Research article

Biomedical Chromatography

Received: 25 January 2011, Accepted: 4 April 2011 Published online in Wiley Online Library: 13 May 2011

(wileyonlinelibrary.com) DOI 10.1002/bmc.1647

Linearization of the MS response function: case study for metformin assay in plasma samples for bioequivalence purposes

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ABSTRACT: Calibration data of LC-MS/MS rarely fit the pure least square regression model, especially for large concentration intervals. The response function of the MS instrument is corrected by weighted regression models or logarithms. The choice of a response linearization method is based on results produced through back-interpolation of experimental data and/or evaluation of correlation coefficients. Two bioequivalence studies carried out for pharmaceutical formulations containing metformin gave us the opportunity to appreciate the impact of the MS response linearization method (logarithm and 1/*x* weighted linear regression) on method quality characteristics. The sample preparation was based on protein precipitation with acetonitrile. Chromatographic separation was achieved on a Zorbax® CN column (mobile phase acetonitrile and aqueous 10 mM ammonium acetate solution, pH 3.5). Tandem MS detection was performed on a triple quadrupole spectrometer equipped with an electrospray source, operated under positive-ion mode. The method was validated and used for evaluation of the bioequivalence of formulations containing 500 and 1000 mg metformin. The 500 mg metformin study used logarithms for linearization of the detector response, while the 1000 mg metformin study was based on 1/*x* linear weighted regression. Data resulting from validations and studies completion were compared with evaluate the impact of the response linearization on the method quality characteristics. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: MS response linearization; metformin; human plasma; LC-ESI-MS/MS; incurred sample reanalysis

Introduction

The response of a tandem MS detector is proportional to the number of ions produced within the source and the collision cell and 'filtered' through the mass analyzers. Based on theoretical assessments, the response of MS equipments should be linearly related to the amount of analyte reaching the source over at least two orders of magnitude, although the various principles of ion production, extraction, analysis and counting may induce significant variance of the dynamic range covered by different instruments (Medvedovici *et al.*, 2010). Undoubtedly, processes directly influencing ionization yield are the major sources of the relatively poor precision of the MS instrumentation and for the restriction of the linear response domain. When uncertainties relating to sample preparation (complex procedures designed for complicated matrixes) and the instrumental response are both considerable with respect to each other, a convenient approach for calibration consists of using the internal standard method together with weighted least square regression, with the weighting containing the contribution of errors for *x* (concentration) and/or *y* (instrumental response; Massart *et al.*, 1988; Brueggemann *et al.*, 2005). However, in some cases, weighted calibration models remain unsuccessful (Georgiță *et al.*, 2008) and log–log representation of peak area ratio to concentration followed by application of an unweighted linear regression may represent a practical solution. The alternative of linearization through logarithms is cited and frequently discussed in the literature (Singtoroj *et al.*, 2006; Szabo *et al.*, 1994, Karnes and March, 1991; Srinivas, 2008, 2009).

Small, highly polar molecules like metformin (logarithm of the partition coefficient between water and octanol is −2) are particularly difficult to assay in biological samples owing to the lack of alternatives for a proper isolation from the complex matrixes and the poor interactions with stationary phases (Tache *et al.*, 2001; Wang *et al.*, 2004; AbuRuz *et al.*, 2003; David *et al.*, 2005).

Several methods for analysis of metformin in plasma using liquid chromatography–tandem mass spectrometry (LC/MS/MS) have been already published (Wang *et al.*, 2004; Chen *et al.*, 2004; Heinig and Bucheli, 2004; Koseki *et al.*, 2005; Swales *et al.*, 2010; Mistri *et al.*, 2007; Marlice *et al.*, 2007; Liu and Coleman, 2009; Zhang *et al.*, 2007; Hsieh *et al.*, 2009; Zhong *et al.*, 2005;

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Abbreviations used: ACN, acetonitrile; ESI, electrospray ionization; HILIC, hydrophilic interaction separation mechanism; ISR, incurred sample reanalysis.

Zhao *et al.*, 2007; Ding *et al.*, 2007; Discenza *et al.*, 2010; Gu *et al.*, 2010) in the last decade. Most of them involve protein precipitation with organic solvent, without any evaporation step; others use protein precipitation combined with liquid–liquid extraction (Wang *et al.*, 2004) or cation exchange solid-phase extraction (Koseki *et al.*, 2005). To enhance chromatographic retention, the normal-phase mechanism involved by supercritical fluid chromatography (Agrawal *et al.*, 2010) or the hydrophilic interaction separation mechanism (HILIC) have been successfully applied (Liu and Coleman, 2009; Hsieh *et al.*, 2009; Huttunen *et al.*, 2009).

The goal of the present work relates to evaluation of the impact of the response linearization method (logarithmation vs $1/x$ weighted regression) on the quality attributes of validated analytical methods occasioned by the assessment of the bioequivalence of pharmaceutical formulations containing 500 and 1000 mg of metformin hydrochloride as the active ingredient. Evidently, application of the least square regression fails to fit the calibration data in both situations. The sample preparation method was based on plasma protein precipitation through the addition of acetonitrile. The chromatographic separation was achieved on an aqueous normal-phase partition mechanism, which was described in a previous publication (Georgiță *et al.*, 2007). Tandem MS detection with electrospray ionization (ESI) was used in the multiple reaction monitoring mode, through monitoring positive ions produced in the source.

Experimental

Materials

All solvents were HPLC grade from Merck (Darmstadt, Germany). Acetic acid was p.a. grade from Merck. Ammonium acetate (extra pure) was purchased from Merck. Water for chromatography (minimum resistivity 18.2 MΩ and maximum total organic compounds (TOC) - 30 ppb) was produced within the laboratory by means of a TKA Lab HP 6UV/UF instrument. Metformin hydrochloride as standard reference substance was purchased from the European Pharmacopoeia, Council of Europe, Strasbourg, France (metformin hydrochloride, batch 2, code. no. EPM0605000). The internal standard (IS), 1-methylbiguanide, was purchased from Mikromol GmbH (Luckenwalde, Germany).

Instrumentation

Experiments were performed with an Agilent 1100 series LC system (Agilent Technology, Waldbronn, Germany) consisting of the following modules: degasser (G1379A), quaternary pump (G1311A), thermostated autosampler (G1329A) and column thermostat (G1316A). The Agilent triple quadrupole mass spectrometric detector model G2571A was used together with the ESI (G1948B) ionization source and controlled using Agilent Mass Hunter Workstation software, version B.00.01. The system had valid operational qualification status on use.

Parameters controlling the MS/MS detector were as follows: drying gas temperature, 350°C; drying gas flow, 10 L/min; pressure of the nebulizer gas, 60 psi; Vcap, 1500 V; fragmentor, 100 V; collision energy, 10 V. The monitored mass transitions were m/z 130.2–60.2 a.m.u. for metformin and m/z 116.2–60.2 a.m.u. for the IS.

Sample preparation procedure

An aliquot of 200 µL from the plasma sample was vortexed for 5 min at 2000 rpm with 400 µL of a 500 ng/mL solution of IS in acetonitrile, followed by 5 min centrifugation at room temperature and 9000***g***. An aliquot from the supernatant was transferred to the injection vial.

Chromatographic method

The chromatographic column was a Zorbax® CN (Agilent Technologies, USA), 150 mm length, 4.6 mm internal diameter and 5 µm particle size, fitted with a Phenomenex C_{18} security guard cartridge (2 × 4 mm). The column was thermostated at 25°C. Elution was isocratic, using as the mobile phase constituents acetonitrile and aqueous 10 mM ammonium acetate solution, adjusted to pH 3.5 with acetic acid, mixed in a volumetric ratio of 1:1, at a flow rate of 0.8 mL/min. The injection volume was set at 5 µL. Separation was controlled through an aqueous normal-phase mechanism, when absolute retention for both target compounds (metformin and IS) exponentially increased with the increase in the percentage of acetonitrile (%ACN) in the mobile phase:

$$t_R = 3.4 + 0.00029\, e^{\%ACN/8.2}; r^2 = 0.9954 \text{ for metformin}$$

$$t_R = 3.15 + 0.00021\, e^{\%ACN/8}; r^2 = 0.9964 \text{ for IS}$$

These relationships were determined in the interval 10–90% ACN in the mobile phase.

Methodology of the bioequivalence studies

The analytical method was applied for assessment of two bioequivalence studies designed for single-dose administration of immediate release formulations (two periods, cross-over, controlled, randomized, fasting conditions) containing 500 and 1000 mg of the active ingredient. Both studies enrolled 26 healthy volunteers each. Seventeen sampling times (including the pre-dose sampling, 0.5 h sampling interval until 5 h from administration, then 6, 8, 10, 12, 16 and 24 h after administration) were made. The protocols of the bioequivalence studies were formally accepted by the evaluation department of the Romanian National Medicines Agency and received the approval of the National Ethics Committee. Pharmacokinetic parameters were determined by means of the Kinetica™ software (version 4.4.1) from the Thermo Electron Corporation, USA.

Results and discussions

Data resulting from the calibration procedures are comparatively presented in Table 1. Validation of the method was based on two different sets of experiments, separately carried out for the 500 and 1000 mg metformin studies. One can observe that the computed correlation coefficients issuing from both linearization models are the same. From the determination of the signal-to-noise (S/N) ratio at different concentration levels, it appears that the instrumental lower limit of quantitation (LLOQ) is around 1.5 ng/mL, far below (~1/20) the lower concentration level used in the calibrations (the choice of the lowest concentration in the calibration was based on counting six halving times from the average maximum plasma concentration level reported in the literature for metformin at both considered dosages, as the pharmacokinetics of the active ingredient were known to be nonlinear). The values computed for LLOQ according to the log-log representation (two –alternative computational modes) were closer to the value obtained through studying the S/N ratio, compared with results produced through application of the weighted linear regression, which indicates the 25 ng/mL threshold as an LLOQ level.

Precision, expressed as relative standard deviation (RSD%) of concentration values obtained through back-interpolation in the regression for independently processed replicates at each level, was more homogeneously distributed for the log–log model rather than the $1/x$ weighted one, as illustrated in Fig. 1.

Table 1. Characteristics of the linearity studies carried out through log–log representation and 1/*x* weighted linear regression

Characteristics	Method 1	Method 2
Independent variable (*x*); *c* represents concentration	log *c*	*c*
Dependent variable (*y*); A_M is peak area for metformin; A_{IS} is peak area for the internal standard	log (A_M/A_{IS})	A_M/A_{IS}
Studied concentration interval (ng/mL)	25–2500	25–4000
No. of calibration levels	8	9
Concentrations levels (ng/mL)	25/50/100/500/1000 1500/2000/2500	25/50/100/500/1000 1500/2000/2500/4000
Independent replicates per concentration level	6	6
Regression	Linear	Weighted 1/*x* linear
Regression coefficient (r_{xy})	0.99907	0.99907
Slope (*B*)	0.93877	0.00101
Intercept (*A*)	−2.59461	0.02027
Standard deviation of the slope (s_B)	0.0165	1.636×10^{-5}
Standard deviation of the intercept (s_A)	0.04428	0.00646
LLOQ [$(5 \times s_A)/B$, ng/mL]	1.7[a]	32
LLOQ (according to,[b] ng/mL)	1.5[c]	15
S/N ratio at 2.5 ng/mL	10.1	9.4
Minimum RSD% at a concentration level	0.96% (25 ng/mL)	
Maximum RSD% at a concentration level	0.18% (2500 ng/mL)	
Minimum averaged %bias per concentration level	−8.9% (2500 ng/mL)	−8.5% (50 ng/mL)
Maximum averaged %bias per concentration level	13.2% (500 ng/mL)	6.7% (500 ng/mL)

[a]Calculated as $10^{[(5\times s_A)/B]}$.
[b]Calculated according to (Liteanu and Rica, 1980) by applying the relationship $X_i = [t \times (s_A + \overline{x} \times s_B)]/[B + 2 \times t \times s_B]$, where $\overline{x}$ is the mean concentration and *t* is the Student coefficient (bilateral) considered for $n - 2$ degrees of freedom and a confidence level of $P\% = 90\%$.
[c]Calculated as 10^{X_i}.

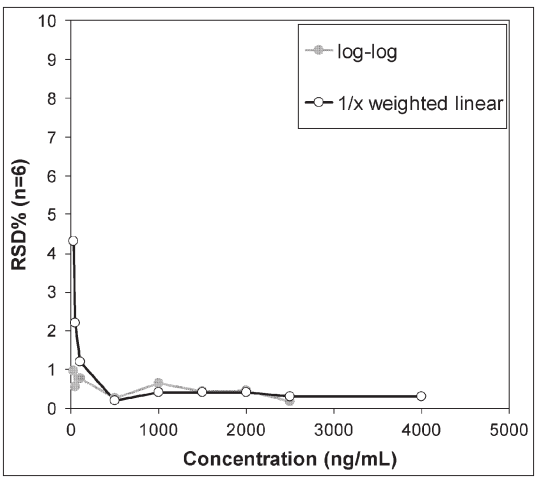

Figure 1. Relative standard deviations (%) calculated at different concentration levels of the linearity study ($n = 6$) for both linearization techniques being applied (log–log transformation and 1/*x* weighted linear regression).

Intra-day ($n = 10$, independently processed replicates) and inter-day precisions ($n = 6$) expressed as RSD% were evaluated at 75, 800 and 1600 ng/mL levels for the log–log linearization method and at 75, 1600 and 3200 ng/mL levels for the 1/*x* weighted regression. RSD% values varied in the 0.3–0.8% interval for the first method and 0.5–3.2% interval for the second one. Percentage biases of the determined concentration values with respect to known ones varied between −5.5 and 7.7% for the first method and from −9.9 to 7.2% for the second one.

On completion of the studies, the analytical sequence consisted of a calibration, the quality control (QC) samples having the same nominal concentrations as those being tested during the evaluation of precision for method validation, and incurred samples from a volunteer. The injection order was as follows: the calibration, one injection from the QC set, incurred samples (injected in increasing order of their sampling time, alternating phase 1 and phase 2 collections), and another QC set, independently prepared. To demonstrate the stability of the MS/MS equipment response over the period taken for running each study, peak area values of the internal standard in the incurred samples ($n = 884$) were characterized by a RSD% of 12.2% for the first study (500 mg) and of 8.3% for the second one (1000 mg).

In both studies, none of the calibration samples failed back-interpolation. In addition, none of the QC samples being analyzed exceeded the ±15% percentage bias interval generally accepted as the accuracy threshold by guidelines in force. Table 2 synthesizes data resulting from calibrations and QC sets analyzed over the analytical sequences during both studies.

From data given in Table 2, it is quite difficult to state which of the linearization methods induces a better interpretation of the experimental data. However, precision and accuracy were slightly more favorable when log–log linearization was applied.

A normalized response was calculated for the incurred samples analyzed over each of the two bioequivalence studies.

Table 2. Summarizing statistics carried out on calibration samples, QC samples and regression parameters obtained over running the analytical sequences during completion of the two bioequivalence studies

		500 mg[a] log–log				1000 mg[a] 1/*x* weighted linear			
Sample type	Concentration (ng/mL)	Mean value	RSD%	Minimum %bias	Maximum %bias	Mean value	RSD%	Minimum %bias	Maximum %bias
Calibration	25	24.8	1.1	−3.5	0.9	26.1	3.9	−3.2	14.0
Calibration	50	49.3	1.0	−3.3	1.6	46.3	3.0	−13.0	2.6
Calibration	100	96.2	0.6	−4.8	−2.5	96.2	1.9	−7.3	−1.0
Calibration	500	551.0	0.8	8.9	11.7	515.7	1.8	−0.1	6.8
Calibration	1000	1070.3	1.0	6.0	12.0	1032.6	1.1	1.7	5.8
Calibration	1500	1530.1	0.5	0.3	2.9	1538.6	0.6	1.1	3.6
Calibration	2000	1898.4	0.6	−6.8	−4.2	2036.9	1.0	0.3	4.1
Calibration	2500	2327.2	0.6	−8.0	−6.0	2453.0	0.6	−3.1	−0.9
Calibration	4000	—	—	—	—	3929.9	0.8	−3.2	−0.4
QC (1)	75	69.3	2.3	−11.1	−4.1	73.3	3.5	−8.5	6.8
QC (2)	800	863.6	2.3	0.4	11.8	—	—	—	—
QC (3)	1500	1601.1	2.5	−3.4	11.6	1576.6	1.0	2.9	7.0
QC (4)	3000	—	—	—	—	3121.1	1.0	0.5	6.5
Slope (*B*)	—	0.9373	0.5	—	—	0.0011	5.7	—	—
Intercept (*A*)	—	−2.5468	0.5	—	—	0.0117	27.7	—	—

[a]Number of analytical sequences (n) = 26.

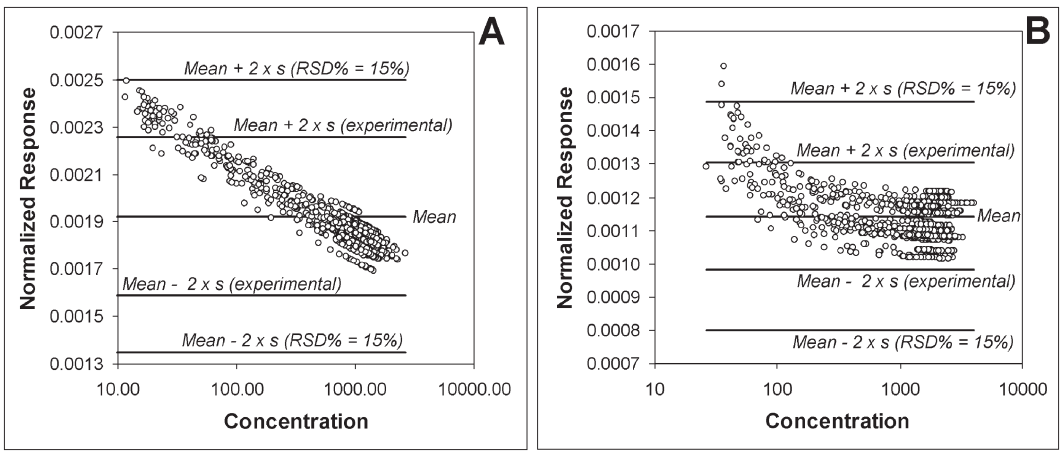

Figure 2. Variation of the normalized responses determined in the incurred samples according to the determined concentration values (A, data obtained after applying the log–log linearization technique; B, data obtained through 1/*x* weighted linear regression).

The normalized response represents the ratio between the response of the detector (peak area ratio between analyte and internal standard) and the concentration value resulting from interpolation in the associated calibration. Theoretically, the normalized response illustrates the sensitivity of the assay (variation of the response per unit of concentration). If the response function used for calculation is linear, the normalized responses for the analyzed samples should be randomly distributed with respect to the mean over the whole concentration domain, with the dispersion illustrating the overall accuracy of the method. When a function is used for calibration forces linearization by means of any adequate mathematical technique (e.g. log–log transformation or 1/*x* weighting), the normalized response should also be functionally distributed over the concentration domain, the distribution pattern specifically reflecting the linearization technique. The plots of the normalized responses according to the calculated concentrations of the incurred samples are illustrated in Fig. 2.

In the case of the log–log linearization, the normalized response decreases linearly with the increase in the concentration values (note that the concentration axis is shown with a logarithmic scale) and is distributed symmetrically with respect to the mean. In the case of 1/*x* weighting, the normalized response decreases logarithmically with the increase in the concentration (the same logarithmic representation was made on the Ox axis). The values of the normalized response are randomly distributed with respect to the mean only for concentrations higher than 500 ng/mL, but exponentially decrease with the

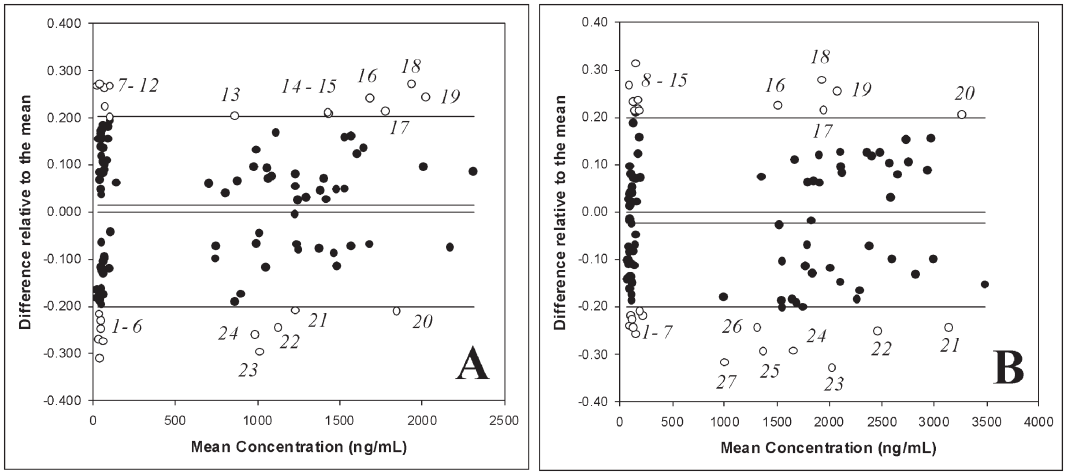

Figure 3. Results of the Bland–Altman approaches on incurred reanalyzed samples after completion of (A) the 500 mg metformin formulation study; and (B) the 1000 mg metformin formulation study. Applied linearization technique was (A) log–log transformation; (B) 1/*x* weighted linear regression.

concentration increase up to this threshold. The RSD% values of the normalized responses are almost equal for the two linearization techniques and fall within the 15% limit (8.7% for the log–log linearization and 7.1% for the 1/*x* weighting).

Enhanced attention was paid during the last period to assessing the reproducibility of results relating to incurred samples. Incurred sample reanalysis (ISR) has been recently discussed by Rocci *et al.* (2007). ISR can be considered as a specific form of cross-validation (Gilbert *et al.*, 1995), having already used regression and reproducibility limits to evaluate experimental results. The Bland–Altman approach is, however, preferred (Bland and Altman, 1986; Lytle *et al.*, 2009) for evaluation of ISR results.

The two bioequivalence studies carried out on 500 and 1000 mg metformin formulations created the opportunity to evaluate not only the reproducibility of the method itself, but also the way in which the linearization of the detector response might influence the final data assessment. Consequently, after each study completion, in a time interval covered by the long-term stability studies contained within the method's validation (a 4 month long-term stability period at −40°C was validated), samples from each volunteer and administration phase corresponding to collections 2 and 16 (sampling times of 1 and 24 h after administration) were reanalyzed. Samples collected 1 h after administration were close to the time corresponding to the maximum concentration (the T_{max} pharmacokinetic parameter; values of 2.69 and 2.83 h were determined for tested and reference formulations, respectively, during the 500 mg study, while for the 1000 mg study T_{max} was 2.48 and 2.38 h, respectively). Consequently, in such samples, the concentration values were close to the upper limit of quantitation (ULOQ). Samples collected 24 h after administration belonged to the late elimination period, their concentration values being closest to the LOQ level. It is worthwhile to note that determined values for the maximum plasma concentration level (the C_{max} pharmacokinetic parameter) were very similar to data found in literature and confirmed the decision of considering 25 ng/mL as the lowest concentration level in the calibrations. Determined C_{max} values were 1497.6 and 1434.6 ng/mL for tested and reference formulations during the 500 mg study and 2228.9 and 2513.7 ng/mL, respectively, during the 1000 mg study. The resulting 104 reanalyzed incurred samples from a study were included in two analytical sequences, each sequence containing calibration and QC samples. The linearization modes used during the study completion were also applied to reanalyzed incurred samples (log–log transformation for reanalysis of samples from the 500 mg metformin formulations study, and 1/*x* weighted linear for the 1000 mg formulations). The Bland–Altman approach was used to assess the experimental data. The results are illustrated in Fig. 3.

The ISR procedures were successful for both studies, as less than 33% of the reanalyzed samples (more precisely 24% for the 500 mg metformin study, and 27% for the 1000 mg metformin study) showed differences with respect to initial assayed values outside the ±20% interval relative to the mean of the determinations.

On average, reanalyzed samples produced results slightly higher than initial ones for the 500 mg metformin study. For the 1000 mg metformin study the situation was reversed. Outliers were evenly distributed between sample populations characterized by low and high concentration values (12 vs 12) for the metformin 500 mg study. For the 1000 mg metformin study, 15 outliers were obtained for samples having concentrations below 100 ng/mL, while 12 other outliers were obtained for concentrations higher than 1000 ng/mL.

Conclusions

Both linearization methods perform similarly, their impact on the quality attributes resulting from validation, completion studies and reanalysis of incurred samples being comparable. It seems that log–log transformation acts in a more uniform way with respect to the whole concentration interval while 1/*x* weighted linear regression induces a slightly reduced precision and accuracy at concentration levels close to LOQ. The lack of significant differences between the two alternative linearization techniques observed through the present study may be also a consequence of the fact that the instrumental LLOQ level was far below the lowest concentration level targeted by the method. Statistical evaluation of the LLOQ level for the log–log transformation led to results close to the instrumental LLOQ level determined on the S/N basis. The 1/*x* weighted linear

regression approach produced a computed LLOQ level close to the lowest concentration level considered in calibrations. Although the monitored response domain was large enough (4 orders of magnitude), the advantages of one linearization method over the other are slight. As most of the quantitative software packages accompanying commercial instruments offer automatic statistical evaluation of weighted linear regression types and the subsequent data treatment (but no log–log transformation features are available), such an approach is far more convenient for bioanalysts in the current practice.

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