903</td>
<td>0.097</td>
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<tr>
<td>1.50</td>
<td>1.824</td>
<td>0.079</td>
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<td>...</td>
<td>...</td>
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</tr>
<tr>
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<td>0.969</td>
<td>0.010</td>
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<td>...</td>
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<td>0.0011</td>
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<tr>
<td>100.00</td>
<td>0.000</td>
<td>0.0011</td>
</tr>
</tbody>
</table>

The resolution or uncertainty from Table 1, in terms of \%T, is 0.25\%. The resolution or uncertainty in terms of absorbance, \( \Delta A \), changes logarithmically with \( A \), as graphically demonstrated in Figure 2, curve A. \%T is divided into about 400 parts in Table 1. This is equal to about a 9-bit digital conversion. Curve B of Figure 2 demonstrates a 12-bit digital conversion, used on GEMSAEC. A 12-bit conversion will be shown to improve precision. The 12-bit conversion is equivalent to dividing the \%T table into 4096 parts.

The uncertainty also depends on the signal-to-noise ratio of the electro-optical system. The effect of these two sources of uncertainty can be seen by making measurements of the combined results of digitization and noise. Figure 3 demonstrates the results of such a measurement by plotting standard deviation of the uncertainty as a function of absorbance from 0 to 2 absorbance.

Curves drawn in Figure 3 were obtained by using a least-squares fit to the exponential function \( \Delta A = Ce^{-A} \) from data obtained by loading dichromate solutions of different absorbances in the GEMSAEC.
the lower the curve on the graph, the better the quality.

This can also be seen from the equations for the curves: the prototype has the equation \( \Delta A = 0.000342 e^{1.876A} \) while the production version (S/N 109) has the equation \( A = 0.000072 e^{1.876A} \).

For the following analysis, linear kinetic reactions are used for the time during which the reaction rates, \( R_o \), are calculated. The linear portion of the reaction is identified and isolated in the ENI GEMSAEC system by proprietary computer curve search routines. The criteria for deviation from linearity, \( \theta \), Figure 4, can be controlled by the operator via computer software.

Mathematical analysis can be accomplished by a simplified graphical model that uses the linear portion of the kinetic reaction. The linear portion, \( T \), of the reaction (Figure 4) is isolated by the computer routine. Figure 5 enlarges this portion to show the effect of the parameters considered. The rectangles represent the maximum uncertainty of the measurement of absorbance, \( A \), and time, \( t \). Note that \( \Delta A \) is larger at \( A_i \) than at \( A_5 \), as indicated by the digitization and noise curves (Figure 3).

While the following simplified analysis is applied for reactions with increasing rate; the same logic can be used for reactions with decreasing rate. The computer search routines operate on either type of reaction.

From Figure 5 the rate would be:

\[
R_o = \frac{A_f - A_i}{t_f - t_i}
\]

which is the slope of the solid straight line drawn between the two points shown.

Because of the uncertainties, the measured rate can be:

\[
R^* = \frac{A_f \pm \Delta A_f - (A_i \pm \Delta A_i)}{t_f \pm \Delta t - (t_i \pm \Delta t)}
\]

i.e., the slope of the curve could be anywhere between dotted lines \( B \) and \( C \) in Figure 5.

On a practical basis, the uncertainty of the time, \( \Delta t \), is negligible in a digital computer system. By ignoring \( \Delta t \), and taking an extreme case, the rate will be:

\[
R^* = \frac{A_f + \Delta A_f - A_i - \Delta A_i}{t_f - t_i}
\]

The maximum error in the computer value of the rate is then:

\[
\text{Error} = R^* - R_o = \frac{A_f + \Delta A_f - A_i + \Delta A_i}{t_f - t_i} - \frac{A_f - A_i}{t_f - t_i}
\]

which yields:

\[
\text{Error} = \frac{\Delta A_f + \Delta A_i}{t_f - t_i}
\]
This term represents the maximum percentage error expected.

It is more appropriate in the clinical laboratory to consider the error in terms of coefficient of variation. Therefore, the standard deviation of the uncertainty is used to provide the coefficient of variation rather than the maximum values, and

\[ E_R,\% = \frac{\sigma DA_i + \sigma DA_i}{R.T} \times 100 \]  

(8)

These values are taken as a conservative approach, rather than taking the root mean square (rms) of the numerator. From the statement of Equation 8 we can conclude that: (a) The coefficient of variation is decreased as the signal-to-noise ratio of the electro-optical system increases and (or) digital resolution of the conversion system becomes smaller, and (b) the coefficient of variation is decreased as the length of time of the reaction and rate of reaction increases.

The second conclusion is valid for most practical cases, but can be deceptive since \( \Delta A \) and \( A \) are not independent. This can be seen more clearly in the family of curves in Figures 6 and 7, obtained by using Equation 8 and data from curve A, Figure 3. These were calculated for linear reaction times of 0.5, 1, 2, and 4 min and starting absorbances \( A_i \) of 0.5, 1.0.

From Figure 6, \( E_R \) begins to increase (at \( R = 0.12A/min \) and \( T = 4 \) min) rather than to decrease. If the scale of the curve extended further to the left, \( E_R \) would increase for all of the times shown, the result of the dependence of \( \Delta A \) on \( A \).

The heavy lines provide a reference of 5% \( cv \) for each curve.

The curves of Figures 6 and 7 can be used to bound the variables in the laboratory to obtain desired levels of precision.

In a practical sense, for a chemical reaction that has an increasing reaction rate, with a starting absorbance of 0.5, we can limit \( cv \) to 1.5\% by configuring analyses for normal or expected ranges to operate at reaction rates above 0.005 \( A/\text{min} \) and 0.015 \( A/\text{min} \) and with linear reactions lasting for 2 to 4 min. (Draw a horizontal line at 1.5% on Figure 6 to determine these bonds).

Modification of the chemical procedure by the clinical chemist, to decrease starting absorbance values or increase rates, would allow for greater precision.

From the curves and Equation 8 we see that the variable under the instrument designers' control is standard deviation of \( \Delta A \). The instrument designer cannot control rates, although we can use time to our advantage. The computer search-routine does this for us automatically.

ENI attacked the problem to decrease \( sd \) of \( \Delta A \) to improve precision. The effect of this effort was seen for uncertainty curves for a prototype unit of an ENI GEMSAEC, Curve B of Figure 3, compared to
the present production version, Curve A (S/N 109) of Figure 3. There is almost a 10-fold difference in performance in terms of the standard deviation of ΔA.

The impact on performance can be demonstrated by calculating and then plotting the CV for the two machines, and comparing the expected precision. This was done for 1- and 2-min reaction times and 1.0A starting absorbance (Figure 8). The effect on CV is clear.

For the prototype we see that we would not obtain a CV of 5% for many practical reaction rates at a starting absorbance of 1.0A. With the present version, S/N 109, a CV of 5% can be achieved for practical, lower rates. The difference is attributable to the lower uncertainty of ΔA for the S/N 109.

Additional System Variables

Two other major variables contribute to the coefficient of variation of the GEMSAEC in this kinetic mode, those due to temperature control and to sample and reagent addition.

Temperature

Typically, enzyme reaction rates double for about every 10°C increase in temperature (2). From this statement, mathematical analyses (see Table 2) show that, to obtain a 1.5% coefficient of variation (\(E_r\), of the kinetic rate) due to temperature, temperature must be controlled to within ±0.3°C.

Typical correction factors for temperature provided by reagent manufacturers and in literature show that many enzyme rates do not quite double for every 10°C (3). Therefore, a broader temperature control for the same error in precision can be allowed for many reactions. The requirement of ±0.30°C was taken as a practical goal and has been achieved.

Fluid Loading

Fluid loading errors originating from fluid loading by careful hand pipetting or by Rotorloader II (a device that automatically loads sample and reagent) has demonstrated a combined CV of less than 1.6% for 30 replicate samples of 20 µl plus 500 µl reagent (see Table 3). A combined CV of 1.6%, owing to fluid loading of serum and reagent via automated or manual techniques, is considered a practical, achievable value.

The three sources of variation in precision can now be assigned as part of an error or precision budget for the GEMSAEC in kinetic operation.

The values used are practical limits, based on analyses and tests noted previously, and are tabulated below.
(\(E_R\) is taken over a range of rates and times from Figures 6 and 7 to provide a cv of 1.5%).

**Error or Precision Budget**

Let \(E_R\) be the electronic/optical computer uncertainty, ±1.5%; \(E_T\) that for the temperature control (±0.3°C), ±1.5%; and \(E_F\) that for the fluid dispensing (Rotoloder or pipetting), ±1.6%.

These variables are independent and are combined (4) by the addition theorem in statistics by taking the square root of the sum of the squares to define the total variation, \(E_s\).

\[
E_s = \sqrt{\text{1.5}^2 + \text{1.5}^2 + \text{1.6}^2} = 2.65\%
\]

We would expect to obtain a cv of 2.65% for the conditions described.

The impact of errors owing to the precision of temperature control and fluid addition, of course, becomes less severe as the precision errors from the instrument system are allowed to increase.

**Experimental Results**

Experiments to test the validity of the error analyses were run on the GEIMSAEC described. For simplicity data are shown only for tests at starting absorbances of 0.5 and 1.0 absorbance units.

The LDH procedure of Wacker, with increasing reaction rates and use of the Boehringer Mannheim Corp. (BMC) kit, Catalog no. 15899 TLL, was modified to enable starting absorbance values of approximately 0.5 and 1.0 absorbance units to be used. Vials from this kit, with 15 mg NAD, are reconstituted with 0.05M pyrophosphate buffer, pH 8.6, containing 0.045 ml of lactate, to yield a final concentration of 7.0 mmol of NAD per liter. 20 µl samples were used.

Absorbance was adjusted by the addition of NADH to vary the starting absorbances. Rates were adjusted by adding “Chemtrol” reference serum (Clinton Laboratories, Los Angeles, Calif. 90019) and diluting with phosphate buffered saline (pH 7.2). To achieve high rates of 0.25A/min small amounts of LDH (from rabbit muscle) were added to the reference serum. The rate was measured experimentally and the reference serum was then diluted to yield approximately 0.25A/min.

Readings were obtained at timed increments by using the ENI multiple end-point program, which provides printout for reactions at selected time intervals. The intervals are set into the computer by a switch on the front of the control module (Figure 1).

Temperature was maintained at 30.5°C ± 0.2°C.

Exact starting absorbances could not be maintained to match the 0.5 and 1.0 for the theoretical data. Actual values ranged between 0.4 to 0.6 and 0.9 to 1.1, respectively. 15 points of data were obtained at each rate and time. The data obtained from the tests are plotted in Figures 9 and 10. Data were not obtained at rates higher than 0.15A/min in most cases since the reactions were found not linear in this region, as had been indicated on the instruction sheets for the reagent kit used.

The experimental data and the calculated values agree in form for most data, as can be seen by inspection.

Quantitative estimates of the agreement, for example, can be obtained by using the data from Figures 6 and 9 (displayed in Figure 11 for a time of 2 min) to calculate the value of \(E_p\), the combined effect of temperature and fluid additions, from the data, and plotting the results. The calculated value of \(E_p\) is shown in Figure 12.

From the data of Error or Precision Budget, \(E_p\) was expected to be 2.2% (\(\sqrt{1.5^2 + 1.6^2}\)). Figure 12 shows that this level of variation, or less, was obtained for most cases for a reaction time of 2 min.

The data from Figures 7 and 10 (for a time of 2 min) show closer quantitative agreement than expected, if similarly calculated.

The causes of the differences are not known, and are presently under investigation. The conservative assumption for Equation 8 may have contributed to the differences.
Table 3
Sample and Reagent Addition Weighings on Mettler Balance: Fluids—as Shown

<table>
<thead>
<tr>
<th>Sample-pooled serum (Lavry pipet), 20 µl</th>
<th>Reagent-LDH test (Eppendorf pipet), 500 µl</th>
<th>Dispensing reagent-LDH test, 300 µl</th>
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</thead>
<tbody>
<tr>
<td>20.45</td>
<td>508.45</td>
<td>21.89</td>
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<td>-2.47</td>
<td>-504.82</td>
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</table>

mean 20.39 503.82 21.76 507.77
SD 0.0734 1.98 0.3091 2.65
CV 0.36% -1.42% 1.42% 0.52%

Discussion

With the variations considered, a coefficient of variation of 5% and lower can be achieved at activity rates of 0.01 A/min and slightly lower for computer selected, linear reaction times that range from 2 to 4 min at starting absorbances in the range of 0.5 A.

The precision expected for other rates and absorbances can be defined from the equations, curves, and data presented.

The precision depends on instrumental measurement uncertainties as well as variables of chemistry such as absorbance, rate, and length of time of linear reaction.

When these variables are minimized, control of temperature and fluid delivery can be maintained within practical values to enable desirable coefficients of variation for the clinical laboratory to be obtained.
Comparison of actual experimental data and theoretical expectations demonstrates agreement between theory and experimental results.

The methods described here for evaluating GEMSAEC precision define the precision to be expected from kinetic analyses based on activity rather than any specific chemical determination. The techniques represent a convenient and practical way to evaluate different kinds of kinetic analyzers as well as a simple method for establishing the limit of precision that can be expected from fast analyzers.

I would like to express appreciation to Mrs. S. Klotzch, Chief Chemist, BMC, New York, N. Y., for technical assistance in setting up the experiment described; Miss P. Gensiardi, Chief Technologist, for setting up and performing the chemistry tests and reducing data; Mr. R. Mazzei for assistance in reducing data and generating the associated curves and graphs; Dr. D. Raichel for providing the program to establish the least-squares fit line, for data, and \( \Delta A = Ce^{\Delta A} \); Messrs. Kunts and Weingart for assistance in making the curves; and Messrs. J. Acker, D. Rohlfing, and R. Saylor for critically reviewing the manuscript.

References