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Calibration and validation of linearity in chromatographic biopharmaceutical analysis*

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Abstract: Calibration in chromatographic biopharmaceutical analysis is a major determinate of method performance and many methods have been proposed to evaluate an appropriate calibration model, to determine the linear range and to evaluate the goodness of fit. Ten chromatographic bioanalytical methods have been evaluated in this work by observation of concentration–response curves, linearity plots, calculation of concentration residuals, correlation coefficients and lack of fit analysis. These methods were applied to univariant linear regression, weighted regression, polynomial regression and power fit models in order to determine the most appropriate way to establish and evaluate calibration functions. It was found that weighted linear regression provided the most appropriate calibration function for eight of the 10 methods studied, whereas unweighted regression and the power fit model proved appropriate for one each of the other two methods. The choice of calibration function was best accomplished through observation of calculated concentration residuals. Linearity and sensitivity plots were of little value for assessment of linearity through the selected calibration range if conventional (±5%) tolerance limits are employed. Validation of the calibration model can be accomplished by demonstrating the concentration residuals and the slope of the log concentration–log response plots are within reasonable tolerance limits or by lack of fit analysis. Correlation coefficients were demonstrated to be of little value for this purpose and the quadratic approach to linearity validation was in disagreement with other methods in four of the 10 methods evaluated.

Keywords: *Calibration; validation; quality control; standardization; linear regression.*

Introduction

Validation of chromatographic bioanalytical methods and control of their quality during analysis of samples has become a very important issue in recent years. This has been brought about in part by recent findings of regulatory agencies [1] and has resulted in a joint conference (Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic Studies Conference, Arlington, VA, USA, December 1990) of the Food and Drug Administration, American Association for Pharmaceutical Scientists, Association of Official Analytical Chemists, Health Protection Branch, and The Federation Internationale Pharmaceutique (FDA, AAPS, AOAC, HPB, and FIP). This conference was held to establish consistency among bioanalytical laboratories in the way methods are validated and analytical results are monitored. Calibration of analytical methods is a major issue to be considered because the choice of calibration function and procedure are the primary determinants of method accuracy, especially at the high and low concentration extremes. This function must also be validated in terms of the concentration range in which it is used and the degree to which observed data fit the model. Historically, there has been a bias in favor of the linear model and data may be rejected according to many standard operating procedures because of a lack of conformance to the linear model. The consensus opinion of the group addressing validation at the recent Analytical Methods Validation Conference, however, was simply that calibration functions should be "defined, and demonstated to be monotonic".

Issues related to the choice of calibration function, validation of linear range and goodness of fit evaluation to the model have been dealt with individually in a variety of works. Plots of log concentration versus log response [2] have been used to evaluate the linear model along with correlation coefficients [3], interpolated standard concentrations [4], polynomial regression [5] and lack of fit analysis [6]. Although nonlinear models have been used less frequently in chromatographic pro-

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cedures, some of these methods may also be valuable for evaluation of the useful concentration range and the goodness of fit for nonlinear data.

In this paper, we have evaluated a number of data sets from 10 different analytical methods. These evaluations were carried out to assess the various procedures for choosing a calibration function, validation of calibration range and goodness of fit of the model.

Experimental

Ten different chromatographic methods for the analysis of drugs in plasma were selected to represent various types of chromatographic detectors, separation modes and extraction techniques. These methods are characterized in Table 1 and all were used for collection of bioanalytical data at the Biopharmaceutical Analysis Laboratory — Virginia Commonwealth University. All calibrations were carried out using a minimum of six concentration points and the number of calibration curves evaluated reflect the number of different analytical runs that were performed for the individual analytical method (see Table 2). Mean data from all calibrations were used to evaluate each calibration function to assess the useful concentration range and to test goodness of fit.

Data from each analytical method were fitted to the univariant linear regression model [7] according to:

$$y = mx + b, \quad (1)$$

where y represents response (peak area for Method H, peak height ratio for Methods I and J, and peak area ratio for the remainder), x represents concentration and m and b represent the best fit slope and y axis intercept for each calibration curve respectively. The same calibration data were then fitted to a weighted linear regression model [6]. Weighting factors of $1/x$ and $1/x^2$ were evaluated as approximations of 1/variance [8] and used in determining concentrations from the coefficients m and b as in the unweighted linear model.

Data were also fit to a power function [9] which allows the calibration curve to display a small degree of curvature over the dynamic range. This was accomplished using the following mathematical expression:

$$y = m^*x^R, \quad (2)$$

where m^* is a constant which represents the calibration slope at an apparent concentration of 1 and the exponent R is the slope of a plot of log y versus log x.

Quadratic fit of the data was determined according to the following equation:

$$y = b_0 + b_1x + b_2x^2. \quad (3)$$

In order to evaluate calibration models for each method, interpolated concentrations were determined by calculating concentrations from individual mean response values using parameters determined from the entire calibration curve. Per cent relative concentration residuals (% RCR) were calculated to compare deviations of individual concentration points as follows:

$$\% \text{ RCR} = 100 \text{ (RC} - \text{NC)/NC}, \quad (4)$$

where RC and NC represent the interpolated and nominal concentrations, respectively.

Visual representation of response versus concentration plots, log concentration versus log response plots and slope versus log concentration plots were constructed using the graphics function of Quattro-Pro [10].

Results and Discussion

Choice of calibration function

The obvious first step in selecting an appropriate calibration function is visual observation of a plot of unfitted concentration versus response data for linearity or degree and type of curvature. One problem associated with this is that slight deviations may not be detected because of the scale at which the plots are normally observed. A plot modelled from a power curve (with an exponent, R, of 0.908) is shown in Fig. 1. This plot appears linear where the entire range of 10–1000 concentration units is displayed (Fig. 1, insert) because of the apparent compression of the low end of the curve and no apparent deviation at the high end of the curve. The low end of the curve, however, shows a clear departure from linearity since both of the lowest points are below the curve. This situation could also be mistaken for a weighting problem since the curve conforms to the high points better than the low points. Although visual representations may serve as a

Table 1
Characteristics of analytical methods used for calibration assessment

Method	Compound type	Extraction	Separation	Detection	Concentration* range ratio
A	Sulphonylurea, 2° amine	Double SPE†	HPLC	UV	300
B	Sulphonylurea, 2° amine	Single SPE	HPLC	UV	100
C	2° amine	Double LLE†	HPLC	FL	50
D	Carboxylic acid	Single LLE	HPLC	UV	300
E	Carboxylic acid, 2° amine	Single LLE	HPLC	UV	160
F	1° amine	Triple LLE	GC	ECD	25
G	2° amine	Triple LLE	GC	ECD	25
H	Carboxylic acid, 2° amine, sulphonylamine	Single SPE	HPLC	FL	16
I	2° amine	Single SPE	GC	Mass	123
J	3° amine	Single SPE	GC	Mass	123

*Highest concentration divided by lowest concentration of the calibration curve.
†SPE = solid phase extraction.
‡LLE = liquid–liquid extraction.

Table 2
Effect of calibration type on per cent relative concentration residuals

	Method A (n = 11) Standard concentrations (ng ml^{-1})						
	10	20	50	100	750	2000	3000
Linear, unwt	28.80	2.53	18.28	0.07	−1.26	−0.80	0.43
Linear, 1/x*	1.51	−10.99	13.12	−2.38	−1.31	−0.61	0.66
Linear, 1/$\hat{x}$2	2.94	−10.47	12.94	−2.68	−1.78	−1.11	0.16
Quadratic	−17.60	−19.45	11.96	−1.82	0.42	−0.14	0.02
Power	5.70	−10.75	10.42	−4.82	−2.14	−0.23	1.60
	Method B (n = 92) Standard concentrations (ng ml^{-1})						
	10	20	75	250	500	1000	
Linear, unwt*	0.75	−0.02	−1.80	−0.67	0.97	−0.19	
Linear, 1/x	1.37	0.28	−1.74	−0.66	0.96	−0.21	
Linear, 1/$\hat{x}$2	0.22	−0.07	−1.50	−0.27	1.39	0.24	
Quadratic	11.60	4.70	−1.45	−1.24	0.27	−0.05	
Power	0.60	0.00	−1.71	−0.58	1.03	−0.16	
	Method C (n = 25) Standard concentrations (ng ml^{-1})						
	1	2	5	10	20	50	
Linear, unwt	−4.05	−5.77	−5.28	1.54	2.21	−0.35	
Linear, 1/x	3.40	−2.30	−4.20	1.79	2.05	−0.73	
Linear, 1/$\hat{x}$2*	1.81	−2.76	−3.97	2.30	2.68	−0.06	
Quadratic	15.00	1.50	−4.60	0.00	0.45	0.02	
Power	14.00	−5.00	−11.80	−3.90	1.45	7.62	
	Method D (n = 19) Standard concentrations (ng ml^{-1})						
	0.5	2	10	25	50	100	150
Linear, unwt	27.95	5.33	−4.98	−0.20	0.23	0.58	−0.26
Linear, 1/x	5.59	−0.09	−5.89	−0.46	0.24	0.70	−0.10
Linear, 1/$\hat{x}$2*	0.67	−0.48	−4.03	0.66	1.44	1.94	1.14
Quadratic	58.00	12.00	−4.50	−0.64	−0.28	0.36	−0.09
Power	12.00	−7.00	−11.40	−2.88	0.80	4.52	5.62

Table 2
Continued

	Method E (n = 21) Standard concentrations (ng ml^{-1})							
	5	10	20	50	100	250	500	800
Linear, unwt	23.44	13.09	4.87	0.32	−2.64	−0.96	0.22	0.04
Linear, 1/x	2.05	2.66	−0.10	−1.38	−3.25	−0.90	0.51	0.41
Linear, 1/$\hat{x}$2*	−0.65	1.76	−0.13	−0.88	−2.59	−0.11	1.34	1.26
Quadratic	13.60	8.70	3.15	0.26	−2.11	0.12	1.77	2.04
Power	−3.60	2.60	1.90	1.00	−1.40	−0.18	0.19	−0.64

	Method F (n = 35) Standard concentrations (ng ml^{-1})					
	5	10	20	50	75	125
Linear, unwt	2.32	−2.94	−1.58	1.40	0.02	−0.17
Linear, 1/x	2.96	−2.65	−1.47	1.39	−0.01	−0.22
Linear, 1/$\hat{x}$2*	1.61	−3.02	−1.33	1.89	0.52	0.36
Quadratic	6.00	−1.80	−1.80	0.88	−0.32	0.02
Power	0.00	−1.50	−1.50	2.28	−0.07	−1.65

	Method G (n = 40) Standard concentrations (ng ml^{-1})					
	5	10	2	50	75	125
Linear, unwt	−31.34	−7.21	2.13	4.17	1.86	−1.30
Linear, 1/x	−9.70	2.14	5.46	3.94	0.92	−2.79
Linear, 1/$\hat{x}$2	−2.86	3.79	4.55	1.63	−1.62	−5.47
Quadratic	5.00	0.30	1.20	0.58	−0.57	0.08
Power*	−0.60	0.00	0.45	1.08	−0.03	−0.95

	Method H (n = 20) Standard concentrations (ng ml^{-1})					
	50	100	200	400	600	800
Linear, unwt	−18.12	−6.69	0.53	5.95	1.10	−1.96
Linear, 1/x	−4.08	−0.83	2.27	5.59	0.18	−3.14
Linear, 1/$\hat{x}$2*	−0.64	0.02	1.79	4.41	−1.11	−4.48
Quadratic	6.16	−1.48	−2.67	1.63	0.54	0.06
Power	−1.94	0.26	2.03	4.91	−0.58	−3.94

	Method I (n = 6) Standard concentrations (ng ml^{-1})						
	0.224	0.448	1.12	2.24	6.72	11.2	27.6
Linear, unwt	37.68	16.21	3.70	5.95	−0.73	−4.21	0.68
Linear, 1/x	2.70	−0.73	−2.41	3.52	−0.80	−3.84	1.55
Linear, 1/$\hat{x}$2*	0.78	−1.36	−2.25	3.98	−0.19	−3.21	2.25
Quadratic	−32.59	−14.73	−3.57	6.25	2.98	−1.61	0.04
Power	4.02	−1.79	−4.46	2.23	−0.74	−3.12	3.80

	Method J (n = 6) Standard concentrations (ng ml^{-1})						
	0.09	0.18	0.45	0.9	2.7	4.5	11.1
Linear, unwt	−51.23	−25.66	−11.77	0.85	5.27	1.02	−0.46
Linear, 1/x	−2.78	−2.21	−3.31	4.20	5.27	0.43	−1.61
Linear, 1/$\hat{x}$2*	0.81	−1.05	−3.58	3.34	4.08	−0.77	−2.83
Quadratic	−1.11	−3.33	−5.78	1.11	−10.63	−1.33	0.09
Power	11.11	−5.56	−11.11	−3.33	2.22	0.02	3.96

*Indicates calibration model selected.

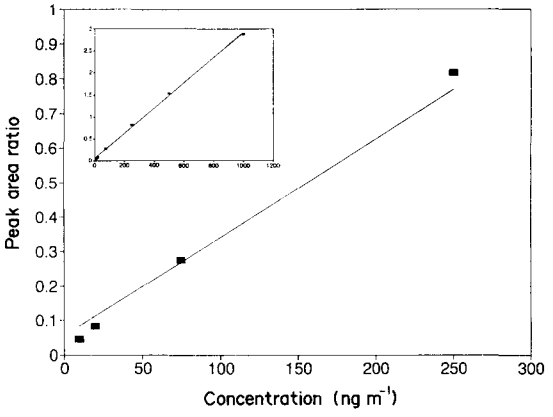

Figure 1
Plot of concentration versus response for data modelled from a power fit $R = 0.908$. Solid line indicates the line of unweighted best fit. Closed squares represent data modelled from a power fit. Insert represents the entire calibration range and the larger graph illustrates the lower four concentration points.

guide, a more sensitive approach is the observation of % RCR data for various calibration functions. The calibration function which demonstrates the lowest % RCR values for the method, especially at the extremes of calibration, would then be selected as the most appropriate model. Calculated % RCR values are listed for each calibration method over the concentrations studied in Table 2.

As can be seen from Table 2, the calibration function judged appropriate most often was the weighted linear function with a weighting factor of $1/x^2$. This may be due to the fact that assumptions of equal variances for linear regression are often not valid for bioanalytical data [11] and x^2 is the most appropriate approximation of these unequal variances [8]. The unweighted linear model was selected one out of 10 times. The method which best fit the unweighted linear model (method B) was one in which a UV detector was used. The % RCR for this data at the low end of calibration was also less for $1/x^2$ weighted calibration giving further evidence for the condition of unequal variances for bioanalytical data. Method G provided a better fit to the power calibration model over the concentration range studied. This could be due to the recognized nonlinear response characteristics of electron capture detectors for gas chromatography [12]. The other electron capture method (method F) also demonstrated a very low % RCR at the low end with power fit calibration. This could be due to the fact that the sensitivity of method F was approximately twice that of method G and a different region of the dynamic range of the detector was being utilized. The quadratic fit provided better data for the two electron capture techniques than for some methods but was not the function of choice for any of the example methods.

Calibration range

The calibration range for each of the analytical methods in question was established through a consideration of the practical range necessary according to the use of the data. Calibration range assessment in this context is not to establish the absolute limits through which the method could be calibrated but rather to establish what selected range is within the limits that may be set for a calibration range. In nonlinear calibration, the range may be taken as the dynamic range (defined as the range of concentrations over which a change in concentration produces a change in response) or an approximation of linearity such as used in the 'almost linear' approach [2]. Tests for dynamic range would involve some demonstration that the slope of calibration maintains a value which is significantly greater than zero. The 'almost linear' approach establishes usual limits for the linear range of the calibration model although a power function is used within that range. Since none of the example data fit the quadratic model well and power function ranges may be set through linear criteria, calibration range assessment was limited to an assessment of the linear range. The linear range is defined as the range of concentration over which the sensitivity (slope of the calibration curve) is constant to within a defined tolerance [13]. The American Society for Testing Materials (ASTM) employs ±5% tolerance limits for linearity of GC detectors and similar definitions are likely to be recommended for HPLC detectors [13]. One way of ensuring conformance to these tolerance limits is observation of % RCR values over the range of interest. It can be seen from Table 2 that all calibration methods selected are within the ±5% tolerance limits at all concentrations tested except for method A. Method A demonstrated a negative bias for the 20 ng ml^{-1} calbrator and a positive bias for the 50 ng ml^{-1} calibrator. All other concentration points for Method A are within 5%, however, and these biases on individual calibration points are

likely to be artifacts unrelated to the calibration function.

Another technique used to assess the linear range within the ±5% tolerance limit is that provided by ASTM Standard Practice E685 [14]. This linearity plot method involves determination of intersection points between a curve drawn through the data points and a line having a slope which is 95% of the best fit linear regression line (Fig. 2). Plots constructed in this way for all methods demonstrated no systematic deviations from linearity as indicated by low or high trends in the data which intersected the 95% best fit linear regression line. The 5 ng ml^{-1} concentration points for Methods E and F fell below the best fit regression line as well as the 20 ng ml^{-1} point for Method A and the 11.2 ng ml^{-1} point for Method 1. These were more likely due to concentration dependent bias rather than nonlinearity because no trend could be observed in the data. This indicates that the 5% lower tolerance limit for this approach may be too conservative for bioanalytical data since 5% is normally well within acceptance limits for method bias.

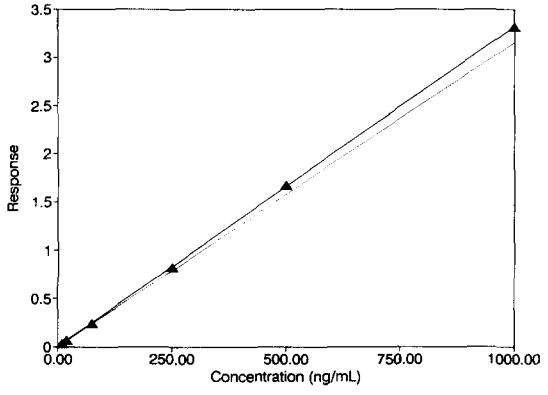

Figure 2
Linearity plot for Method B relating response to concentration showing the best fit line (solid); the 95% best fit line (dotted) and the individual data points (filled triangles).

Another approach to assessment of linear range is to plot sensitivity versus log concentration. In this method, referred to as a sensitivity plot, individual response values are divided by their corresponding concentration. Constancy of this plot indicates linearity and tolerance limits for the linear range can be established by choosing a mid-concentration point slope and multiplying by 0.95 and 1.05 to determine upper and lower limits. Tolerance limits may vary depending on the concentration point chosen so the mid-point concentration was systematically chosen for this work if there were an odd number of calibrators. If there were an even number, the calibrator on the high concentration side of the mid-point was chosen. The calibration range is considered to be within the linear range if there are no intersections of the sensitivity plot and the upper and lower tolerance limits as shown in Fig. 3 for Method B. Method G which demonstrated the lowest % RCR values for the power fit shows a typical nonlinear sensitivity plot where the slope trends from one concentration extreme to the other (Fig. 4).

The sensitivity plot was established to evaluate the linear range of detectors. There were

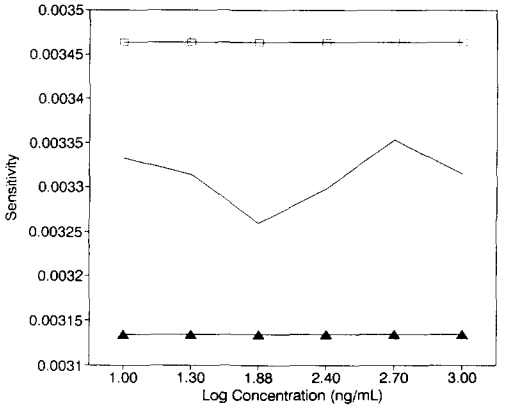

Figure 3
Plot of sensitivity versus log concentration for Method B showing apparent calibration within the linear range. Tolerance limits of +5% and −5% are indicated by open squares and filled triangles, respectively.

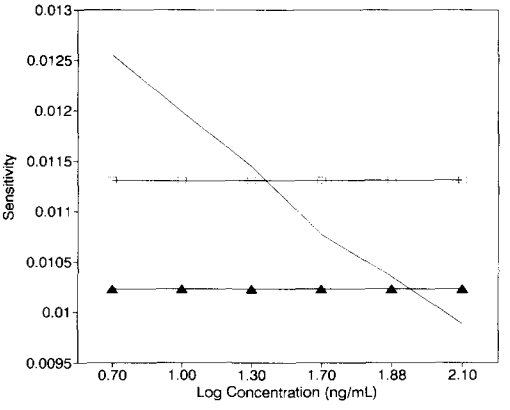

Figure 4
Plot of sensitivity versus log concentration for Method G showing lack of linearity and apparent calibration outside the linear range. Tolerance limits of +5% and −5% are indicated by open squares and filled triangles, respectively.

therefore, no allowances for sources of nonlinearity other than detector response alone considered in this approach. In bioanalytical methods, there may be adsorption and complexation processes occurring in sample processing that potentially adds both variability and departure from linearity that would not occur in clean solution. Tolerance limits and expectations regarding slope consistency should therefore be different for bioanalytical processes. This may account for the observation that only one of the methods studied was calibrated within the linear range as determined by this approach with tolerance limits set for detector response linearity alone.

Goodness of fit

The correlation coefficient (r) has been recognized as a poor indicator of how well a linear regression equation fits the linear model [3, 15]. In spite of this, correlation coefficients are probably the most widely used linearity test in chromatographic biopharmaceutical analysis. The correlation coefficient is of benefit for demonstrating a high degree of relationship between concentration–response data once the linear model has been established but is of little value in documenting adherence to the linear model, since a highly correlated curvilinear function can show high correlation coefficients [16]. Correlation coefficients for the methods evaluated are presented in Table 3. All of the methods demonstrated good correlation and since the acceptance criteria was 0.99 or greater, all methods would demonstrate acceptable linearity by this test. It is noteworthy that even Method G which demonstrated lower % RCR data for the power fit and Method A which demonstrated inconsistency in % RCR demonstrated correlation coefficients greater than 0.999.

Observation of % RCR data for individual standard points has been shown to be a more sensitive assessment of goodness of fit to the linear model than correlation coefficients [4]. A linearity test through the range of calibration could be performed by application of acceptance limits to individual % RCRs and these limits should be on the order of acceptance limits established for method accuracy. If limits of ±15% were taken as acceptable, it can be seen from Table 2 that selected calibration types in all methods would demonstrate acceptable conformance to the model chosen for calibration. If ±10% was chosen as acceptance limits, selected calibration types for all methods except Method A would be acceptable. It should be noted that observation of % RCR data is beneficial for observation of goodness of fit for both linear and nonlinear models. The sensitivity of this test as a criterion for goodness of fit of course depends on the acceptance criteria established with the utility of the technique being more appropriate for choosing the model rather than demonstrating conformance to the model.

In the polynomial regression approach, linearity is determined through establishment that the coefficient on the concentration squared term is not significantly different from zero [5]. Although this method has the advantage of not requiring replication, it can be seen from Table 3 that only five of the 10 methods tested (Methods B, C, D, E and F) would provide acceptable results for conformance to the linear model. This indicates that this test may not be practical for chromatographic bioanalytical data, and it likely has benefit only

Table 3
Comparison of results for tests of goodness of fit

Method	Log–log slope	Polynomial regression*	Lack of fit†	Correlation coefficient
A	0.9868	Nonlinear ($P = 0.006$)	Linear ($P > 0.10$)	0.99996
B	1.0004	Linear ($P = 0.28$)	Linear ($P > 0.10$)	0.99998
C	0.8951	Linear ($P = 0.066$)	Linear ($P > 0.10$)	0.99990
D	0.9544	Linear ($P = 0.26$)	Linear ($P > 0.10$)	0.99998
E	0.0169	Linear ($P = 0.29$)	Linear ($P > 0.10$)	0.99998
F	1.0348	Linear ($P = 0.32$)	Linear ($P > 0.10$)	0.99997
G	0.9270	Nonlinear ($P = 0.002$)	Linear ($P > 0.10$)	0.99943
H	1.0003	Nonlinear ($P = 0.015$)	Nonlinear ($P = 0.001$)	0.99889
I	0.9839	Nonlinear ($P = 0.028$)	Linear ($P > 0.10$)	0.99975
J	0.9340	Nonlinear ($P = 0.040$)	Linear ($P > 0.10$)	0.99983

*Linearity tested by comparing t calculated for the x^2.
†Linearity tested by comparing F calculated to tabulated F at $\alpha = 0.05$.

to determine whether or not the quadratic model is statistically valid.

It can be shown for linear data that a plot of log concentration versus log response will ideally provide a slope equal to one [16]. The slope of such a plot for each method studied is shown in Table 3. The question again arises as to what tolerance limits to establish for acceptance of the linear model. If a confidence interval approach were applied to the nine methods where a linear model was chosen, the acceptance range for the slope (±2SD) would be 0.8810–1.0648 and all methods tested would fall within this range. The problem with this approach is that one must assume that the nine methods evaluated were indeed linear and a more absolute criterion for tolerance limits would be desirable.

The most accepted statistical method for determination of linearity is the F test for lack of fit [6]. This test has the disadvantage of requiring replicates and the possibility that very precise data may be judged nonlinear if an inadequate number of replicates are employed in the data analysis. Table 3 shows the results of lack of fit analysis for each of the methods tested. The only method demonstrated to be nonlinear by this technique is Method H where a power fit was chosen according to % RCR data. This would indicate that lack of fit analysis provides a good assessment of linearity providing there are enough replicates. It is good analytical practice to carry out sufficient replicates of calibration during validation of an analytical method. This requirement should not constitute a practical limitation in most cases.

Conclusion

The preceding treatments indicate that not all assessments of calibration may be appropriate for chromatographic bioanalytical data. Many of these procedures were developed for clear solutions and bioanalytical variables were not allowed for in the conventional tolerance limits. The choice of procedures for calibration assessment must be put into the context of method establishment where the goal is to determine the most appropriate calibration function, method validation where the goal is to assess the quality of the method and quality control where the goal is to determine whether or not the method is operating properly. Although these goals cannot be strictly separated, they must be dealt with differently and the tests which are applied should be chosen appropriately.

For establishment of the calibration function it appears that observation of the concentration response curve combined with an assessment of % RCR data would be most helpful for choosing the appropriate calibration function. Once the calibration function is established, it should be tested with replicate validation data for absolute conformance to the model. This can be done most efficiently by either log–log plots or lack of fit analysis for linear data and conformance to tolerance limits of % RCR data for nonlinear models. During quality control where the calibration function has been established and tested, correlation coefficients and % RCRs should be evaluated with appropriate acceptance criteria to determine the acceptability of individual calibration points and calibration curves during the analysis process.

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