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Non-linear heteroscedastic regression model for determination of methotrexate in human plasma by high-performance liquid chromatography

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Abstract

Generalized least squares regression with variance function estimation was used to derive the calibration function for measurement of methotrexate plasma concentration and its results were compared with weighted least squares regression by usual weight factors and also with that of ordinary least squares method. In the calibration curve range of 0.05 to 100 μ*M*, both heteroscedasticity and non-linearity were present therefore ordinary least squares linear regression methods could result in large errors in the calculation of methotrexate concentration. Generalized least squares regression with variance function estimation worked better than both the weighted regression with the usual weight factors and ordinary least squares regression and gave better estimates for methotrexate concentration.

Keywords: Weighted least squares regression; Variance function estimation; Methotrexate

1. Introduction

A completely validated, accurate and reproducible bioanalytical method is an important requirement in pharmacokinetic and biopharmaceutical studies. The quality of bioanalytical data is highly dependent on the quality of calibration model used to generate the standard curve; therefore, the choice of an appropriate calibration model is necessary for reliable quantification. However, unlike the pharmaceutical analysis, the concentration range in the bioanalysis test samples (being influenced by many factors such as absorption, distribution, metabolism, excretion, etc.) is dynamic and broad, normally of the order of three or more [1]. Although using two or more standard curves with different calibration ranges is common [2,3], a single standard curve that encompasses the entire dynamic concentration range in a pharmacokinetic study is of great use during routine analysis. Usually, linear models are preferable, but, if needed, the use of non-linear models should be considered [4]. On the other hand, one of the basic assumptions of the ordinary least squares (OLS) regression method (which is usually used for de-

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velopment of a calibration function) is constancy of variance or homoscedasticity for all response values. Many examples in analytical chemistry (including high-performance liquid chromatographic methods) indicate that this assumption is not often fulfilled, i.e. the variability of the response often increases with the response level. In such a situation, some remedial actions like transformation or using weighted least squares regression must be done in order to stabilize the variance of the response and thus heteroscedasticity can be accounted for [1,5,6].

Various weighting schemes have been used to remove the heterogeneity of response variability [1,5–8]. Ordinarily the weights are unknown and must be estimated. If replicates at all design points are available, a simple and common approach is to calculate the variance of the responses at each concentration level and choose the weighting factor to be inversely proportional to sample variance. In case of a small number of replicates, this approach can be unreliable. It has been recommended to use at least 10 replicates for the calculation of sample variance that may be time-consuming and expensive. Since variability usually changes smoothly with the response level, it is reasonable to try to find a relationship between responses (or concentration) and error terms. If a sensible relationship (a so-called variance function) can be found, knowledge about the response variance will be incorporated into fitting [5]. Different types of variance function and methods for estimation of them have been introduced. Generalized least squares regression is a weighted least squares method with estimated weights (rather than known weights). In this method information about the variance function is incorporated into the fitted model [5,8].

Methotrexate is a folic acid antagonist, which has been widely used in the treatment of neoplastic and non-neoplastic diseases. Various methods including high-performance liquid chromatography have been used to determine methotrexate concentration in biological fluids for pharmacokinetic studies and therapeutic drug monitoring purposes [2,3,10–20]. Most of the time, it is necessary to quantify methotrexate over a wide concentration range in plasma or serum after high-dose infusion of this drug. This fact can result in heteroscedasticity of response, so that the ordinary least squares regression methods cannot be used [1,5,6]. In the majority of the published HPLC methods for determination of methotrexate concentration, ordinary linear least squares regression has been used for construction of the calibration curve [10–14,16]. In some other methods, the concentration range was divided to low and high concentration regions [2,3,18,19]. There are also other studies that used ordinary linear least squares regression to define the calibration curve for high-performance liquid chromatographic determination of methotrexate over a very wide concentration [15,21]. The use of OLS in these situations could lead to inaccurate estimation of methotrexate concentration. Cociglio et al. developed a HPLC method and used a bi-logarithmic transformation to define the calibration model and deal with the heteroscedasticity of methotrexate peak area [17]. A few other methods used weighted least squares linear regression for constructing standard curves [3,20].

The aim of the present study was to define a calibration function for determination of methotrexate in human plasma by a HPLC method over a 2000-fold methotrexate concentration range (0.05 to 100 μM). In this regard it was shown that the simple linear equation is not a suitable model. To do this, statistical methods such as linear and non-linear weighted least squares regression and generalized least squares regression (GLS) were used and their results were compared [5,6,9]. In order to select the best model, standard statistical tests for checking the validity and fitness of the models were used [22,23].

2. Experimental

The data of an assay validation for the quantitation of methotrexate in plasma of patients receiving high-dose methotrexate infusions were used in this study. The determination of methotrexate was done by a simple high-performance liquid chromatographic method. To each 225 μl of plasma samples, 25 μl of *p*-aminoacetophenone (10 μg/ml) were added as internal standard. The samples were then deproteinized using 40 μl of trichloroacetic acid 2 *M* in ethanol. After vortex mixing and centrifugation at 10 000 *g*, the supernatant was directly injected onto a Eurospher-100 octadecylsilane column (4 I.D.×125 mm, particle size 5 μm). The mobile phase consisted of phosphate buffer (pH 3.9) and acetonitrile (87:13) and was delivered at the rate of 1 ml/min. The UV

detection was done at 307 nm and ambient temperature. The retention times of methotrexate and internal standard were 5.6 and 8.4 min, respectively. The mean recovery of methotrexate was 70% and that of *p*-aminoacetophenone 78%.

In total, eight standard curves in human blank plasma in the range of 0.05 to 100 μ*M* (11 levels of methotrexate concentration) from independently spiked samples were analyzed. The peak area ratio of methotrexate to internal standard was considered as the response. Data analysis was conducted on the pooled data (totally 88 data points) using S-Plus and SPSS statistical packages. *Levene*'s test was used in order to check for the presence of heteroscedasticity in the response data [24]. Because of the wide concentration range of methotrexate, various types of models (defining the relationship of peak area ratio and methotrexate concentration) and weighting schemes were considered. The models were:

A: $y = ax + b$

B: $y = ax^2 + bx + c$

C: $y = ax^b$

D: $y = ax^b + c$

where y is peak area ratio of methotrexate to internal standard and x is methotrexate concentration and a, b and c are parameters of the models. Weighting factors (w) were $1/x$, $1/x^2$, $1/y$, $1/y^2$, and $1/\sqrt{x}$. In addition, generalized least squares regression with different variance function was used to fit models to data. The variance functions were:

(a) $\text{var}(y_i) = \sigma^2(\theta_1 + x_i^{\theta_2})^2$; $w_i = \dfrac{1}{(\theta_1 + x_i^{\theta_2})^2}$

(b) $\text{var}(y_i) = \sigma^2(\theta_1 + y_i^{\theta_2})^2$; $w_i = \dfrac{1}{(\theta_1 + y_i^{\theta_2})^2}$

(c) $\text{var}(y_i) = \sigma^2 x_i^{2\theta}$; $w_i = \dfrac{1}{x_i^{2\theta}}$

(d) $\text{var}(y_i) = \sigma^2 y_i^{2\theta}$; $w_i = \dfrac{1}{y_i^{2\theta}}$

(e) $\text{var}(y_i) = \sigma^2 e^{2\theta x_i}$; $w_i = \dfrac{1}{e^{2\theta x_i}}$

(f) $\text{var}(y_i) = \sigma^2 e^{2\theta y_i}$; $w_i = \dfrac{1}{e^{2\theta y_i}}$

in which x_i and y_i are concentration and peak area ratio at the ith design points, respectively, σ^2 is an unknown scale factor for variance; $v\text{ar}(y_i)$ is variance of response at y_i and w_i is the corresponding weight factor using the estimated variance function. θ, θ_1 and θ_2 are unknown parameters to be estimated during the variance function estimation.

To select the best variance function in generalized least squares regression the log-likelihood values were compared. The summary of notations used for models fitted to data is given in Table 1.

The method proposed by Tse was used for model selection. After the general procedure of regression

Table 1
Notations for calibration models with different equations and weighting schemes

Model notation	Equation	Weight
A1	$y = ax + b$	1
A2	$y = ax + b$	$1/x$
A3	$y = ax + b$	$1/x^2$
A4	$y = ax + b$	$1/y$
A5	$y = ax + b$	$1/y^2$
A6	$y = ax + b$	$1/\sqrt{x}$
B1	$y = ax^2 + bx + c$	1
B2	$y = ax^2 + bx + c$	$1/x$
B3	$y = ax^2 + bx + c$	$1/x^2$
B4	$y = ax^2 + bx + c$	$1/y$
B5	$y = ax^2 + bx + c$	$1/y^2$
B6	$y = ax^2 + bx + c$	$1/\sqrt{x}$
C1	$y = ax^b$	1
C2	$y = ax^b$	$1/x$
C3	$y = ax^b$	$1/x^2$
C4	$y = ax^b$	$1/y$
C5	$y = ax^b$	$1/y^2$
C6	$y = ax^b$	$1/\sqrt{x}$
D1	$y = ax^b + c$	1
D2	$y = ax^b + c$	$1/x$
D3	$y = ax^b + c$	$1/x^2$
D4	$y = ax^b + c$	$1/y$
D5	$y = ax^b + c$	$1/y^2$
D6	$y = ax^b + c$	$1/\sqrt{x}$
Aa	$y = ax + b$	$1/(0.1270 + x_i^{1.2002})^2$
Ab	$y = ax + b$	$1/(0.0153 + y_i^{1.1706})^2$
Ba	$y = ax^2 + bx + c$	$1/(0.0677 + x_i^{1.0202})^2$
Bb	$y = ax^2 + bx + c$	$1/(0.0029 + y_i^{0.9121})^2$
Ca	$y = ax^b$	$1/(0.1855 + x_i^{1.0119})^2$
Cb	$y = ax^b$	$1/(0.0151 + y_i^{0.9005})^2$
Da	$y = ax^b + c$	$1/(0.0588 + x_i^{0.9909})^2$
Db	$y = ax^b + c$	$1/(0.0033 + y_i^{0.8977})^2$

modeling or doing generalized least squares regression to fit the candidate models to the data, the adequacy of the models was assessed by the lack-of-fit test and the significance of model parameters. Those models with significant lack of fit test or non-significance of the parameters were eliminated. *R*-squared values were calculated for the remaining models. The ratio of the R^2 to R^2_{max} (maximum observed R^2 among different models fitted to data) was calculated for each model and called *r*. The models with *r*-values less than a predetermined value were rejected. The recommended value for this ratio was 0.8 as proposed by Tse et al. Among the remaining models, the best model was identified by comparing the MSEs (mean squared prediction error) of the estimated samples for given target responses. These target responses were arbitrarily selected to cover the entire range of concentration. Here the selection of y_0s was done so that the expected x_0 values were equal to the concentrations used for construction of the calibration curve (i.e. 0.05, 0.1, 0.25, etc.). Calculation of bias and MSE at various values of y_0 was done using the model parameters and residual sum of the squares (SSE) as explained by Tse et al. [22]. The best model was considered as the one with the smallest MSEs [22].

3. Results and discussion

The homogeneity of response variance at different levels of methotrexate concentration was rejected through *Levene's test* ($P<0.00001$). This test is less sensitive to departure from normality than the Bartlett test [24]. The same result could be obtained when a one-tailed *F*-test was used for comparison of response variance between highest and lowest concentrations of the calibration curve as proposed by ISO (International Organization for Standardization) [5]. Also, examination of the residual plots of various models without any weighting factor confirmed the heterogeneity of variance (Fig. 1). This fact led to the use of the usual weighting plans that have been already proposed in such situations. Therefore, each model was fitted again to the pooled data using weighted linear (or non-linear) least squares regression.

The estimated parameters and the results of lack of fit test for models fitted by weighted least squares regression method to data are shown in Table 2.

For most of the models the biases of the estimates are relatively small in comparison with their corresponding MSE values (Table 3). This suggests that the dominant component of the MSE is the variance of the estimate. Among the unweighted models, the quadratic equation (B1) showed the least mean of both bias and MSE, but for the lower end of the calibration range it is not the case, that is the methotrexate concentration estimate had relatively large bias and MSE at values near the quantitation limit for this model. On the other hand, non-significance of two parameters of this model confirmed that it is overparametrized. Similarly, comparison of models C1 and D1 showed that model redundancy due to not-significant parameters led to a great increase in MSE and bias. The large difference between the point estimate of concentration at $y_0 = 0.0078$ for these two models indicates that the validity of models would affect the MSE [22]. According to the results, most of the models should be discarded either because of their non-significance of parameters or failing the lack-of-fit test. The maximum observed *R*-squared value (R^2_{max}) was 0.9927, therefore all the models had *r* values greater than 0.8. Out of 24 different equations fitted with weighted least squares regression using various weighting factors, only models C1, C4 and C6 showed significance of all parameters and their lack of fit tests were not significant at $P=0.05$ (Table 2). All these three models had rather good and very close *R*-squares and showed acceptable MSE and bias at the lower end of the calibration curve, but model C6 had the minimum MSE and bias for the upper limit of the range and also the least values for the mean bias and MSE of the entire range.

Reconsideration of Studentized residual plots for all of the four types of models, especially for the above three models, showed that the use of these weight factors could not lead to stabilization of variance even for the model C6 (Fig. 1) as it could be seen from the wedge shape of the residual pattern. It seemed that these weighting factors were not properly chosen. Generalized least squares regression with variance function estimation was conducted on the data in order to get better estimation of both model parameters and methotrexate concentration.

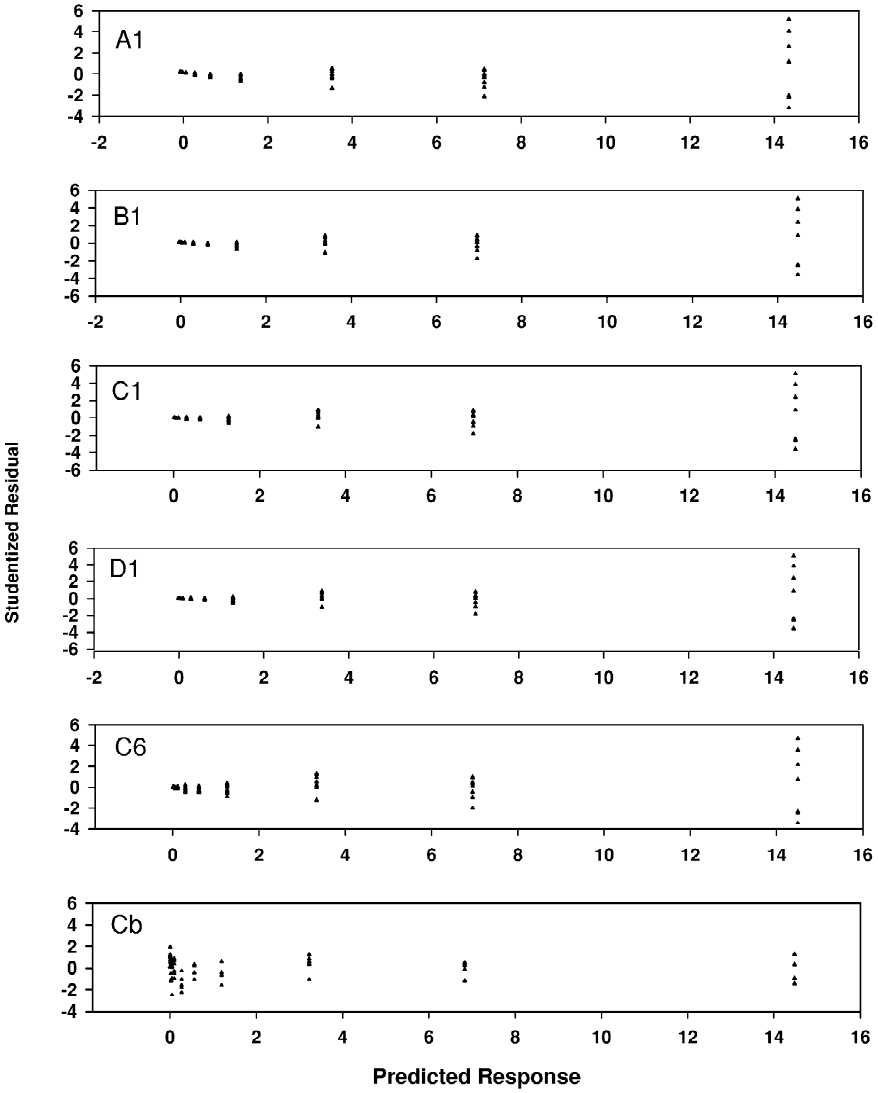

Fig. 1. Studentized residual plots for some of the models fitted to data.

The models with the variance functions (a) and (b) had the greatest log-likelihood values among the other models (Table 4), thus in each class of models these two variance functions (and hence two new weighting factors) were used and the results were considered for further assessment. The statistical results of generalized least squares regressions using these two variance functions are summarized in Table 5.

According to Table 5, when concentration (x_i) was considered as the covariate of variance function, the models failed the lack of fit test. Again, linear and quadratic models had not-significant parameters and should be eliminated. Between models Cb and Db, the former values for bias and MSE of concentration estimate were smaller than that of the latter. Although all the parameters of model Db are significant, the greater number of parameters for this model results in larger values of MSE (Table 3). Thus, the simpler model was chosen.

Table 2
Summary of the estimated parameters and results of lack-of-fit test for models fitted to data through weighted least squares regression using usual weight factors

	Model											
	A1	A2	A3	A4	A5	A6	B1	B2	B3	B4	B5	B6
Parameter estimates[a]	*a*: 0.1443 (0.0016) ($P<0.0001$) *b*: −0.0875 (0.0542) ($P=0.1097$)	*a*: 0.1398 (0.0256) ($P<0.0001$) *b*: −0.0066 (0.0580) ($P=0.9095$)	*a*: 0.1157 (0.1230) ($P=0.3495$) *b*: 0.0004 (0.0179) ($P=0.9825$)	*a*: 0.1382 (0.0095) ($P<0.0001$) *b*: −0.0078 (0.0196) ($P=0.6906$)	*a*: 0.1064 (0.0138) ($P<0.0001$) *b*: 0.0000 (0.0022) ($P=0.9984$)	*a*: 0.1425 (0.0093) ($P<0.0001$) *b*: −0.0242 (0.0972) ($P=0.8039$)	*a*: 0.0001 (0.0001) ($P=0.0724$) *b*: 0.1345 (0.0056) ($P<0.0001$) *c*: −0.0421 (0.0590) ($P=0.4772$)	*a*: 0.0002 (0.0007) ($P=0.7912$) *b*: 0.1262 (0.0570) ($P=0.0295$) *c*: −0.0037 (0.0591) ($P=0.9504$)	*a*: 0.0005 (0.0038) ($P=0.8895$) *b*: 0.1037 (0.1516) ($P=0.4975$) *c*: 0.0013 (0.0190) ($P=0.9464$)	*a*: 0.0002 (0.0003) ($P=0.4396$) *b*: 0.1242 (0.0204) ($P<0.0001$) *c*: −0.0046 (0.0200) ($P=0.8202$)	*a*: 0.0006 (0.0005) ($P=0.2267$) *b*: 0.0969 (0.0158) ($P<0.0001$) *c*: −0.0008 (0.0023) ($P=0.7293$)	*a*: 0.0001 (0.0003) ($P=0.6638$) *b*: 0.1314 (0.0270) ($P<0.0001$) *c*: −0.0139 (0.1001) ($P=0.8898$)
R^2	0.9901	0.9892	0.9435	0.9881	0.9340	0.9916	0.9904	0.9916	0.9642	0.9910	0.9570	0.9924
Lack of fit												
F-ratio	0.5142	5.5004	14.2905	6.3519	16.2190	12.9567	0.1960	2.7641	6.6642	3.1574	8.4442	0.7630
P-value	0.8601	0.0000	0.0000	0.0000	0.0000	0.0000	0.9907	0.0097	0.0000	0.0038	0.0000	0.6361
	C1	C2	C3	C4	C5	C6	D1	D2	D3	D4	D5	D6
	a: 0.1133 (0.0110) ($P<0.0001$) *b*: 1.0532 (0.0219) ($P<0.0001$)	*a*: 0.1054 (0.0962) ($P=0.2764$) *b*: 1.0701 (0.2200) ($P<0.0001$)	*a*: 0.1124 (0.1164) ($P=0.3366$) *b*: 1.0414 (0.3792) ($P=0.0073$)	*a*: 0.1032 (0.0325) ($P=0.0021$) *b*: 1.0736 (0.0767) ($P<0.0001$)	*a*: 0.1034 (0.0121) ($P<0.0001$) *b*: 1.0584 (0.0488) ($P<0.0001$)	*a*: 0.1101 (0.0538) ($P=0.0439$) *b*: 1.0597 (0.1130) ($P<0.0001$)	*a*: 0.1157 (0.0139) ($P<0.0001$) *b*: 1.0488 (0.0263) ($P<0.0001$) *c*: −0.0194 (0.0636) ($P=0.7609$)	*a*: 0.1047 (0.1032) ($P=0.3133$) *b*: 1.0717 (0.2364) ($P<0.0001$) *c*: 0.0012 (0.0619) ($P=0.9842$)	*a*: 0.0940 (0.1726) ($P=0.5875$) *b*: 1.1018 (0.5969) ($P=0.0684$) *c*: 0.0034 (0.0225) ($P=0.8808$)	*a*: 0.1026 (0.0355) ($P=0.0048$) *b*: 1.07491 (0.0835) ($P<0.0001$) *c*: 0.0009 (0.0212) ($P=0.9679$)	*a*: 0.0905 (0.0171) ($P<0.0001$) *b*: 1.1091 (0.0697) ($P<0.0001$) *c*: 0.0029 (0.0026) ($P=0.2732$)	*a*: 0.1110 (0.0588) ($P=0.0627$) *b*: 1.0580 (0.1215) ($P<0.0001$) *c*: −0.0041 (0.1045) ($P=0.9692$)
R^2	0.9905	0.9927	0.9547	0.9925	0.9539	0.9925	0.9905	0.9927	0.9766	0.9925	0.9717	0.9927
Lack of fit												
F-ratio	0.1519	0.9718	9.7586	0.8823	8.7472	0.3893	0.1601	1.0742	0.9978	0.9818	2.3389	0.4293
P-value	0.9977	0.4698	0.0000	0.5449	0.0000	0.9368	0.9972	0.3914	0.4491	0.4619	0.0218	0.9154

[a] Standard error of parameter estimate is given in the first parenthesis below the parameter value.

Table 3
Bias and MSE values for estimated concentrations with various models at selected target responses

Model	$y_0 = 0.0078$[a]			$y_0 = 15.2443$			Mean for the whole range[c]	
	$\hat{x}_0$[b]	Bias ($\times 10^{-6}$)	MSE ($\times 10^{-4}$)	$\hat{x}_0$	Bias ($\times 10^{-6}$)	MSE ($\times 10^{-4}$)	Bias ($\times 10^{-6}$)	MSE ($\times 10^{-4}$)
A1	0.66	−1924.000	1359.800	106.22	11 000.000	10 873.100	136.846	2279.200
A2	0.10	−43.370	29.230	109.12	225.800	230.700	1.660	47.967
A3	0.06	−10.330	6.951	132.76	66.900	82.225	2.595	14.355
A4	0.11	−345.100	232.600	110.36	1822.000	1882.100	17.455	386.691
A5	0.07	−969.600	652.900	143.26	6919.000	99 250.400	4.823	1601.030
A6	0.22	−2.534	172.000	107.16	1304.000	1310.300	6.445	277.373
B1	0.37	−1051.000	201.000	104.94	4110.000	1460.000	−23.122	382.109
B2	0.09	−180.900	33.074	104.00	471.100	171.700	−29.937	54.058
B3	0.06	−38.140	6.486	98.24	24.430	10.611	−14.041	7.242
B4	0.10	−1415.000	258.400	104.47	36.460	1325.800	−239.425	419.155
B5	0.07	−3593.000	598.300	97.58	1706.000	768.000	−1383.200	622.300
B6	0.17	−1154.000	214.800	104.70	3858.000	1381.400	−101.399	384.473
C1	0.08	5.762	0.006	105.06	11 000.000	15 253.400	1864.293	1780.164
C2	0.09	0.007	0.001	104.39	132.500	192.900	22.223	22.507
C3	0.08	0.002	0.000	111.50	37.070	50.509	6.218	5.895
C4	0.09	0.654	0.007	104.90	1278.000	1900.100	214.348	221.694
C5	0.09	1.364	0.001	111.91	2663.000	3953.000	446.666	461.205
C6	0.08	0.058	0.006	104.86	1142.000	1592.200	210.631	204.379
D1	0.25	−2377.000	2037.500	105.11	12 000.000	15 348.300	57.091	3273.209
D2	0.08	−27.000	2575.950	104.33	140.400	192.500	1.016	41.223
D3	0.06	−3.911	4.316	101.35	20.020	30.671	0.093	6.746
D4	0.08	−272.200	263.800	104.85	1429.000	1999.100	12.445	425.182
D5	0.07	−330.900	378.400	101.77	1711.000	2722.100	10.700	595.064
D6	0.12	−243.400	217.500	104.90	1265.000	1634.200	8.927	348.900
Aa	0.07	−2.321	2.525	140.87	10.170	13.710	−0.230	3.498
Ab	0.08	−371.100	403.900	145.68	1697.000	2355.200	−25.100	576.627
Ba	0.07	−18.250	3.128	98.56	12.890	5.554	−6.589	3.584
Bb	0.07	−1972.000	337.400	99.80	1497.000	619.700	−700.946	386.764
Ca	0.09	0.004	0.000	104.01	6.846	10.340	1.148	1.206
Cb	0.09	0.004	0.000	104.90	7.189	11.176	7.350	1.304
Da	0.07	−3.019	3.277	102.73	15.500	23.487	0.080	5.142
Db	0.06	−182.100	202.000	102.77	949.800	1483.400	7.295	320.845

[a] y_0, selected target response.
[b] $\hat{x}_0$, estimated concentration at y_0.
[c] Mean of bias and MSE values for all the estimated concentrations.

Though the point estimates of methotrexate concentration were close for models Cb and C6, MSE and bias of model Cb were much smaller than model C6 over the entire range of the standard curve. On the other hand, application of generalized least squares regression with the weight factor estimate as $1/(\theta_1 + y^{\theta_2})^2$ could lead to stabilization of variance in model Cb (Fig. 1). Therefore the final equation for determining the concentration of methotrexate in human plasma over this range of concentration would be as follows:

$$\text{Cb: } y = 0.0987C^{1.0832};$$

$$\left(\text{var}\,(y_i) = \sigma^2(0.0151 + y_i^{0.9005})^2;\right.$$

$$\left. w_i = \frac{1}{(0.0151_1 + y_i^{0.9005})^2}\right)$$

Thus the concentration could be calculated using the log-transformation of this equation.

In general, weighted least squares regression

Table 4
Summary of different estimated variance functions

	Model											
	Aa	Ab	Ac	Ad	Ae	Af	Ba	Bb	Bc	Bd	Be	Bf
Parameters of variance function	θ_1: 0.1270 θ_2: 1.2002	θ_1: 0.0153 θ_2: 1.1706	θ: 0.9461	θ: 0.9540	θ: 0.0417	θ: 0.2813	θ_1: 0.0677 θ_2: 1.0203	θ_1: 0.0029 θ_2: 0.9121	θ: 0.9116	θ: 0.8851	θ: 0.0672	θ: 0.4428
Log-likelihood value	131.9	128.7	128.5	126.5	59.8	58.5	152.8	150.0	151.1	149.9	77.32	72.6
	Ca	Cb	Cc	Cd	Ce	Cf	Da	Db	Dc	Dd	De	Df
Parameters of variance function	θ_1: 0.1855 θ_2: 1.0120	θ_1: 0.0151 θ_2: 0.9005	θ: 0.8176	θ: 0.8071	θ: 0.0569	θ: 0.3885	θ_1: 0.0588 θ_2: 0.9909	θ_1: 0.0033 θ_2: 0.8977	θ: 0.9029	θ: 0.8687	θ: 0.0579	θ: 0.3931
Log-likelihood value	163.9	163.2	156.0	161.6	85.5	80.3	174.9	173.0	172.6	172.7	85.7	80.4

Table 5
Summary of estimated parameters and results of lack-of-fit test for models fitted to data using generalized least squares regression with variance function estimation

	Model							
	Aa	Ab	Ba	Bb	Ca	Cb	Da	Db
Parameter estimates			*a*: 0.0005 (0.0001) ($P<0.0001$)	*a*: 0.0005 (0.0001) ($P<0.0001$)			*a*: 0.0949 (0.0025) ($P<0.0001$)	*a*: 0.0921 (0.0025) ($P<0.0001$)
	a: 0.1082 (0.0027) ($P<0.0001$)	*a*: 0.1046 (0.0025) ($P<0.0001$)	*b*: 0.1058 (0.0026) ($P<0.0001$)	*b*: 0.1031 (0.0027) ($P<0.0001$)	*a*: 0.1018 (0.0021) ($P<0.0001$)	*a*: 0.0987 (0.0022) ($P<0.0001$)	b: 1.0988 (0.0082) ($P<0.0001$)	b: 1.1029 (0.0083) ($P<0.0001$)
	b: -0.0002 (0.0006) ($P=0.7668$)	*b*: -0.0008 (0.0008) ($P=0.3066$)	*c*: 0.0005 (0.0001) ($P=0.3689$)	*c*: 0.0001 (0.0006) ($P=0.8442$)	*b*: 1.0783 (0.0071) ($P<0.0001$)	*b*: 1.0832 (0.0071) ($P<0.0001$)	*c*: 0.0032 (0.0005) ($P<0.0001$)	*c*: 0.0029 (0.0005) ($P<0.0001$)
R^2	0.9500	0.9500	0.9739	0.9724	0.9850	0.9862	0.9851	0.9832
Lack of fit								
F-ratio	13.0515	0.0760	9.4779	0.1037	11.2000	0.1278	8.6702	0.1100
P-value	0.0000	0.9999	0.0000	0.9995	0.0000	0.9988	0.0000	0.9994

methods are superior to ordinary least squares methods. Baumann et al. stated that if the ratio of response standard deviation within the concentration range is greater than 5, the error resulting from ordinary least squares methods will be considerable. They also recommended the use of weighted least squares methods for smaller ratios of standard deviations at highest and lowest concentration. Weighted least squares regression works well if the weights are known [24]. Most of the time some estimations of weights are used. The use of inverse of response variance is advisable only when there are at least 10 replicates for each concentration level [5,24]. There is also an algorithm for weight selection in choosing the standard curve [6]. Recently, application of generalized least squares regression has been considered. In fact, in this method, the variance function is estimated as well as the parameters of the model [5,24,25]. Furthermore, Mulholland et al. have studied limitations of least squares linear calibration. It was shown in their study that a correlation coefficient of 0.999 or greater can hide exceptionally large errors when data come from a non-linear function [26]. This fact has also been shown in our study. Comparison of MSE and bias values established that the magnitudes of prediction errors for linear models were very large in comparison to those of power models.

In most of the HPLC methods that have been described for determination of methotrexate, ordinary linear least squares regression was used for derivation of the calibration curve [10–14,16]. In certain methods the calibration curve was split into lower and upper range [2,3,18,19]. In a few other methods, weighting factors such as $1/x$ or $1/x^2$ were used for the simple linear model [15,21]. None of the published methods used non-linear heteroscedastic models for the measurement of methotrexate. In addition, based on the results of this study, application of the usual weighting factors did not result in stabilization of response variance in our study. In such situations, application of generalized least squares regression could lead to better estimates of model parameters and thus better estimates of drug concentrations. The use of GLS with variance function estimation in this study led to better estimates for model parameters (as could be seen from the standard errors of parameters in Table 5). Among the different variance functions that can be used in generalized least squares regression, the forms (a) or (b) are particularly appealing, since they model the variance of response at small response values with

component θ_1, whereas component y_i^{θ} describes the error relationship at larger response values. A corresponding error behavior is known from several chromatography and capillary electrophoresis methods as stated by Baumann et al. Near the detection limit a constant noise level is prevailing, while at higher concentration the injection errors become dominant [5]. In our study for all of the different types of models, this variance function and so the corresponding weight factors worked better than the others as it could be shown from their log-likelihood values in Table 4.

4. Conclusion

In summary, whenever heteroscedasticity and nonlinearity present concurrently, the use of generalized least squares regression with variance function estimation can lead to better estimates of model parameters and hence better estimation for drug concentrations in test samples than weighted least squares method using the usual weight factors.

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