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Linear regression for calibration lines revisited: weighting schemes for bioanalytical methods

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Abstract

When the assumption of homoscedasticity is not met for analytical data, a simple and effective way to counteract the greater influence of the greater concentrations on the fitted regression line is to use weighted least squares linear regression (WLSLR). The purpose of the present paper is to stress the relevance of weighting schemes for linear regression analysis and to show how this approach can be useful in the bioanalytical field. The steps to be taken in the study of the linear calibration approach are described. The application of weighting schemes was shown by using a high-performance liquid chromatography method for the determination of lamotrigine in biological fluids as a practical example. By using the WLSLR, the accuracy of the analytical method was improved at the lower end of the calibration curve. Bioanalytical methods data analysis was improved by using the WLSLR procedure. © 2002 Published by Elsevier Science B.V.

Keywords: Linear regression; Heteroscedasticity; Weighting schemes; Bioanalytical methods

1. Introduction

A well-designed and interpreted calibration curve is essential in any analytical methodology. In fact, the quality of bioanalytical data is highly dependent on the quality of the standard curve used to generate it. Analyte concentrations in unknown samples are typically evaluated by using the regression results obtained from calibration curves and although some analytical procedures may require a non-linear calibration approach, linear regression is the most commonly adopted model.

However, the condition of equal variances, termed homoscedasticity, is frequently not met for analytical data. Obviously, when the range in x-values is somewhat larger—usually a concentration range of more than one order of magnitude—it might be expected that the variance of each data point might be quite different [1]. Larger deviations present at larger concentrations tend to influence (weight) the regression line more than smaller deviations associated with smaller concentrations, and thus the accuracy in the lower end of the range is impaired [1–3]. A simple and effective way to counteract this situation is to use weighted least squares linear regression (WLSLR) [1,2,4–7]. The aim of the present paper is to stress the relevance of weighting schemes for linear regression analysis and to show how this approach can be used and be useful in the bioanalytical field. Although statistical considerations are not new for mathematical experts, we believe the present paper may be of great utility for

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practising bioanalysts. The steps to be taken are described and illustrated with a data set obtained during the validation process of a high-performance liquid chromatographic (HPLC) method [8].

2. Simple and weighted linear regression models—background

The objective of a regression analysis is to find a deterministic model which allows a prediction of the values assumed by the dependent variable (y) when independent variables (x_n) are known or fixed. That model defines the kind of relationship between variables. Since the experimental values hardly fit the mathematical model, the methodology of minimising the sum of squares (SS) of the deviations between the data and the assumed model should yield the best estimate of the model parameters. This is called the ''method of least squares'' and the expression to be minimised is:

$$\mathrm{SS} = \sum (y_{\mathrm{observed},i} - y_{\mathrm{predicted},i})^2 \quad (1)$$

In the simple linear regression model, the relationship between variables is established by a straight line, mathematically expressed as $y = a + bx$, where y is the dependent variable (measured with error), x is the independent variable (known without error), a is the y-intercept of the regression equation and b is the slope of the regression equation.

Usually, the simple least squares method considers that, for each value of x, there is a subpopulation of y-values normally distributed, that the means of all the subpopulations of y lie on the same straight line and that all the subpopulations of y-values have equal variances [9].

However, it is very common for the standard deviation (SD) of the measurement to alter with x (heteroscedasticity). In many cases, SD rises proportionally to the concentration, leading to a constant coefficient of variation. Nevertheless, taking into account that random error is caused by noise and noise sources may be a function of signal or concentration or other factors, different behaviours may be observed. Despite observing this, the most common occurrence of heteroscedasticity is an increase of variance as a function of concentration [1,2,4–6,10].

In order to counteract the greater influence of the greater concentrations on the fitted regression line, the weighted least squares linear regression is used.

The expression to be minimised now takes the following form [1]:

$$\mathrm{SS} = \sum \frac{(y_{\mathrm{observed},i} - y_{\mathrm{predicted},i})^2}{\sigma_i^2} \quad (2)$$

where σ_i^2 is the variance of the standard point.

Taking the objective of the WLSLR into consideration, the most appropriate weighting factor, w_i, is the inverse of the variance of the standard point:

$$w_i = \frac{1}{\sigma_i^2} \quad (3)$$

However, this weight is usually impractical, and other empirical weights based on x-variable (concentration) or y-variable (response) may provide a simple approximation of variance [1,2].

3. Performance

3.1. Test of homoscedasticity

The homoscedasticity assumption should be tested in any linear regression analysis. It can be performed by plotting residuals versus concentration [1,2,4,7,11] and by applying an F-test in accordance with the following statistics [12–14]:

$$F_{\mathrm{exp}} = \frac{s_2^2}{s_1^2} \quad (4)$$

$$F_{\mathrm{tab}}\,(f_1, f_2; 0.99)$$

where the experimental F-value is expressed as the ratio between the variances obtained at the lowest (s_1^2) and at the highest (s_2^2) concentration level of the working range, and the tabled F-value is obtained from the F-table at the confidence level of 99% for $f_1 = f_2 = (n - 1)$ degrees of freedom.

If variance is constant over the whole calibration range, residuals will fall more or less randomly around the x-axis and F_{exp} will be lower than F_{tab}.

3.2. Choice of the weighting factor

In the light of the evidence of the heteroscedastic situation, the following step should be the choice of the weighting factor, w_i. As it is not suitable to calculate the inverse of variance in laboratory routine, taking into account the fact that it requires several determinations for each calibration point and a fresh calibration line should be performed each time the method is used, other empirical weights such as $1/x^{1/2}$, $1/x$, $1/x^2$, $1/y^{1/2}$, $1/y$ and $1/y^2$ should be studied.

The best weighting factor is chosen according to a percentage relative error (%RE), which compares the regressed concentration (C_{found}) computed from the regression equation obtained for each w_i, with the nominal standard concentration (C_{nom}):

$$\%\text{RE} = \frac{C_{\text{found}} - C_{\text{nom}}}{C_{\text{nom}}} \times 100 \tag{5}$$

The %RE, evaluated by plotting %RE versus concentration as well as by calculating %RE sum, defined as the sum of absolute %RE values, is a useful and sensitive indicator of goodness of fit in the evaluation of the effectiveness of a weighting factor for WLSLR [1].

The best w_i will be that which gives rise to a narrow horizontal band of randomly distributed %RE around the concentration axis and presents the least sum of the %RE across the whole concentration range.

3.3. Weighted straight line equation

The model parameters (a and b) of the weighted straight line equation can now be estimated. Conversion of the relations for unweighted least squares into their weighted counterparts can easily be done by adding a term w_i to any sum and changing any term n into Σw_i [1,15]. Knowing that estimated values for the a and b parameters for unweighted least squares may be obtained by the following formulas:

$$b = \frac{n \cdot \sum x_i y_i - \sum x_i \cdot \sum y_i}{n \cdot \sum x_i^2 - \left(\sum x_i\right)^2} \tag{6}$$

$$a = \frac{\sum x_i^2 \cdot \sum y_i - \sum x_i \cdot \sum x_i y_i}{n \cdot \sum x_i^2 - \left(\sum x_i\right)^2} \tag{7}$$

estimated values for the a and b parameters of the weighted regression equation can be obtained by the following modified formulas:

$$b = \frac{\sum w_i \cdot \sum w_i x_i y_i - \sum w_i x_i \cdot \sum w_i y_i}{\sum w_i \cdot \sum w_i x_i^2 - \left(\sum w_i x_i\right)^2} \tag{8}$$

$$a = \frac{\sum w_i x_i^2 \cdot \sum w_i y_i - \sum w_i x_i \cdot \sum w_i x_i y_i}{\sum w_i \cdot \sum w_i x_i^2 - \left(\sum w_i x_i\right)^2} \tag{9}$$

where (x_i, y_i) is the ith data pair of n total data pairs and w_i is the weighting factor chosen.

The degree of dependence established between the two variables, expressed by the correlation coefficient (r-value), can be obtained by the following modified formula:

$$r = \frac{\sum w_i \cdot \sum w_i x_i y_i - \sum w_i x_i \cdot \sum w_i y_i}{\sqrt{\sum w_i \cdot \sum w_i x_i^2 - \left(\sum w_i x_i\right)^2} \cdot \sqrt{\sum w_i \cdot \sum w_i y_i^2 - \left(\sum w_i y_i\right)^2}} \tag{10}$$

A generic diagram of the process described is represented in Fig. 1.

4. Practice

To exemplify the procedure described, we selected the intra-day assay data set generated during the validation process of a recently developed HPLC method for the determination of lamotrigine in plasma [8]. Regression parameters were obtained by introducing the respective formulas on a Microsoft Excel® worksheet.

4.1. Test of homoscedasticity

The plot of residuals versus concentration obtained for the intra-day assay chromatographic data is shown in Fig. 2. The residual plot clearly showed that error was not randomly distributed around the concentration axis. The F-test (Table 1) also revealed a significant difference between the variances, when the experimental F-value ($F_{\text{exp}} = 1.45 \times 10^5$) was compared to the tabled one ($F_{\text{tab}} = 15.98$).

There was evidence that variances were significantly different, thus homoscedasticity was not met.

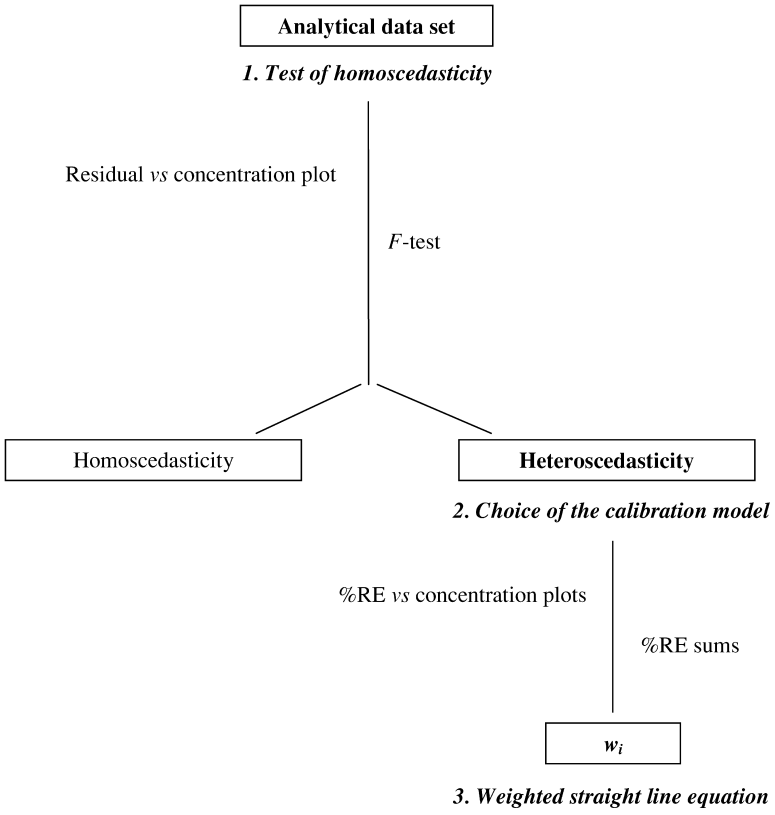

Fig. 1. Generic diagram of weighting schemes for linear regression analysis.

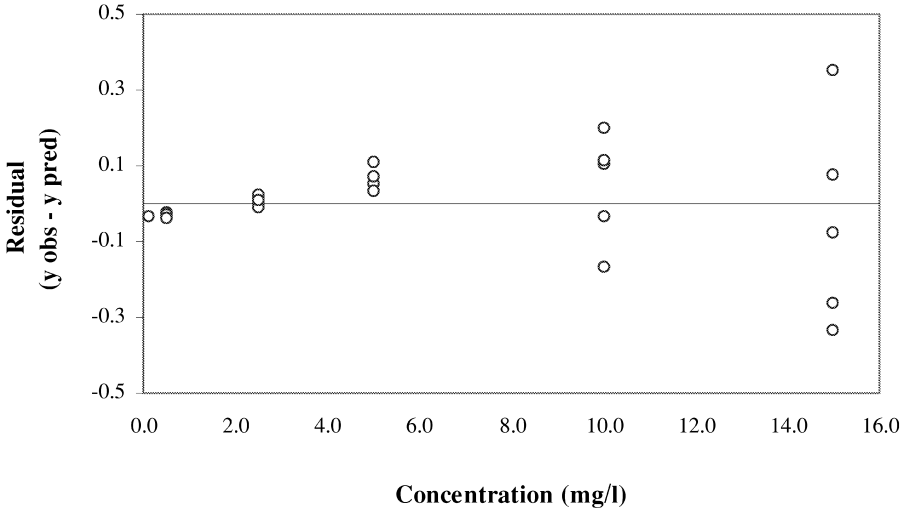

Fig. 2. Residuals plotted against lamotrigine concentrations for the validation intra-day assay.

Table 1
Test of homogeneity of variances (s^2): F-test

Standard (mg/l)	A	$A_{I.S.}$	Response ($A/A_{I.S.}$)	s^2
0.1	75381	3373583	0.022	5.25×10^{-7}
	62834	2744443	0.023	
	68050	2863618	0.024	
	73212	3190782	0.023	
	75930	3144582	0.024	
15.0	12209191	3244845	3.763	7.60×10^{-2}
	12953689	2911392	4.449	
	11682576	2903904	4.023	
	10940196	2852877	3.835	
	11147470	2672128	4.172	

$$F_{exp} = \frac{s^2_{15.0}}{s^2_{0.1}} = \frac{7.60 \times 10^{-2}}{5.25 \times 10^{-7}} = 1.45 \times 10^{+5}$$

$F_{tab}(4, 4, 0.99) = 15.98$

A, lamotrigine peak area; $A_{I.S.}$, internal standard peak area.

4.2. Choice of the weighting factor

The %RE plots for unweighted (model 1) and weighted (models 2–7) regressions of the lamotrigine intra-day assay data across the whole concentration range are shown in Fig. 3. Model 1 clearly underestimated the concentrations in the lower range of the calibration curve, near the limit of quantification (LOQ). Models 4 and 7 presented the best %RE distribution scatter at the lower end of the calibration curve.

The regression parameters of the calibration curve generated for each weighting factor and the respective sums of the relative errors are summarised in Table 2. The weighting factor $1/x^2$ (model 7) produced the least sum for this data set providing the most adequate approximation of variance. Thus, the $1/x^2$ weighting factor was chosen.

4.3. Weighted straight line equation

For the intra-day assay data the calibration curve obtained with the weighting factor $1/x^2$ was $y = 0.2804x - 0.0049$, with $r = 0.999$. The accuracy of the data, expressed by bias value, was evaluated across the whole concentration range using weighted (model 7) and unweighted (model 1) linear regression. The results are shown in Table 3.

5. Discussion

Unlike pharmaceutical analysis, the concentration range in bioanalytical methods is usually dynamic and broad, presenting three or more orders of magnitude, in order to monitor concentrations effectively [16]. When the range in data values is large, it might be expected that the variance of each data point might be quite different. In the present case, the concentration data ranged between 0.1 and 15.0 mg/l. Therefore, the test of homoscedasticity was carried out.

Residual plots can be used to evaluate the need for weighting when unweighted LSLR is applied. If the data adequately fit the linear model, then the residuals should be randomly distributed in a horizontal band centred on the concentration axis [2]. In this study, the residual plots for unweighted LSLR clearly showed that the residuals were not randomly distributed around the concentration axis. Instead, an increase in variance as a function of concentration was observed. This need of weighting LSLR was confirmed with the results of the F-test.

The best weighting factor was chosen taking into account either the plots or the sums of the %RE calculated for each weighting factor. In the present study, the $1/x^2$ weighting factor produced the smallest %RE sum, and the most random distribution around the x-axis at the lower end of the calibration

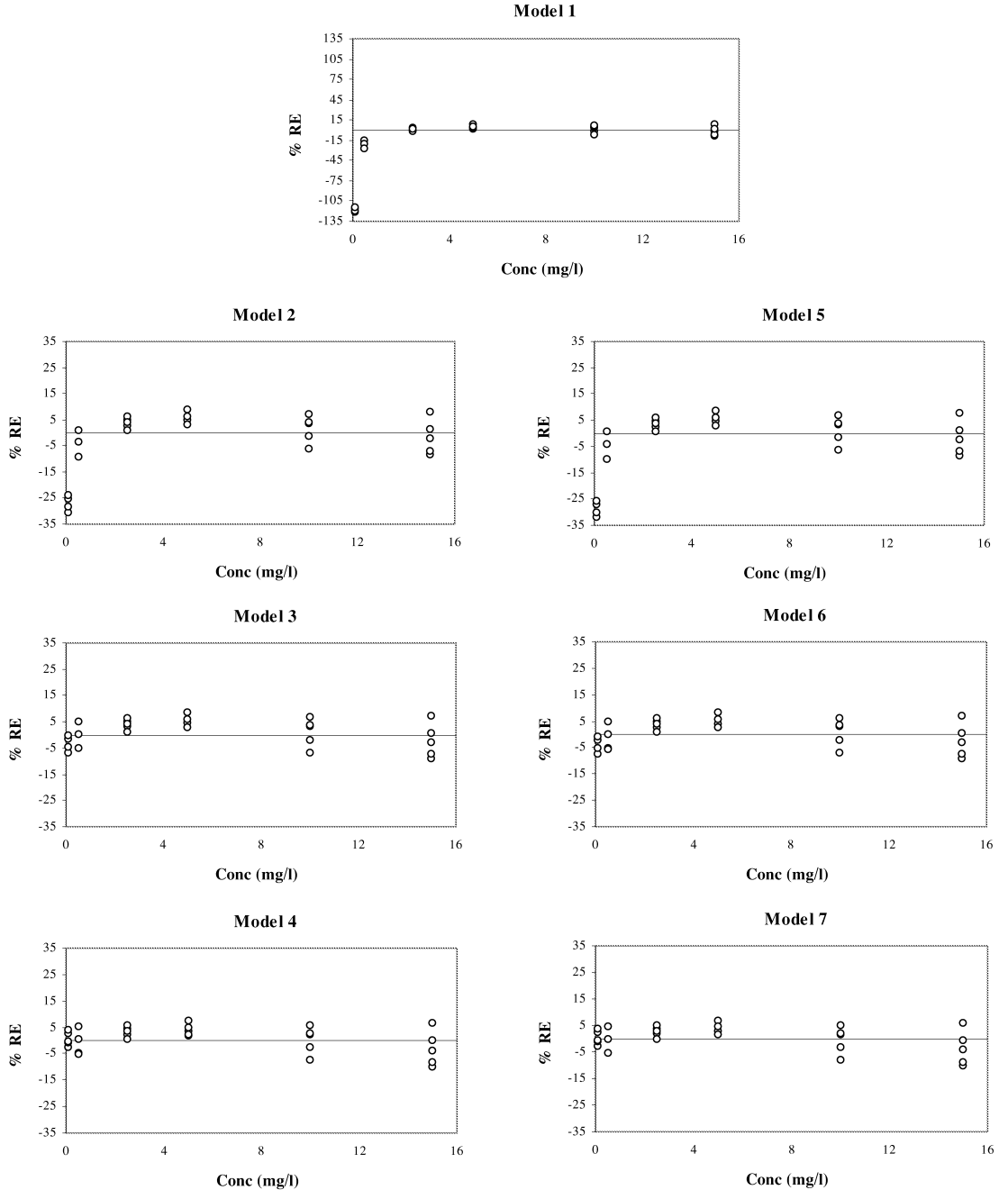

Fig. 3. Percentage of relative error (%RE) versus concentration obtained for model 1 ($w_i = 1$), model 2 ($w_i = 1/y^{1/2}$), model 3 ($w_i = 1/y$), model 4 ($w_i = 1/y^2$), model 5 ($w_i = 1/x^{1/2}$), model 6 ($w_i = 1/x$) and model 7 ($w_i = 1/x^2$).

Table 2
Regression parameters of the calibration curve generated for each weighting factor (w_i) and the respective sum of the relative errors (Σ%RE) for the intra-day assay data; $n=30$

Model	w_i	b	a	r	Σ%RE
1	1	0.2713	+0.0281	0.997	788.90
2	$1/y^{1/2}$	0.2743	+0.0033	0.998	264.31
3	$1/y$	0.2762	−0.0034	0.998	133.48
4	$1/y^2$	0.2789	−0.0048	0.998	116.47
5	$1/x^{1/2}$	0.2747	+0.0037	0.998	271.34
6	$1/x$	0.2770	−0.0033	0.998	133.71
7	$1/x^2$	0.2804	−0.0049	0.999	112.38

curve (Table 2 and Fig. 3). Additionally, as happens with the majority of bioanalytical methods [10], the SD of the response was proportional to the concentration (Fig. 2). According to all these circumstances, the chosen weighting factor was $1/x^2$.

In this work, the calibration model was chosen during validation. Although the choice of the calibration model is currently normally included in the pre-validation phase [17,18], it has been more recently recommended to include it during the validation procedure, by using all validation samples and individual calibration curves in several batches in order to simulate the real conditions of routine analysis [19].

The comparison study between the weighted least squares procedure and the conventional least squares calibration revealed useful improvements in accuracy, which was particularly evident at the lower end of the range, as expected. Percentage bias was considerably greater than acceptable limits of ±20% [20], when simple least squares regression was used, especially at the lower end of the calibration range (Table 3). Consequently, weighted linear regression analysis was able to show a lower limit of quantification (LOQ), the lowest concentration on the standard curve that can be measured with acceptable accuracy and precision [20]. Szabo et al. also referred to a reduction of LOQ by using weighted linear analysis [3]. A broad linear dynamic calibration range could be used by this means, with a higher degree of accuracy [1,2], which may contribute to improve precision in laboratorial routine analysis [4]. This is of the utmost importance, taking into account that the accurate quantification of low serum concentrations versus time is particularly relevant in pharmacokinetic and pharmacodynamic studies.

Although linear regression is the most frequently used approach for determining a best-fit calibration line, providing a most efficient way to fit experimental data to an appropriate model, it should be assured that correctness of the mathematical model is assumed. A more complete analysis of the regression approach (WLSLR) should be considered, taking into account the error pattern of the data. Although WLSLR is more complex and laborious than ordinary linear regression, involving the use of statistical tests and mathematical operations, it should be performed in order to obtain more realistic results.

In addition, this paper brings out an issue referred to but not specified in the most recent FDA guidelines for bioanalytical methods validation [21]. According to these guidelines, the ''selection of weighting and use of a complex regression equation should be justified''. The present paper may contribute to

Table 3
Comparison of the accuracy obtained by using unweighted ($w_i=1$) and weighted ($w_i=1/x^2$) linear regression in lamotrigine assay validation[a]

Nominal concentration (mg/l)	% Bias	
	Model 1 Unweighted ($w_i=1$) $y=0.2713x-0.0281$	Model 7 Weighted ($w_i=1/x^2$) $y=0.2804x-0.0049$
0.1	−117.99	+0.28
0.5	−23.38	−2.33
2.5	+1.31	+2.73
5.0	+4.50	+3.46
10.0	+1.64	−0.48
15.0	−1.21	−3.63

[a] Values represent %bias obtained from five replicates; $\%\text{bias}=(C_{\text{mean}}-C_{\text{nominal}})/C_{\text{nominal}}\times 100$.

the justification required by the regulators, as it shows when, why and how to use weighting schemes for practising bioanalysts.

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