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Research Article

High-Performance Liquid Chromatographic Determination of Propofol in Human Plasma: Comparison of Different Heteroscedastic Calibration Curve Models

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Abstract
Purpose: The aim of this study was to select the best calibration model for determination of propofol plasma concentration by high-performance liquid chromatography method.
Methods: Determination of propofol in plasma after deproteinization with acetonitrile containing thymol (as internal standard) was carried out on a C_{18} column with a mixture of acetonitrile and trifluoroacetic acid 0.1% (60:40) as mobile phase which delivered at the flow rate of 1.2 mL/minute . Fluorescence detection was done at the excitation and emission wavelengths of 276 and 310 nm, respectively. After fitting different equations to the calibration data using weighted regression, the adequacy of models were assessed by lack-of-fit test, significance of all model parameters, adjusted coefficient of determination ($R^2_{adjusted}$) and by measuring the predictive performance with median relative prediction error and median absolute relative prediction error of the validation data set.
Results: The best model was a linear equation without intercept with median relative prediction error and median absolute relative prediction error of 4.0 and 9.4%, respectively in the range of 10-5000 ng/mL. The method showed good accuracy and precision.
Conclusion: The presented statistical framework could be used to choose the best model for heteroscedastic calibration data for analytes like propofol with wide range of expected concentration.

Introduction

Propofol (2, 6-diisopropylphenol) is a rapid acting anesthetic drug which is commonly used for induction and maintenance of anesthesia and for sedation in intensive care unit patients.[1,2] High clearance and large apparent volume of distribution of this drug make it a good controllable intravenous anesthetic agent.[3]
Pharmacokinetics of propofol has been the subject of several studies. It shows a high degree of inter-individual variability and could be affected by factors such as patient age, sex and genetic polymorphism.[4-11]
A fully validated, accurate and precise method for measurement of propofol in biological fluid is necessary for pharmacokinetic investigations on this drug. Various high-performance liquid chromatography methods with ultraviolet,[12-14] fluorescence,[14-18] mass spectrometry[12,14,19,20] and electrochemical[12] detection have been reported for determination of propofol concentration in biological fluids.
Since the quality of the bio analytical data is completely under the influence of the calibration model, a well-designed and interpreted calibration curve in required for any analytical methodology.[21,22] Although the unknown concentrations of the analytes in biological samples are usually determined using linear calibration equations, in some cases the use of nonlinear models should be considered especially when the concentration range in the test samples is broad.[23,24]
Homoscedasticity or the equality of response uncertainty (or variances) over the entire concentration range is one of the basic assumptions of ordinary least squares regression method that is usually used to derive the calibration equations. However, this condition is not usually fulfilled and weighted regression is used to account for the heteroscedasticity of the measured response. It is clear that when the concentration range is broad, the variances of response values at different levels of concentration might be quite different.[22,25]
Very wide ranges of concentrations have been observed during pharmacokinetic studies of propofol.[5,7,9,26] In some studies, two calibration curves were constructed for lower and higher ranges of propofol concentration.[16] Although this approach is common,[27-29] using a single

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standard curve that encompasses the entire concentration range is preferred.

This study was designed and conducted to set a high-performance liquid chromatography method for determination of propofol in human plasma with the focus on selecting the best calibration equation. To do this, we assessed different linear and nonlinear models using several usual weighting schemes. Standard statistical approaches for checking the validity and models goodness of fit were used to choose the best calibration model as described in experimental section.

Materials and Methods

Chemicals

Propofol (≥97%) was obtained from Sigma-Aldrich, USA. Thymol, trifluoroacetic acid and acetonitrile (HPLC-grade) were purchased from Merck, Germany.

Preparation of standard solutions and plasma standards

Stock standard solutions of 1mg/mL propofol and 0.1 mg/mL thymol (as internal standard) were prepared in methanol and acetonitrile, respectively and kept refrigerated. Then, a solution of 150 ng/mL thymol were made by further dilution of its stock solution with acetonitrile and used as the working internal standard and precipitating agent. Standard solutions of propofol at the concentrations of 100, 200 ,500 ,1000 ,2500 ,5000 ,10000 ,25000 and 50000 ng/ml were made by dilution of proper volumes of stock standard with methanol. Plasma standards of propofol were then prepared freshly by spiking 900 µL of human blank plasma with the above standards to give the concentration range of 10 to 5000 ng/mL. Plasma standards were stored at 4 °C until the time of analysis.

Chromatography conditions

Chromatography condition was similar to those reported by Knibbe et al with some modifications.[16] The high-performance liquid chromatography system consisted of an Agilent 1260 Infinity quaternary pump and Agilent 1260 Infinity fluorescence detector (Agilent, USA). A Capital ODS-H-Optimal ® column (150 mm × 4.6 mm, 5 µm particle size) (Capital HPLC Limited, UK) was used as stationary phase. The mobile phase, a mixture of acetonitrile and trifluoroacetic acid 0.1% (60:40, v/v), was degassed by ultra-sonication for 15 minutes before using and delivered at the flow rate of 1.2 mL/minute. The excitation and emission wavelengths were set at 276 and 310 nm, respectively. Chromatography was carried out at ambient temperature.

Sample pretreatment

Four hundred microliter of the working internal standard solution was added to 200 µL of plasma standard or real sample and vortex-mixed for 2 minutes. Samples were then centrifuged at 10000 g for 5 minutes. The supernatant was separated and centrifuged for another 5 minutes .Fifty microliter of the clear supernatant was injected onto the chromatography column.

Modeling the calibration curve and statistical analysis

Calibration curves were constructed using the peak area ratios of propofol to internal standard (PAR) as the response variable. Five replicates of independently spiked propofol plasma standards in the concentration range of 10 to 5000 ng/mL were analyzed and the results were pooled together for regression analysis. Homoscedasticity of PAR values were assessed using Levene's test.[30] The following linear and nonlinear models[31] were fitted to the PAR–propofol concentration (C) data:

a: $PAR = \alpha.C + \beta$

b: $PAR = \alpha.C$

c: $PAR = \alpha.C^2 + \beta.C + \gamma$

d: $PAR = \alpha.C^{\beta}$

e: $PAR = \alpha.C^{\beta} + \gamma$

f: $PAR = \alpha.e^{\beta.C}$

Five different weighting schemes including 1 (no weight), 1/C, $1/C^2$, 1/PAR and $1/PAR^2$ were used for weighed regression analysis and denoted by subscripts 1-5, respectively.[22,32] After fitting the candidate models to the calibration data, method proposed by Tse *et al* with some modifications were employed for selection of the best model.[31] The adequacy of the models were first assessed by the lack-of-fit test and significance of all model parameters. Normality of the residuals were also checked by D'Agostino-Pearson normality test.[33] Models that showed significant lack-of –fit test, substantial deviation of residuals from normal distribution, had non-significant parameters[31] or with high parameters dependency (greater than 0.99) [34] were excluded from further analysis. Among the remaining models, those with the ratio of adjusted R-squared ($R^2_{adjusted}$) to the maximum observed $R^2_{adjusted}$ greater than a predefined value (0.8) were chosen.[31]

Another five replicates of propofol plasma standards at the concentrations of 10, 1000 and 5000 ng/mL were prepared and analyzed. The calculated concentrations with each of the selected models were compared with the nominal concentrations and the relative prediction error (PE%) was determined as follows:

$$PE\% = \frac{C_{predicted} - C_{nominal}}{C_{nominal}} \times 100$$

In which $C_{predicted}$ and $C_{nominal}$ are the calibration model predicted and nominal concentrations of propofol, respectively. Median of relative prediction errors and median absolute relative prediction errors were used as measures of bias and precision. These metrics of predictive performance were compared by constructing 95% confidence interval around them.[35]

Results and Discussion

Chromatograms of human blank plasma, propofol plasma standard at the concentration of 500 ng/mL as well as a real sample obtained 3 minutes post termination

of a propofol infusion at the rate of 50 µg/minute (equal to 373 ng/mL) are shown in Figure 1 (A-C). The retention times of propofol and thymol are 5.00±0.09 (SD) and 9.17±0.05 minutes, respectively. Analytical recovery of propofol at different concentrations was greater than 90%.

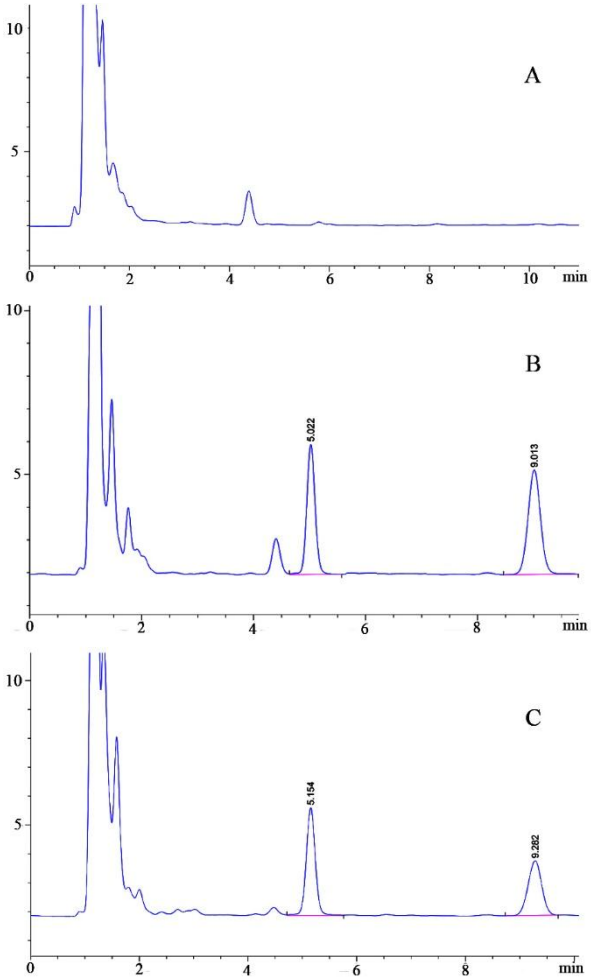

Figure 1. Chromatograms of human blank plasma(A), propofol plasma standard at the concentration of 500 ng/mL(B) and a sample obtained 3 minutes post termination of a propofol infusion at the rate of 50 µg/minute(equal to 373 ng/mL)(C)

Homoscedasticity of PAR values at different concentrations of propofol was rejected (Levene statistic = 28.7, p-value<0.0001). Plot of residuals against propofol concentration (model $b_1$in Figure 2) obtained after ordinary regression analysis of PAR-concentration data further confirms the heterogeneity of response variance. Heteroscedastic nature of the PAR values at the different levels of propofol concentration make the use of weighted least squares regression method to fit the calibration models inevitable. Fitting of the commonly used linear model (a_1) by ordinary least squares regression method (with no weighting factor) led to calibration equation that predict propofol concentration at the limit of the quantitation of the method (10 ng/mL) with substantial relative prediction error (greater than 348%). Although this model ,like the majority of the models in Table 1, has a rather good adjusted R-squared value and the ratio of its $R^2_{adjusted}$ to the maximum observed $R^2_{adjusted}$ is greater than 0.8, its predictive performance is not acceptable. As stated by other investigators, coefficient of determination (R^2) might be quite misleading if it used as the only measure of the goodness of fit and quality of a calibration equation without reference to factors such as pattern of calibration data points, number of observations , etc.[36,37]

Due to the wide range of propofol concentration that may be encountered in real samples, nonlinear calibration equations were also considered in the current study.[24] Among different models suggested elsewhere,[31] and fitted to the propofol HPLC calibration data, the exponential models (f_1-f_5) showed significant lack-of-fit (p-value <0.0001) and low values of $R^2_{adjusted}$, therefore these models were left out.

The intercepts of the linear equations (a_1-a_5) were not significantly different from zero, in other words it seems that these models are overparametrized and simpler models should be taken into account. Overparametrization of the calibration model lead to instability of the estimated parameters which in turn increase the variance of the calculated concentrations.[31] As could be seen from Table 1, the same is true for the constants of the quadratic models (c_1-c_5). Also, the quadratic terms of these equations have very small values and in case of models c_3 and c_5 do not differ significantly from zero. Therefore, the quadratic calibration equations could not be used as predictor of propofol concentration in the unknown samples.

Of the two power models (d and e) that were fitted to the data, models e_1-e_5 had a non-significant parameter (γ) and could be simplified to models d (Table 1). Models d_1-d_5 all showed high R^2 but the parameters of d_1 had dependency values greater than 0.99 that is a sign of model redundancy.[31]

It is now generally accepted that the least squares regression of heteroscedastic data needs proper weighting factor to account for inequality of uncertainty in response variable that is very common in analytical methods such as high-performance liquid chromatography. To remove the heterogeneity in response variability, different weight factors have been used. If enough replicates (a minimum of ten) of response variable are available at all levels of analyte concentrations, the common approach is to calculate the variance of the response and select the weight as the reciprocal of the response variance at each concentration. Due to the lack of such a large number of replicates, other empirical weights such as the ones used in the present study may be considered.[22,32,36]

A good weighting factor should remove the heterogeneity of the response variance and results in models with acceptable predictive performance as measured by median relative prediction error (bias) and median absolute relative prediction error (precision) of the estimated concentrations in the validation data set.[22]

Statistically significant bias was not observed for any of the models as could be found from the 95 % confidence interval around the median relative prediction errors in Table 1. Selection of the best weighting factors was carried out according to the method proposed by Almeida et al with some modifications.[22] Assessment of the results of applying different weighting schemes on median relative prediction error of propofol concentration (Table 2) reveals that for all models (a-e) the reciprocal of squared propofol concentration ($1/C^2$) or peak area ratio of the propofol to internal standard ($1/PAR^2$) led to the minimum bias and best precision of the estimation. It has been reported that the use of relative prediction error as the quality coefficient for choosing the best weight factor is predisposed to find proportional error in the data,[32] therefore in the current study, in addition to assessing the relative prediction error of the concentration in the validation set, selection of the best weight factor was also based on statistical judgments such as the ability of the weighting scenario in removing the heterogeneity in PAR variance (stabilization of the response variance), having the least standard error of the estimate (Sy.x) and passing the normality test of the residuals (Table 1). As could be seen from Tables 1 and 2, models b_3, b_5, d_3 and d_5 which where fitted to the calibration data with weights equal to $1/C^2$ or $1/PAR^2$ have the least standard error of estimates among all models and their residuals do not significantly deviate from normal distribution. Plots of residuals against propofol concentration are shown in Figure 2 for the above mentioned models. Although all these fitted equations (b_3, b_5, d_3 and d_5) could stabilize the variance of the response (PAR), the minimum residual values (Figure 2) were observed for models with reciprocal of squared concentration as the weighting factor. This fact could be also realized from Sy.x values (Table 1).

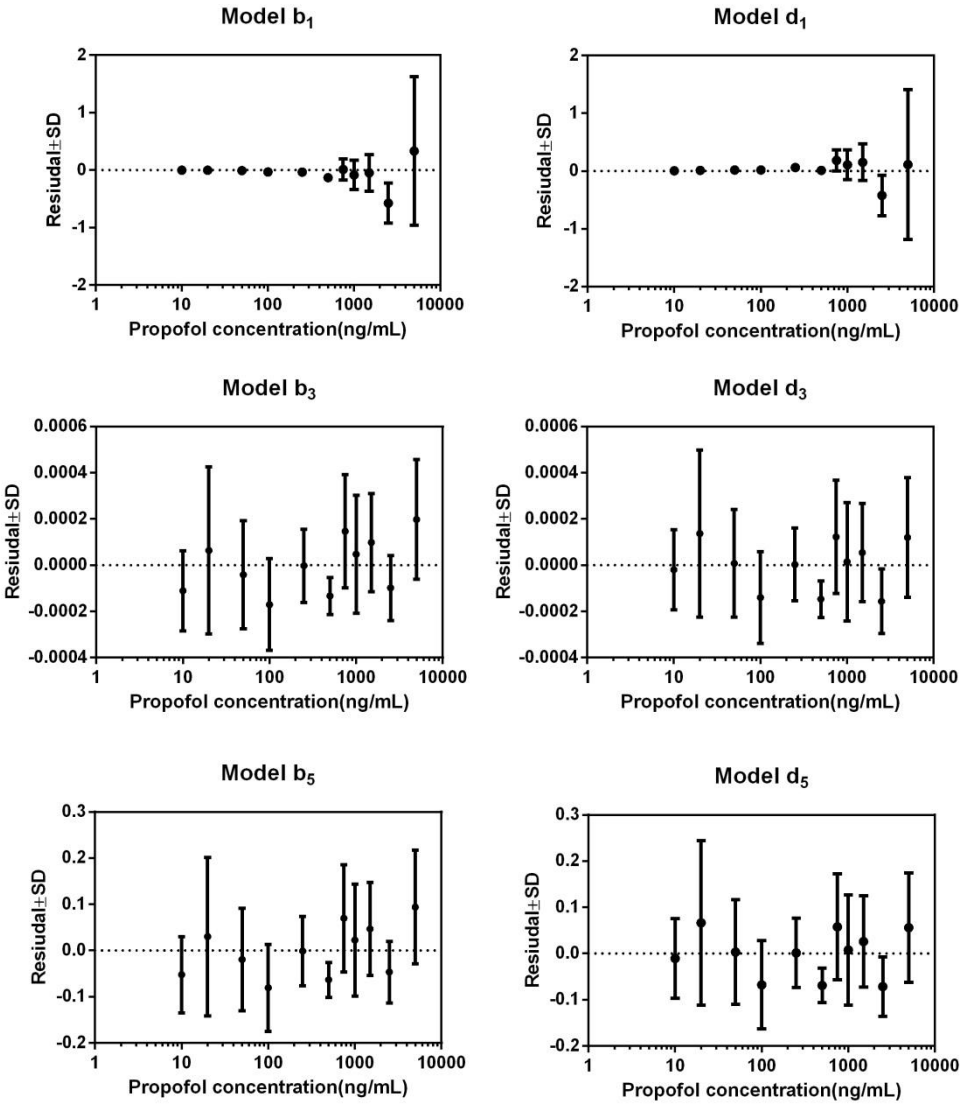

Figure 2. Plots of mean residuals ± SD of models b_1, b_3, b_5, d_3 and d_5 against propofol concentration

Table 1. Summary of the estimated parameters of models fitted to the propofol calibration data and the goodness of fit results (values in the parentheses are the 95% confidence intervals

Model	Weight	Model Parameters			Lack-of-fit		§Sy.x	Normality of residuals (p-value)	$R^2_{adjusted}$	Parameter dependency		
		α	β	γ	F	p-value				α	β	γ
a_1	1	0.00227 (0.00219, 0.00235)	-0.0801 (-0.2270, 0.0668)	-	1.30	0.2669	0.4374	< 0.0001	0.9828	0.351	0.351	-
a_2	1/C	0.00220 (0.00213, 0.00226)	-0.0051 (-0.0216, 0.0115)	-	1.91	0.0760	0.0078	< 0.0001	0.9878	0.055	0.055	-
a_3	$1/C^2$	0.00213 (0.00206, 0.00220)	-0.0011 (-0.00327,0.00105)	-	1.53	0.1666	0.0002	0.1461	0.9846	0.251	0.251	-
a_4	1/PAR	0.00220 (0.00213, 0.00226)	-0.0046 (-0.0199, 0.0107)	-	1.91	0.0756	0.1669	< 0.0001	0.9878	0.050	0.050	-
a_5	$1/PAR^2$	0.00213 (0.00205, 0.00220)	-0.0011 (-0.0031, 0.0010)	-	1.51	0.1742	0.1101	0.1372	0.9844	0.260	0.260	-
b_1	1	0.00224 (0.00218 , 0.00231)	-	-	1.29	0.2649	0.4382	< 0.0001	0.9827	-	-	-
b_2	1/C	0.00219 (0.00213, 0.00226)	-	-	1.76	0.0973	0.0078	< 0.0001	0.9879	-	-	-
b_3	$1/C^2$	0.00211 (0.00205, 0.00217)	-	-	1.49	0.1751	0.0002	0.1825	0.9846	-	-	-
b_4	1/PAR	0.00219 (0.00213, 0.00226)	-	-	1.76	0.0973	0.1659	< 0.0001	0.9879	-	-	-
b_5	$1/PAR^2$	0.00211 (0.00205, 0.00217)	-	-	1.49	0.1751	0.1101	0.1825	0.9846	-	-	-
c_1	1	7.1×10^{-8} (1.8×10^{-8}, 1.2×10^{-7})	0.0019 (0.0017, 0.0022)	0.0453 (-0.1224, 0.2130)	0.61	0.7655	0.4138	< 0.0001	0.9846	0.926	0.942	0.553
c_2	1/C	4.6×10^{-8} (1.0×10^{-8}, 8.1×10^{-8})	0.0021 (0.0019, 0.0022)	0.0004 (-0.0159, 0.0167)	1.27	0.2824	0.0074	0.0015	0.9890	0.755	0.767	0.119
c_3	$1/C^2$	4.0×10^{-8} (-7.0×10^{-9}, 8.6×10^{-8})	0.0021 (0.0020, 0.0022)	-0.0003 (-0.0026, 0.0020)	1.34	0.2489	0.0002	0.1527	0.9852	0.455	0.592	0.372
c_4	1/PAR	4.5×10^{-8} (9.5×10^{-9}, 8.0×10^{-8})	0.0021 (0.0019, 0.0022)	0.0003 (-0.0156, 0.0162)	1.31	0.2635	0.1584	0.0021	0.9890	0.746	0.759	0.120
c_5	$1/PAR^2$	3.9×10^{-8} (-9.8×10^{-9}, 8.8×10^{-8})	0.0021 (0.0020, 0.0022)	-0.0003 (-0.0025, 0.0019)	1.34	0.2508	0.1083	0.1328	0.9849	0.445	0.591	0.382
d_1	1	0.00128 (0.00068, 0.00189)	1.068 (1.010, 1.125)	-	0.81	0.6097	0.4199	< 0.0001	0.9842	0.996	0.996	-
d_2	1/C	0.00168 (0.00125, 0.00211)	1.034 (1.002, 1.067)	-	1.41	0.2131	0.0075	0.0004	0.9887	0.988	0.988	-
d_3	$1/C^2$	0.00196 (0.00178, 0.00214)	1.013 (0.9976, 1.028)	-	1.32	0.2539	0.0002	0.1038	0.9851	0.902	0.902	-
d_4	1/PAR	0.00169 (0.00127, 0.00211)	1.033 (1.002, 1.065)	-	1.43	0.2066	0.1605	0.0005	0.9887	0.987	0.987	-
d_5	$1/PAR^2$	0.00196 (0.00178, 0.00214)	1.013 (0.9974, 1.028)	-	1.30	0.2661	0.1085	0.0846	0.9848	0.897	0.897	-
e_1	1	0.00108 (0.00035, 0.00182)	1.087 (1.007, 1.167)	0.0663 (-0.1211, 0.2536)	0.85	0.5637	0.4219	< 0.0001	0.9840	0.998	0.998	0.628
e_2	1/C	0.00161 (0.00113, 0.00209)	1.040 (1.002, 1.078)	0.0050 (-0.0134, 0.0234)	1.55	0.1689	0.0076	0.0003	0.9885	0.991	0.991	0.279
e_3	$1/C^2$	0.00187 (0.00153, 0.00221)	1.019 (0.9929, 1.046)	0.0011 (-0.0024, 0.0046)	1.44	0.2081	0.0002	0.1034	0.9850	0.973	0.967	0.731
e_4	1/PAR	0.00162 (0.00114, 0.00210)	1.039 (1.001, 1.076)	0.0050 (-0.0134, 0.0233)	1.56	0.1635	0.0004	0.0004	0.9885	0.991	0.990	0.290
e_5	$1/PAR^2$	0.00188 (0.00154, 0.00221)	1.019 (0.992, 1.046)	0.0010 (-0.0024, 0.0045)	1.42	0.2173	0.1093	0.0844	0.9846	0.972	0.966	0.738
f_1	1	1.0440 (0.8158, 1.2710)	0.00049 (0.00044, 0.00053)	-	25.35	< 0.0001	0.9672	0.0182	0.9159	0.907	0.907	-
f_2	1/C	0.1584 (0.0725, 0.2444)	0.00087 (0.00075, 0.00099)	-	213.90	< 0.0001	0.0443	0.0003	0.6068	0.851	0.851	-
f_3	$1/C^2$	0.0312 (0.0181, 0.0442)	0.00112 (0.00105, 0.00135)	-	334.10	< 0.0001	0.0017	0.0015	0.1884	0.325	0.325	-
f_4	1/PAR	0.7612 (0.5751, 0.9473)	0.00057 (0.00050, 0.00064)	-	118.80	< 0.0001	0.7629	< 0.0001	0.7236	0.693	0.693	-
f_5	$1/PAR^2$	0.3889 (0.2831, 0.4947)	0.00098 (0.00082, 0.00113)	-	245.90	< 0.0001	0.8099	< 0.0001	0.4610	0.351	0.351	-

§ Standard error of estimate

Although the difference of median relative prediction error and median absolute relative prediction error between the above four models could not be considered statistically significant, model with the lower median values of the above predictive performance parameters are preferred.[31,35] However, the 95% confidence interval for the parameter β of model d_3 includes 1 and thus the model could be reduced to a more simplified form or b_3.

Application of the weighted least squares regression method with a proper weighting factor could result in better estimation of the unknown concentration near the lowest level of the analyte in the calibration curve (limit of quantitation).[22] Model b_3 predicts the limit of quantitation (10 ng/mL) with median absolute prediction error of 7.7 % (Table 2).

On the other hand with the above mentioned weighted regression model, it is possible to cover the entire range of calibration curve (up to 500 fold) using one simple equation with good accuracy and precision. Table 3 shows the accuracy and precision of the reported high-performance liquid chromatography method for quantitation of propofol in human plasma.

Since selection and using the weighted and/or more complex equation for the calibration curve should be justified,[24] the presented approach of choosing appropriate weighted model for determination of propofol could address this issue.

Table 2. Predictive performance parameters of the different propofol calibration models

Model	Relative prediction error (%)			Absolute relative prediction error (%)			Median absolute relative prediction error(%) at 10 ng/mL
	Median	Lower confidence limit	Upper confidence limit	Median	Lower confidence limit	Upper confidence limit	
a_1	-2.5	-11.9	9.8	11.9	9.5	15.0	348.0
a_2	-0.1	-10.2	12.7	12.3	10.2	15.4	21.8
a_3	3.1	-7.5	16.0	10.1	7.5	16.0	8.4
a_4	-0.1	-10.2	12.7	12.4	10.2	15.4	19.6
a_5	3.1	-7.5	16.0	10.1	7.5	16.0	8.4
b_1	-2.1	-12.2	10.1	11.6	10.1	14.7	13.1
b_2	0.1	-10.3	12.5	11.8	9.6	12.9	11.2
b_3	4.0	-6.8	17.0	9.4	6.0	17.0	7.7
b_4	0.1	-10.3	12.5	11.8	9.6	12.9	11.2
b_5	4.0	-6.8	17.0	9.4	6.0	17.0	8.4
c_1	6.5	4.1	12.2	6.5	4.1	12.2	[§]not estimated
c_2	-3.5	-11.5	7.4	7.6	6.1	14.1	7.2
c_3	-3.1	-11.8	8.2	8.5	6.7	13.8	7.5
c_4	-3.4	-11.6	7.4	7.7	6.3	14.0	7.1
c_5	-3.0	-11.8	8.3	8.6	6.7	13.8	7.6
d_1	-2.5	-9.0	7.7	9.0	6.1	15.4	41.0
d_2	-2.5	-11.6	9.5	11.6	7.8	14.6	18.6
d_3	0.3	-9.3	12.6	11.5	9.3	12.9	8.9
d_4	-2.4	-11.5	9.6	11.5	7.9	14.6	18.0
d_5	0.4	-9.2	12.7	11.4	9.2	13.0	8.8
e_1	7.3	3.8	12.5	7.3	3.8	12.5	not estimated
e_2	-2.7	-11.7	9.0	9.2	7.0	13.8	17.1
e_3	-0.6	-10.0	11.5	11.5	8.0	13.2	7.4
e_4	-2.6	-11.7	9.0	9.3	7.1	13.8	17.1
e_5	-0.5	-10.0	11.6	11.6	7.9	13.1	7.4
f_1	7.8	1.8	19.6	7.8	1.8	18.3	not estimated
f_2	10.3	2.7	56.3	5.1	0.3	183.2	not estimated
f_3	33.9	29.4	60.1	4.0	0.3	241.2	not estimated
f_4	5.1	0.3	183.2	10.3	2.7	56.3	not estimated
f_5	4.0	0.3	241.2	33.9	29.4	60.1	not estimated

[§] Concentration could not predicted by the model

Table 3. Results of accuracy and precision of the method

Nominal concentration (ng/mL)	Accuracy (%)		Precision (%)	
	Intra-day	*Inter-day*	*Intra-day*	*Inter-day*
10([§]LOQ)	94.8±4.5	109.6±10.4	8.7	12
1000	102.3±3.6	93.3±10.1	3.5	11.8
5000	109.4±5.8	114.5±9.0	5.4	11.2

[§] Limit of quantitation

Conclusion

In summary, with the presented approach of constructing calibration equation, it is possible to choose the best model when the response variable is heteroscedastic especially over a broad range of concentration for propofol and any other analyte with such a wide expected range of concentrations in real samples.

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Conflict of Interest
There is no conflict of interest to be reported.

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