Statistical Analysis of the Stability of the Standard Curve for Some Syva EmT Assays

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Derivation of standard curves for the EmT® therapeutic drug monitoring system involves several mathematical algorithms, all of which can be rewritten in the form of a linear equation \( y = mx + b \). We examined the stability of the standard curve in terms of slope and y-intercept for three drug assays (procainamide, gentamicin, and carbamazepine) by generating calibration curves intermittently for periods as long as 90 days. Controls at three concentrations were assayed after each calibration to validate the standard curves. On the basis of 98% confidence intervals, the slopes of standard curves for procainamide, gentamicin, and carbamazepine were stable for 89, 80, and 57 days, respectively. Control values generated from standard-curve manipulations (adjustments to the y-intercept) indicated consistent accuracy and precision throughout the entire study, as compared with control values determined after each calibration. The increased utility of the standard curve and reagents suggests that full recalibration on a regular basis is not always necessary.

**Additional Keyphrases:** drug assay - economics of laboratory operation - assay optimization - enzyme immunoassay

The "enzyme-multiplied immunoassay technique" (EmT®; Syva Co., Palo Alto, CA 94304), a popular system for measuring drug concentrations in serum because of its ease of operation, small sample requirements, and nonradioactive methodology, has the major drawback of high cost, primarily for frequent calibration. Because of "aging" or deterioration of reagents after reconstitution, daily calibration is necessary to ensure accurate assay performance. Attempts at cost reduction have taken several forms. Initially, the EmT reagent system was adapted to centrifugal analyzers, with costs being reduced through use of microvolumes of reagents. More recently, investigators have added excess substrates and coenzyme to the reagents manufactured for centrifugal analyzers to increase the number of determinations available per assay kit (I). Reagents in these procedures are drastically altered from those supplied by the manufacturer. Other researchers have documented standard-curve stability of two weeks for reagents stored at ambient temperature (2, 3), their intent being to minimize the degree of reagent deterioration and the resulting alterations in the standard curve. Although controlling the reagent environment may slow the deterioration of reagents, eventual aging of reagents is unavoidable. We therefore investigated the possibility of dealing with the reagent-aging process by mathematically examining the response patterns of the standard curves in repeated calibrations, using the slope and y-intercept values to evaluate curve stability.

Data reduction procedures used with EmT therapeutic drug monitoring assays incorporate different models or mathematical algorithms to best fit a calibration curve to a set of points by means of a least-squares regression formula. Three models are currently in use (4):

Model 1: Rate = \[ \text{rate}_0 + K \left( \frac{1}{1 + e^{-\text{ln} a + b \ln C}} \right) \]

Model 2: Rate = \[ \text{rate}_0 + K \left( \frac{1}{1 + e^{-\text{ln} a + b \ln C + c \ln C^2}} \right) \]

Model 3: Rate = \[ \text{rate}_0 + K \left( e^{\text{ln} C + a \ln C + b + c \text{ln} C^2} \right) \]

For all these models:
- Rate = response (rate of change or absorbance)
- C = concentration
- \text{rate}_0 = theoretical rate for a calibrator with zero concentration (y-intercept of linear equation)
- K = scale parameter (slope of linear equation)
- \( a', b, c' \) = parameters that define the nonlinear elements of each model

The particular model chosen by Syva personnel for each assay is based on the least-squares criterion for goodness of fit. All three models are nonlinear and require the Gauss-Newton algorithm for nonlinear least squares, an iterative algorithm that calculates new sets of parameters until determining the ones that minimize the residual sum of squares. Initially, one uses the given parameters (\( a', b, c \)) and assumes a "best fit." By using the first-order Taylor series, the nonlinear function is estimated by producing new parameters that approach the best-fit parameters. The cycle is repeated for each new set of parameters until those parameters producing the best-fit curve are obtained. Although the nonlinear models are more flexible in fitting data, they require greater software capabilities than the simpler linear-regression model. By using the linear-regression model, each Syva model can be written as a linear equation of the form \( y = mx + b \), where m is the slope and b is the y-intercept.

**Materials and Methods**

We examined the stability of standard curves generated for Syva EmT reagents by performing multiple calibrations of three different assays, each utilizing a different mathematical algorithm, during as many as 90 days. The specific assays we used were: model 1, procainamide (lot N01); model 2, gentamicin (lot N01); and model 3, carbamazepine (lot N01). In our experience, these were the least stable of the various EmT assays.

For each assay, reagent for approximately 400 test cycles was required. Syva provided four gentamicin kits (100 tests...
per kit) and eight kits each for procainamide and carbamazepine (50 tests per kit). All were in lyophilized form, and all were reconstituted at the start of the study as recommended by the manufacturer. We pooled the buffer solutions from all the kits because the same buffers are used for all EMT therapeutic drug kits. Each set of assay kits was labeled alphabetically, and each assay was calibrated every few days with the next sequentially labeled kit in the set until all reagents were exhausted.

We performed the calibrations according to the manufacturer's specifications, using the Syva EMT Autolab 5000 System (a spectrophotometer and sample carousel coupled to a Syva CP-5000 Clinical Processor).

We assayed "Stratus Tri-level" controls (American Dade, Div. of American Hospital Supply Corp., Miami, FL 33152), which span the range of clinical interest, immediately after calibration to verify each curve.

During the course of the study, we performed routine maintenance of the AutoLab 5000 System and operation procedures according to recommended instructions. Reagents were stored at temperatures below 8 °C when not in use, and allowed to reach room temperature before use. We made no attempt to place unusual restrictions on reagent use or storage. Assay conditions were intended to simulate the daily work environment and to subject the reagents to expected normal handling.

For all computations we used the Hewlett-Packard Model HP-86 computer, after having written appropriate programs to accommodate the necessary calculations. We derived slopes and y-intercepts (with appropriate 95% confidence intervals) for all calibration curves through a method of linear least-squares regression (5). In this simple linear regression all given parameters (a', b, and c) remained constant throughout the calculations, unlike the Syva software, in which nonlinear least-squares regression is used with parameters continuously changing as the curve approaches a "best fit." The "best-fit" curve we generated through the linear regression approach may be improved by using a nonlinear regression method if there is undetermined variability affecting the calibration curve.

Results

We generated 37 procainamide calibration curves during 89 days, using eight assay kits; 30 gentamicin calibration curves during 80 days, using four assay kits; and 34

![Graphs of slopes and y-intercepts for procainamide, gentamicin, and carbamazepine assays.](https://example.com/graphs)

Fig. 1. (Left) Slopes with 95% confidence intervals and (right) y-intercepts with 95% confidence intervals on a series of days for all three EMT algorithms. (Left) Slopes and y-intercepts, with appropriate confidence intervals, calculated from curves similar to those presented in Fig. 2. (Left) Linear-regression lines for daily slope values (showing slopes of zero and areas of overlapping confidence intervals) indicate the slope was constant for 86, 80, and 57 days, respectively, for the procainamide, gentamicin, and carbamazepine assays. (Right) Linear-regression lines for the daily y-intercept values show non-zero slopes across time and indicate changing y-intercepts for all three assays.
carbamazepine calibration curves during 88 days, using eight assay kits.

Figure 1 (left) illustrates the slopes and confidence intervals observed with time for all three assays. Based on overlapping 95% confidence intervals, there is no statistically significant change in the slope of the calibration curves for procainamide and gentamicin (models 1 and 2) during the study. The slope of the carbamazepine calibration curves (model 3) remained statistically unchanged for 57 days.

Figure 1 (right) illustrates the y-intercepts and confidence intervals observed with time for all three assays. The relative instability of y-intercepts for all three models (as demonstrated by non-overlapping 95% confidence intervals) is evident early in the study. The linear-regression lines for the daily y-intercepts show a positive slope and indicate an overall gradual upward drift of the y-intercept for all three assays.

Figure 2 graphically shows the 37 calibration curves for procainamide when the model 1 algorithm was used. Similar curves can be constructed for gentamicin and carbamazepine showing the general parallelism associated with lines of similar slopes and differing y-intercepts. Slopes, y-intercepts, and 95% confidence limits derived from this and similar curves are plotted in Figure 1.

Table 1 shows statistics for the control material analyzed immediately after each calibration and for calculated values derived from the y-intercept update procedure. The means, standard deviations, and coefficients of variation of control material analyzed after each calibration reflect optimal values and precision when assayed according to the manufacturer's recommendations. We selected three slopes for each assay, representing the lowest, the mean, and the highest slopes from valid curves. With the slope held constant, the y-intercept was adjusted, using a single target calibrator to produce control values from raw control absorbance rates. We compared control results from these curve manipulations to the optimal values previously determined. We applied Student's t-test to examine the statistical significance of the differences between control values determined both ways.

Only the low control (Stratus 1) was shown to yield statistically significant differences in value (i.e., p < .01) as a result of y-intercept manipulations. These differences, although statistically significant, are less than 10% and are clinically insignificant. The control data show the precision of both methods to be comparable. CVs for all control results

Table 1. Comparison, with Use of Student's t-Test, of Calculated Controls Via y-Intercept Updates and Controls Assayed Immediately after Calibration

<table>
<thead>
<tr>
<th>Control no.</th>
<th>Model 1 (Procainamide n = 37)</th>
<th>Model 2 (Gentamicin n = 30)</th>
<th>Model 3 (Carbamazepine)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Mean, mg/L</td>
<td>2.2432</td>
<td>5.1703</td>
<td>11.4514</td>
</tr>
<tr>
<td>SD, mg/L</td>
<td>0.0959</td>
<td>0.2847</td>
<td>0.5848</td>
</tr>
<tr>
<td>CV, %</td>
<td>4.27</td>
<td>5.51</td>
<td>5.11</td>
</tr>
<tr>
<td>Slope b</td>
<td>233.66603</td>
<td>232.8435</td>
<td>282.31400</td>
</tr>
<tr>
<td>Mean, mg/L</td>
<td>2.1351</td>
<td>5.0838</td>
<td>11.5946</td>
</tr>
<tr>
<td>SD, mg/L</td>
<td>0.0789</td>
<td>0.1756</td>
<td>0.3519</td>
</tr>
<tr>
<td>CV, %</td>
<td>3.70</td>
<td>3.46</td>
<td>3.04</td>
</tr>
<tr>
<td>p</td>
<td>&lt;.01</td>
<td>&gt;.05</td>
<td>&gt;.10</td>
</tr>
<tr>
<td>Slope c</td>
<td>245.9167</td>
<td>271.5477</td>
<td>30.35884</td>
</tr>
<tr>
<td>Mean, mg/L</td>
<td>2.2514</td>
<td>5.1541</td>
<td>11.4948</td>
</tr>
<tr>
<td>SD, mg/L</td>
<td>0.0937</td>
<td>0.2364</td>
<td>0.5270</td>
</tr>
<tr>
<td>CV, %</td>
<td>3.72</td>
<td>4.59</td>
<td>4.58</td>
</tr>
<tr>
<td>p</td>
<td>&gt;.34</td>
<td>&gt;.39</td>
<td>&gt;.36</td>
</tr>
<tr>
<td>Slope d</td>
<td>249.00240</td>
<td>281.98110</td>
<td>31.92524</td>
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<tr>
<td>Mean, mg/L</td>
<td>2.2730</td>
<td>5.1568</td>
<td>11.2162</td>
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<tr>
<td>SD, mg/L</td>
<td>0.0652</td>
<td>0.1642</td>
<td>0.3716</td>
</tr>
<tr>
<td>CV, %</td>
<td>2.87</td>
<td>3.18</td>
<td>3.31</td>
</tr>
<tr>
<td>p</td>
<td>&gt;.06</td>
<td>&gt;.40</td>
<td>&gt;.02</td>
</tr>
</tbody>
</table>

* a n = 34 for controls analyzed after each calibration; n = 30 for calculations with adjusted y-intercept. b Lowest slope of all calibrations for that particular model. c Average for all calibrations for that particular model. d Highest slope of all calibrations for that particular model.
are well below the expected standards of 8 to 10% stated in Syva's EMIT literature.

Discussion

We rewrote the mathematical algorithms used by Syva so they would reflect the least-squares linear regression equation as part of the calibration process. We considered the estimate of error associated with each calibration and assigned a degree of confidence for slope and y-intercept parameters. Based on 98% confidence intervals, there is curve stability relative to the slope of the regression equation. The y-intercept is not stable for the same period of time. The apparent upward drift of the y-intercept appears to be linear; however, we made no attempt to define the accuracy of predicting the y-intercept. This instability may very well be associated with changes in the reactant constituents as a result of aging, producing higher baseline absorbance rate changes.

We demonstrated viable curve stability beyond the time suggested both by the manufacturer and other investigators, showing slope stability for all three Syva algorithms of 89, 80, and 57 days, respectively, for procainamide, gentamicin, and carbamazepine. We improved utilization of reagents and calibration curves by replacing the full calibration procedure with a standard-curve manipulation. Curve editing of the y intercept is possible with Syva Autolab software. With use of this method, full calibration may be limited to a one-time procedure with subsequent y-intercept updates to compensate for reagent aging.

We observed no significant differences in assay results whenever a y-intercept update is used. We showed the minor variations in assay results as a function of curve manipulations to be statistically and clinically insignificant. This procedure of y-intercept updating is simple, quick, and cost effective, and it makes full recalibration procedures unnecessary. An assay can be calibrated on day 1 and validly stored for longer than 50 days. A single standard may be assayed to ascertain the extent of y-intercept change. Full calibration can be replaced by y-intercept updates followed by validation with appropriate controls. This can be utilized on a daily basis for low-volume assays and, more significantly, upon reconstitution of subsequent assay kits of the same lot. The y-intercept has merely to be entered into the memory of the CP-5000 microprocessor. Once verified with appropriate controls, results can be generated for patients.

Minimizing calibration is important when one considers labor and reagent cost. At the current cost of $2 per cycle, EMIT calibration curves cost approximately $20 per calibration for a seven-point curve with tri-concentration control validation. This amounts to a cost of approximately $700 for an average 33 calibration curves for each of the models we examined. By using our method, a laboratory may save hundreds of dollars per month for any single high-volume assay. Savings are even more significant for low-volume assays, in which disproportionately large amounts of reagents currently are consumed during calibrations.

The underlying principles, chemical formulations, manipulations of reagent components, and the system used for monitoring the progress of the EMIT assays are analogous, irrespective of new therapeutic drug monitoring assays in the Syva EMIT methodology (6). Therefore, our results may be applicable to all Syva EMIT therapeutic drug monitoring assays that make use of models 1, 2, and 3. Consistency in the lot and algorithm is necessary in order to realize curve stability. One can expect substantially greater savings by combining y-intercept updates with other approaches to optimize reagent use.

References