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Analytical Survey

Evaluation of calibration routines in pharmaceutical and bioanalytical separations, with reference to Monte Carlo simulations

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Abstract: A Monte Carlo method for the estimation of the bias and inter-assay component of precision attributable to given combinations of assay and calibration routine is described. Simulations are performed by a computer program which uses the actual concentration versus response and concentration versus intra-assay precision characteristics of the assay under investigation as its database. In operation, a calibration routine is selected, the calibrators defined and the analytical process of calibration followed by the analysis of unknowns is reproduced. In this way the mean bias and inter-assay component of precision at a number of pre-defined concentrations covering the analytical range of the assay is estimated. The program is intended for use by analysts as a practical aid in the selection and optimization of appropriate calibration routines in an experimental environment.

Keywords: *Monte Carlo simulations; assay calibration; accuracy prediction.*

Introduction

Choice of the most appropriate calibration routines for use in drug analysis involving high-performance liquid chromatography (HPLC) or gas chromatography (GC) is often a source of disagreement between analysts involved in the same type of studies. For example, one survey revealed that over half those responding used unweighted regressions and that of those using weighted regressions a variety of weighting factors were employed [1]. The construction of calibration curves has long been part of the analytical chemist's routine, but the traditional use of graph paper has been superseded by the widespread introduction of computer-based data systems which define the calibration curve mathematically. Although there is an abundance of literature concerning least-squares linear regression, the differences between one routine and another is probably not apparent to the majority of analysts who lack detailed training in mathematics. On the other hand, the examples presented by the mathematicians to illustrate their theories often do not reflect the real situations met by the analyst.

A comparison of the analytical situations frequently used as examples in the literature illustrating the use of regressions in assay calibration yielded a series of differences from the assays used in this laboratory, which is primarily concerned with quantitative chromatographic drug analysis for biological fluids.

These differences are listed below and, while possibly not significant when taken alone, when considered together call into question the validity of the application of particular calibration routines when applied to assays with markedly different characteristics.

(I) Some of the literature examples used to illustrate the validity of regressions are limited to a 10-fold dynamic range, whereas in drug bioanalysis concentrations are commonly determined over ranges of 100 or 1000.

(II) The number of calibrators used to define the curve may be quite large (>30) in the literature examples but most analysts limit the number of calibrators to around 10 in order to maximize the number of samples analysed in a single routine batch. Reducing the number of calibrators will increase the variability between individual calibration curves.

(III) A change in the distribution pattern of the concentrations of the calibrators from an arithmetic series (e.g. 1,2,3 . . .), found in the literature, to a geometric series (e.g. 1, 10, 100 . . .) is sometimes employed by analysts

working with assays covering wide dynamic ranges. It has been shown that the precision with which a calibration curve is defined is dependent upon the distribution of the calibrators [2].

(IV) The commonly used linear regression routines are derived with the assumption that the errors are either homoscedastic (i.e. standard deviation is independent of concentration) or that the relative standard deviation is independent of concentration. In practice it is often found that the standard deviation (σ_c) at concentration (c) can be expressed by $\sigma_c = \sigma_o + kc$ where σ_o is the standard deviation at zero concentration and k is a constant [3, 4]. The question arises as to whether there is any advantage in using more robust, but more complex, regression routines which involve the estimation of individual weighting factors in this situation [5, 6].

(V) HPLC UV photometric detectors with a linear range of $>10^5$ have been shown to exhibit changes in the specific response or sensitivity (i.e. response/concentration) of $\pm 5\%$ of the mean value over a range of 9.52 [7]. Experience in this laboratory has also shown that the specific response of both HPLC and GC systems can vary by up to $\pm 10\%$ of the mean value over a range of 1000. The effect of such variation in specific response, while still generally accepted as being within the allowed bounds of linearity, is not discussed in the literature.

Two other factors can influence the choice of calibration routine used by the analyst. The first is the constraints imposed by the chromatographic data system. Some only provide unweighted regressions and at least one manufacturer reverses the normal convention of x representing the concentration and y representing the response in the regressions used in its integrators. In these cases the analyst is therefore faced with the prospect of being limited by the applications available, reprogramming the data system or recalculating the data off-line. Finally, there is the necessity in the pharmaceutical industry to satisfy the requirements of the regulatory authorities.

Considering the factors outlined above, it became evident that some practical means was required whereby an analyst could estimate the errors associated with particular combinations of assay characteristics and calibration routine. As a quantitative evaluation was required the process would of necessity be mathematical in nature but the mathematics should be largely transparent to the analyst and produce data output that is readily understandable. Monte Carlo simulations offered a solution by which the analytical process could be mimicked, i.e. generation of calibration data, definition of a calibration line and estimation of the concentration of unknowns. As the name implies, Monte Carlo methods involve the use of random numbers to simulate the production of experimental data, e.g. the generation of random numbers between 1 and 6 to represent the rolling of a dice.

Measurements in analytical chemistry are subject to random fluctuations, albeit usually following a Gaussian distribution, and make ideal subjects for Monte Carlo methods. A review of the technique made no mention of its use in the simulation of the calibration process though it has been used to compare least-squares and robust regressions in calibration and to test the robustness of three regression methods [8–10].

Errors in Analysis

Before describing the Monte Carlo simulations it is worth making an examination of the errors associated with the reported drug concentrations of samples from pharmacokinetic studies. Generally the analyses are performed over a period of time with fresh calibration curves being generated for each batch of samples. The total error in any determined value can be broken down into a number of components as follows:

(I) Intra-assay precision: this is a characteristic of the assay due to the pure errors of measurement associated with the instrumentation and the skill of the analyst. It is largely independent of the calibration curve as can be demonstrated by considering a general concentration (x) versus response (y) curve of the type $y = ax^2 + bx + c$, which effectively approximates to $y = bx$ when the degree of curvature and intercept are small. A change in the response (y) of, say 5% will result in a corresponding 5% change in the measured concentration (x), i.e. independent of the calibration curve.

(II) Inter-assay component of the overall precision: the total precision (σ_T) is composed of the intra-assay (within-run) precision (σ_{WR}) and an inter-assay (between-run) component of precision (σ_{BR}) according to $\sigma_T^2 = \sigma_{WR}^2 +$

σ_{BR}^2 [11]. If the between-assay changes in the performance of the analyst, etc. are small, then the inter-assay component of precision can be regarded as being solely due to fluctuations in the calibration curves brought about by the normal random distribution of errors of the responses of the sets of calibrators. Its value will reflect the number and distribution of the calibrators and the weighting factor used in the regression.

(III) Accuracy: it is unlikely that the calibration curve will exactly match the actual concentration versus response function over the entire assay range and this lack of fit will give rise to a concentration dependent bias in the determined values.

(IV) Others: there is another source of pure errors which may affect a few individual samples within a batch and include cross contamination by adjacent samples of much higher concentration and gross errors such as pipetting the wrong volume for analysis or loading the chromatographic autosampler out of sequence. These errors should be rare in a well conducted assay and are independent of the calibration curve.

Thus it is only errors II and III affecting the estimated concentrations which can be attributed to the choice of calibration curve and it is these factors which are estimated by the Monte Carlo simulations described.

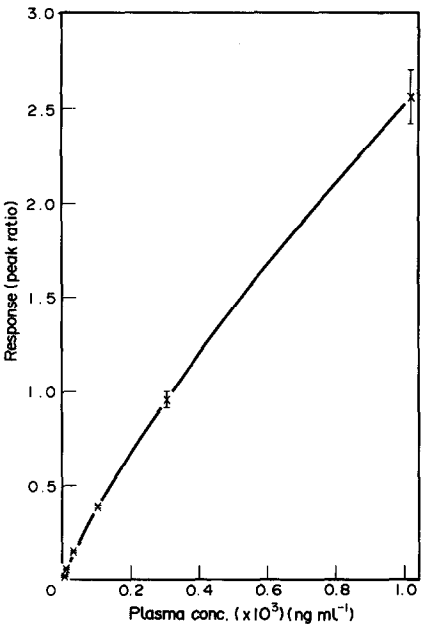

Figure 1
A calibration curve for a gas chromatographic assay of a drug development candidate in plasma using electron capture detection.

Monte Carlo Simulations

The design of the program was aimed at duplicating the analytical process and is illustrated in the following example describing the stages involved in the simulations.

(1) As a first requirement the concentration versus response and concentration versus intra-assay precision characteristics of the assay must be known. In this laboratory, these are normally determined as part of the assay validation by replicate analysis ($n \geq 6$) of a series of samples of the appropriate blank matrix fortified with known amounts of analyte covering the proposed analytical range. The results are expressed in graphical form as smoothed curves from which data may be interpolated for use in the calculations. The normal calibration curve plot of response versus concentration (Fig. 1) is of limited value at low concentrations either for making a critical examination of the fit of the curve or for interpolating values and the data should be replotted as in Figs 2 and 3.

(2) Because the calibration errors will be concentration dependent, a number of concentrations covering the assay range are evaluated and the values entered into the program database together with the corresponding values of mean specific response (i.e. response/concentration) taken directly from Fig. 2. These points represent a series of unknowns in an assay batch and, for example, if a wide concentration range of 1–1000 was to be examined the calibration errors at concentrations of 1, 2, 5, 10, 20 . . . 1000 might be determined.

(3) The number and distribution of the calibrators is defined and their concentrations are entered into the database together with their corresponding values of the mean specific response (Fig. 2) and relative standard deviation (Fig. 3).

(4) A calibration function is selected for evaluation and individual values of the response (y_i) are generated for each of the calibrators at every concentration (x) using the corresponding values of the mean specific response (y/x) from Fig. 1 and relative standard deviation (RSD) from Fig. 3 according to the formula $y_i = y(1 + 0.01\ x \times \text{RSD} \times g)$, where g is a Gaussian or normal random

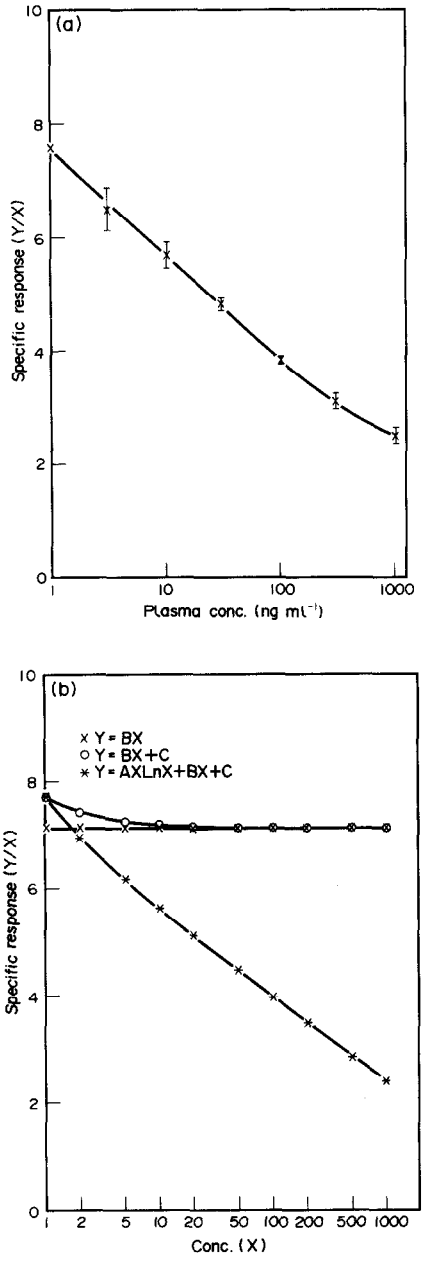

Figure 2
(a) A curve showing the specific response (i.e. gradient of calibration graph) versus concentration of a drug development candidate, assayed by GC, assay used to provide data for the simulations. (b) Curves of three functions based on the same GC data, for comparison with (a) The mean coefficients were derived from six independent calibration experiments of the drug development candidate in plasma x–x, $y = bx$; O–O, $y = bx + c$; *–*, $y = ax\ln x + bx + c$.

number from a set of numbers with a mean of zero and standard deviation of 1.

(5) Once individual responses have been generated for all the calibrators the coefficients of the equation of the calibration curve are calculated.

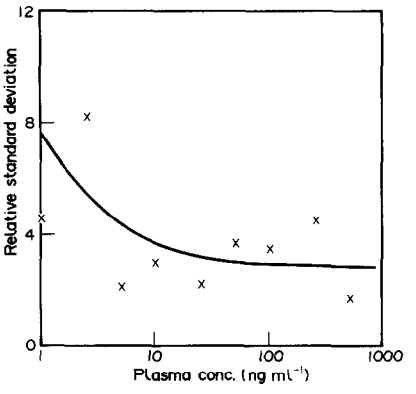

Figure 3
A graph showing the variation of the precision of a gas chromatographic drug assay with plasma concentration.

(6) The fit of the chosen function to the calibration data is examined by estimation of the residuals.

(7) Having determined the calibration function, estimates of the calibration error or bias at each of the concentrations selected in stage 2 are made as illustrated in Fig. 4.

(8) Stages 4–7 are repeated until 10 simulations have been performed so that the mean and inter-assay variability of the calibration bias are determined as a function of concentration.

(9) Having completed the simulations similar evaluations may be made either by selection of a different calibration function or by varying the number or distribution of the calibrators.

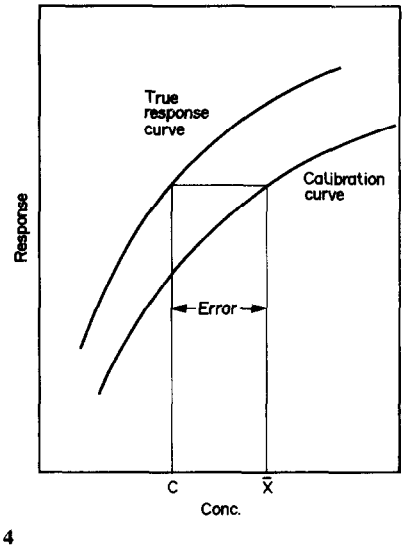

Figure 4
An enlarged section of a calibration curve showing the estimation of the error between an actual concentration (c) in a sample and the mean value ($\bar{x}$) determined from the calibration curve.

The actual pool of 200 Gaussian random numbers initially used in the program had a mean and standard deviation of 0.03242 ± 0.98576, close to the ideal value of 0 ± 1. However nine values outside the range 0 ± 2, i.e. 2 standard deviations were removed to correspond to the common analytical practice of considering such values as outliers although they do form part of the normal statistical population. This changed the mean and standard deviation to −0.01427 ± 0.87793 but it was felt unnecessary to compensate for the reduction of the standard deviation from 1 by refining the calculations as the experimentally determined values of intra-assay precision used in the calculations were subject to greater error. Although not performed in this study, outliers could be selectively placed into the pool of random numbers to deliberately test the effects of such values on the robustness of the various calibration routines.

Application

To test the validity of the program the results from an unpublished capillary GC assay were selected for evaluation. This assay involved the use of an electron capture detector which caused the response to show a marked deviation from true linearity and would therefore provide a more exacting test in prediction of large errors associated with linear calibrations. The assay was used in this laboratory over a range of 1–500 ng ml^{-1} and was essentially free of background interference. The mean of six calibration curves is shown in Fig. 1 and plainly shows some deviation from linearity, although application of linear regression to the data produced a correlation coefficient of 0.9969. Transforming the data (Fig. 2) provides a much more critical review of the curve and allows accurate interpolation of values of the specific response (or sensitivity) for use in the program database.

Similarly, values of the intra-assay RSD were obtained from the curve in Fig. 3. In this case the data are more scattered due to the small populations involved ($n = 6$), but the general shape of the curve was confirmed by other data. A good fit of a curve to the precision data is not considered as important to successful predictions by the program as a good fit of the response data.

The curve in Fig. 1 suggested that a quadratic function may have been appropriate to describe the concentration versus response curve. However, the virtual log–linear plot in Fig. 2(a) showed the curve to be better described by an equation of the type $y = ax\ln(x) + bx + c$, as indicated in Fig. 2(b). This equation was developed as part of this work. Neither of these regressions were available on the data system and so in routine use the assay was calibrated using seven duplicate calibrators at nominal concentrations of 1, 3, 10, 30 . . . 1000 ng ml^{-1} using a point-to-point calculation routine. The number and distribution of the calibrators had been chosen on a purely subjective basis a compromise between adequately defining the curve on a daily basis and keeping the numbers of calibrators within practical limits. Validation of the assay using this routine was performed by the analysis of a series of fortified blank plasma samples on six

Table 1
Comparison of predicted and actual assay performance using point-to-point calibration

Drug added (ng ml^{-1})	Drug found (% of amount added)*		
	Simulations† ($\pm \sigma_{BR}$)	Actual‡ assay ($\pm \sigma_T$)	Actual‡ intra-assay $\pm(\sigma_{WR})$
1.03	99.7 ± 4.2	92.1 ± 5.0	85.8 ± 3.9
2.59	100.9 ± 3.1	97.8 ± 4.2	93.2 ± 7.6
5.13	102.9 ± 1.6	105.0 ± 2.8	98.9 ± 2.1
10.4	100.9 ± 1.5	100.0 ± 1.4	100.3 ± 3.0
26.0	103.0 ± 3.4	100.7 ± 2.6	102.1 ± 2.2
51.9	104.8 ± 1.9	103.7 ± 4.9	105.6 ± 3.9
104	99.7 ± 2.3	101.9 ± 2.9	100.5 ± 3.5
260	100.1 ± 1.5	102.6 ± 3.3	103.4 ± 4.7
521	100.4 ± 1.6	100.8 ± 2.9	104.0 ± 1.8

*Using seven duplicate independent calibrators as described in the text.
†Mean ± standard deviation of 10 simulations.
‡Mean ± standard deviation of six determinations.

separate occasions (overall-assay performance) and on one occasion when six of each of the samples were analysed (intra-assay performance). On all occasions the calibration was performed using independently prepared calibrators as described above. A summary of these results is shown in Table 1.

The same raw data, i.e. the peak height ratios of the drug and internal standard of the calibrators and validation samples, were reprocessed off-line using four different regression procedures and the results from these are shown in Tables 2–5 as the 'actuals' and represent the experimental assay performance when using these calibration routines. Equations for weighted (weighting factor = $1/x^2$) regressions for non-linear curves were developed for this assessment as none could be found in the literature.

The characteristics of the assay, interpolated from Figs 3 and 4, were entered into the program database and the predicted errors estimated at the same concentrations as the validation samples using each of the four calibration routines.

The concentrations and numbers of calibrators were also the same in the simulations as in the experimental validation. These results are also presented in Tables 2–5. Comparison of the actual and simulated data shows that:

(I) There is good agreement between the actual accuracy and the simulations for all four calibration routines despite the diversity and often unsuitability of the routines evaluated in some cases.

(II) As predicted, the intra-assay precision is largely independent of the calibration routine.

Table 2
Comparison of predicted and actual assay performance using unweighted linear regression calibration

Drug added (ng ml^{-1})	Drug found (% of amount added)*		
	Simulations† ($\pm \sigma_{BR}$)	Actual‡ assay ($\pm \sigma_T$)	Actual‡ intra-assay $\pm(\sigma_{WR})$
1.03	−2310 ± 172	−2403 ± 322	−2531 ± 7.3
2.59	−767 ± 66	−805 ± 115	−867 ± 17
5.13	−274 ± 32	−282 ± 48	−329 ± 4.2
10.4	−30.9 ± 14.0	−34.5 ± 16.6	−57.9 ± 5.8
26.0	93.7 ± 4.2	95.0 ± 7.1	81.2 ± 3.6
51.9	112.8 ± 1.6	125.1 ± 6.2	119.1 ± 5.4
104	126.3 ± 1.4	130.9 ± 2.8	125.3 ± 4.1
260	118.1 ± 1.7	121.9 ± 4.4	122.4 ± 5.3
521	106.0 ± 1.7	107.6 ± 3.2	110.6 ± 1.6

*Using seven duplicate independent calibrators as described in the text.
†Mean ± standard deviation of 10 simulations.
‡Mean ± standard deviation of six determinations.

Table 3
Comparison of predicted and actual assay performance using weighted linear regression calibration

Drug added (ng ml^{-1})	Drug found (% of amount added)*		
	Simulations† ($\pm \sigma_{BR}$)	Actual‡ assay ($\pm \sigma_T$)	Actual‡ intra-assay $\pm(\sigma_{WR})$
1.03	87.2 ± 6.5	77.0 ± 10.1	51.7 ± 9.2
2.59	129.2 ± 2.0	123.2 ± 6.9	112.1 ± 11.3
5.13	135.7 ± 1.3	136.1 ± 5.8	127.2 ± 2.7
10.4	131.9 ± 1.5	130.4 ± 6.8	130.2 ± 3.7
26.0	118.9 ± 1.5	120.8 ± 4.6	119.6 ± 2.4
51.9	106.8 ± 1.5	108.6 ± 2.1	110.6 ± 3.5
104	93.8 ± 1.3	96.8 ± 2.1	97.9 ± 2.6
260	79.7 ± 1.2	81.9 ± 1.8	85.9 ± 3.4
521	69.1 ± 1.0	69.9 ± 2.8	74.9 ± 1.0

*Using seven duplicate independent calibrators as described in the text.
†Mean ± standard deviation of 10 simulations.
‡Mean ± standard deviation of six determinations.

Table 4
Comparison of predicted and actual assay performance using weighted quadratic regression calibration

Drug added (ng ml^{-1})	Drug found (% of amount added)* Simulations† ($\pm \sigma_{BR}$)	Actual‡ assay ($\pm \sigma_T$)	Actual‡ intra-assay $\pm(\sigma_{WR})$
1.03	93.1 ± 5.6	85.4 ± 8.4	66.8 ± 7.7
2.59	117.4 ± 1.7	112.9 ± 5.3	105.0 ± 9.7
5.13	119.4 ± 1.4	122.4 ± 2.6	113.7 ± 2.3
10.4	114.6 ± 1.7	115.8 ± 2.0	114.2 ± 3.3
26.0	103.1 ± 1.7	104.5 ± 2.9	104.5 ± 2.1
51.9	93.5 ± 1.7	94.8 ± 1.2	97.4 ± 3.2
104	83.7 ± 1.5	86.2 ± 2.8	89.4 ± 2.7
260	75.6 ± 1.4	77.5 ± 2.2	82.0 ± 3.7
521	73.8 ± 1.3	74.1 ± 3.5	80.4 ± 1.5

*Using seven duplicate independent calibrators as described in the text.
†Mean ± standard deviation of 10 simulations.
‡Mean ± standard deviation of six determinations.

Table 5
Comparison of predicted and actual assay performance using weighted regression $y = ax\ln x + bx + c$

Drug added (ng ml^{-1})	Drug found (% of amount added)* Simulations† ($\pm \sigma_{BR}$)	Actual‡ assay ($\pm \sigma_T$)	Actual‡ intra-assay $\pm(\sigma_{WR})$
1.03	98.8 ± 4.2	90.6 ± 6.2	80.8 ± 5.6
2.59	101.6 ± 1.5	96.3 ± 3.7	92.7 ± 7.7
5.13	102.4 ± 1.8	103.7 ± 2.2	97.8 ± 2.1
10.4	102.0 ± 2.1	102.1 ± 1.3	101.5 ± 3.1
26.0	100.0 ± 2.1	100.6 ± 3.0	100.4 ± 2.2
51.9	98.0 ± 1.9	98.4 ± 1.7	100.8 ± 3.7
104	95.4 ± 1.6	97.5 ± 2.4	97.8 ± 3.1
260	96.0 ± 1.1	97.4 ± 2.6	100.4 ± 5.0
521	97.5 ± 1.3	96.7 ± 3.6	100.4 ± 1.8

*Using seven duplicate independent calibrators as described in the text.
†Mean ± standard deviation of 10 simulations.
‡Mean ± standard deviation of six determinations.

(III) The relationship between the total precision (σ_T), intra-assay precision (σ_{WR}) and the inter-assay component of precision (σ_{BR}), i.e. $\sigma_T^2 = \sigma_{WR}^2 + \sigma_{BR}^2$ was only approximate. This was partly due to the small populations involved in the estimations of the standard deviations. However, the large value of the total imprecision observed at low concentrations using unweighted linear regression (Table 2) was predicted, although exaggerated by probable overestimates of the values of σ_{WR} used in the calculations.

(IV) The intuitive choice of a point-to-point calibration, for this assay, was confirmed both by the Monte Carlo simulations and the much more laborious recalculation of the raw data. The weighted regression $y = ax\ln(x) + bx + c$, which was developed in hindsight as a result of these simulations being available, also shows a good fit to the data. Mean values of $a = -0.685$, $b = 7.12$ and $c = 0.587$ were obtained for the coefficients in the six inter-assay validation runs. The actual underestimates at low concentrations compared with the simulations, observed for both routines, probably reflects differences in the small background response between the calibrators and validation samples.

Discussion

The main benefit of Monte Carlo simulations is that the effects of the choice of an individual calibration routine when applied to a real analytical situation are quantified without any real resort to mathematics by the analyst. Although the primary aim of the project was to improve the quality of the reported data, the non-mathematical aspect of the simulations also provide a teaching aid. It

was shown that the error associated with an estimated value could be broken down into four components, namely, bias, intra-assay precision, inter-assay component of precision and other, non-quantifiable, effects. Intra-assay precision is a characteristic of an assay which must be determined experimentally and was used in the database to provide estimates of the bias and inter-assay components of precision, two components of error which are frequently overlooked. Thus the quantifiable errors in a given assay were estimated as a function of concentration by considering a number of individual concentrations over the assay range. It is important to note that these were chosen independently of the calibrators and the calibration routine under investigation. In the example given Monte Carlo simulations were used to select the most appropriate calibration function for the assay, but equally well the number and distribution of the calibrators, the weighting factors used in the regressions and the effect of outliers could have been assessed.

The approach taken in this study was fundamentally different from that generally taken in that the total error associated with a reported value was assessed. Assays are often validated by analysis of a large number of calibrators and, once the parameters for the calibration function have been determined, the accuracy and precision are estimated by back calculation. However, on a routine basis a much smaller number of calibrators are used. It is then that the limitations of inappropriate weighting factors used in the calibration regression can arise producing large errors associated with the inter-assay component of precision which was not assessed by this method. Another technique is to monitor the residuals from the calibrators to the individual calibration curves and to apply different weighting factors to the regression until the best fit is obtained. This implies that the characteristics of the assay are changing on a daily basis when perhaps what is really happening is that atypical distributions are occurring because of normal random variations and small population sizes. Both of these effects can be shown by Monte Carlo simulations.

Construction of curves of the response and precision as functions of concentration to be used as a source of data for the program was felt to have several advantages. These curves were considered as being a real representation of the underlying characteristics of the assay and there was no need to define them in terms of an assumed mathematical function. No undue weighting was given to any individual value, outliers could be eliminated and there was no need to assume that the concentrations were free of errors.

Furthermore, the simulations were designed to duplicate the actual analytical process so that individual calibration techniques can be evaluated using real analyses rather than under ideal conditions.

In the example given, the degree of non-linearity of the response was greater than normally experienced in chromatographic drug assays. However, this served to illustrate the power of the technique which showed good agreement between the predicted and estimated plasma concentrations even when large values of bias were involved. It is worth emphasizing that the performance of the program is dependent upon the quality of the data from the response and precision curves. The most critical curve from which the assay characteristics are taken is the plot of specific response versus concentration (Fig. 3). Usually there is little scatter of the data from the smooth curve constructed by eye and the robustness of the technique can always be tested by construction of several curves. Estimates of the intra-assay precision function are less clearly defined because of the greater scatter of the data (Fig. 4). However, this only becomes important when large values of the inter-assay component of precision are estimated and the error is amplified, as in the example of unweighted linear regression.

Monte Carlo simulations provide a means whereby diverse calibration routines can be quickly evaluated under standard conditions thereby allowing valid comparisons to be made. In routine use, it is envisaged that having performed an initial evaluation, further refinements to the calibration in terms of the numbers, concentrations and distribution of the calibrators would be investigated. Alternatively, if none of the available regression routines were found to be suitable, as in the example given, other regressions could be derived or obtained from the literature. These are then easily inserted into the program for evaluation.

Another benefit of the use of Monte Carlo simulations is that they provide documentary evidence for the rationale behind the choice of

a particular calibration routine for a given assay. This may be of importance in the pharmaceutical industry or in similar situations where data is subject to review by various regulatory authorities.

In the introduction, questions were raised as to the validity of the application of various calibration routines to chromatographic drug assays. Monte Carlo simulations have been shown to provide a means to answer these questions in real analytical situations.

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