Analytica Chimica Acta 815 (2014) 33–41

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Impact of calibrator concentrations and their distribution on accuracy of quadratic regression for liquid chromatography–mass spectrometry bioanalysis

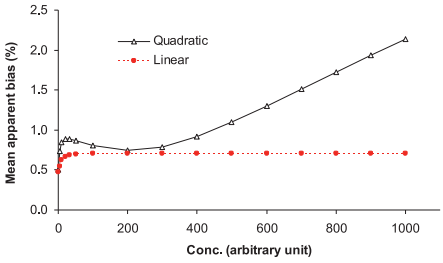
Aimin Tan*, Kayode Awaiye, Fethi Trabelsi

BioPharma Services Inc., Toronto, ON, Canada

HIGHLIGHTS

- Different calibrators have different impacts on accuracy of quadratic regression.
- The lowest & highest concentrations plus their geometric mean are three critical ones.
- Using these critical concentrations can improve the accuracy of quantitation.
- Using these critical concentrations can significantly save time and costs.
- Upper limit quality control must be used in each batch for quadratic regression.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 25 October 2013
Received in revised form 9 January 2014
Accepted 10 January 2014
Available online 19 January 2014

Keywords:
Quadratic calibration
Quadratic regression
Calibration standard
Non-linear regression
Bioanalysis

ABSTRACT

Despite the common use of quadratic regression in LC–MS bioanalysis, how calibrator concentrations should be determined is still vague. Both the number and concentrations of calibrators are usually selected arbitrarily to each one's preference. The purposes of this research were to evaluate the impact of calibrator concentrations and to find new approaches with improved accuracy and reduced cost for LC–MS bioanalysis. It was found for the first time that the lower and upper limits of quantitation plus their geometric mean are the three critical concentrations for quadratic regression. When different concentration ranges, different response precisions, and various degrees of downward quadratic responses were simulated, the best accuracy was obtained by including these critical concentrations and using fewer calibrator concentrations with more replicates per concentration, instead of using more calibrator concentrations in duplicate. In many cases, when the aforementioned three concentrations are used, as few as two replicates per concentration are enough for routine use and up to 20% of time and cost can be saved. Furthermore, downward quadratic response should be eliminated or reduced as much as possible and upper limit quality control must be included in each batch to monitor the accuracy at the high concentration end. The retrospective data analysis of published experimental results corroborates the aforementioned findings. Finally, the typical "concerns" and potential applications of the new quadratic regression approaches are discussed.

1. Introduction

Non-linear or quadratic regression/calibration, the use of weighted or non-weighted least-squares regression for fitting response–concentration data with a second order polynomial

* Corresponding author. Tel.: +1 416 747 8484; fax: +1 416 747 8480.
E-mail addresses: atan@biopharmaservices.ca, aimintan@hotmail.com (A. Tan).

0003-2670/$ – see front matter © 2014 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.aca.2014.01.036

(quadratic) calibration model, is quite commonly used in LC–MS based bioanalysis [1–10]. The possible causes for quadratic response in LC–MS bioanalysis include ionization/detector saturation, formation of dimer/multimer/cluster ions, wide dynamic concentration range, matrix effect, low mass accuracy or selectivity, and even inadequate internal standard (IS) concentration when there is cross-signal contribution from the analyte due to chemical or isotopic interferences [1–3,11].

Despite its common use in LC–MS bioanalysis, it is, however, very confusing as how many calibrators (calibration standards, CSs) to be used and at what concentrations for quadratic regression. There was very limited research on this topic and contradictory recommendations were made. For example, Boulanger et al. recommended three concentrations, the lower (LLOQ) and upper (ULOQ) limits of quantitation plus the middle concentration [12,13]. Hartmann et al. suggested four concentrations without mentioning what the concentrations should be [14,15]. Regulatory guidelines [16–19] specify a minimum of six calibration concentrations together with a general principle that more should be used for quadratic regression than for linear one. Since no consensus has been reached, in reality, both the number of calibrators (typically 7 to 10 and occasionally even 11) and their concentrations are usually selected arbitrarily to each bioanalytical scientist's preference, without necessary regards to their potential impact on the accuracy of quadratic regression [1,5–7]. For example, Liu et al. [1] used eight calibration concentrations for a 1000-fold dynamic concentration range (1 ng mL^{-1} to 1000 ng mL^{-1}) while Xu et al. [7] from the same company utilized nine even for a shorter dynamic range of 250-fold. More interestingly, in the methods developed by the same group of authors for the same analyte over the same concentration range, the number of calibrators and their concentrations were quite different (from eight calibrators in the original method [6] to 10 in the improved method for chromatography [5]) with no reason given in this regard. In some articles, the authors just gave the numbers of calibrators without mentioning their concentrations, leaving an apparent impression that calibrator concentrations are not important in the authors' mind or they are self-evident to readers [4,9].

Based on the above, it is very much desirable to evaluate the impact of calibrator concentrations and their distribution on the accuracy of quadratic regression, particularly when exists an ever growing desire in the global bioanalytical community for scientifically sound practices and guidelines where the rational behind each requirement is given [20,21]. The purposes of this research were to perform such an important evaluation through both simulations and retrospective data analysis of published experimental results, and to find out new approaches that may be more accurate yet still save time and cost, just like the ones proposed for linear regression in LC–MS bioanalysis [22–25]. It is our hope that the findings from this research would be helpful for a more purposeful and scientific selection of calibrators in quadratic regression.

2. Experimental

2.1. Generation of response raw data

The theoretical responses for CS and quality control (QC) samples were first calculated using the following equation, $y = ax^2 + bx + c$, where y is the response and x represents the concentration. Once the theoretical responses were calculated, a random error was added to the calculated response of each replicate of CS or QC samples using the "RAND()" function in Excel program (version 2007). The maximum magnitudes of random error set for poor precision scenario were ±20% at the LLOQ and ±15% at others. In good precision scenario, the corresponding percentages of error were reduced to ±12% and ±7%, respectively. For the very good precision scenario, the magnitudes of error were further reduced to ±6% and ±4%, respectively. A total of 30 batches were simulated for each combination of dynamic concentration range, degree of quadratic response, and precision scenario. Each batch included six QCs and each with six replicates. They were LLQC (QC at the LLOQ), low QC (QC1, 3 × LLOQ), intermediate QC (QCint, geometric mean), medium QC (QC2, 35% of ULOQ), high QC (QC3, 75% of ULOQ), and ULQC (QC at the ULOQ).

2.2. Evaluation of the impact of calibrator concentrations

The theoretical linear or quadratic responses were first calculated according to the corresponding response–concentration equations in Table 1. Then, multi-concentration quadratic or linear regressions were performed using the following CS concentrations: 1, 5, 10, 20, 31.62, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1000 (arbitrary unit). To evaluate the impact of calibrator concentrations, 10% or −10% of error in response was introduced to one calibrator at a time. The resulted individual biases (ε_i) for each unit concentration in this range (1, 2, 3, . . ., 999, 1000) were used to calculate mean apparent bias (MAB) [22], MAB $= \sqrt{1/n\left(\sum_{i=1}^{n}\varepsilon_i^2\right)}$, which was used as an indicator for overall accuracy of quantitation.

2.3. Reconstruction of experimental response data

The nominal (theoretical) concentrations of CS and QC samples, the individual measured/back-calculated concentrations, and the quadratic regression parameters (a, b, and c) for three core batches were all reported in a published research paper for the quantitation of posaconazole in human plasma [5]. Based on these data, the original response data, i.e. the ratios of analyte peak area/IS peak area for all samples, were reconstructed using $y = ax^2 + bx + c$.

2.4. Quadratic regressions

The weighted ($1/C^2$) quadratic regression was performed using an in-house built Excel program, the accuracy of which had been successfully verified against that of the Analyst software (versions 1.4.2 and 1.5.1, AB Sciex, Concord, Ontario, Canada) prior to its application.

Six different calibration schemes were compared, 10 × 2 (10 CS levels in duplicate), $7 + 6_{gm} + 7$ (seven replicates at the LLOQ and ULOQ levels plus six for geometric mean), $7 + 6_{am} + 7$ (same as $7 + 6_{gm} + 7$ except using arithmetic mean), 5 × 4 (five CS levels in quadruplet), 10 × 1plus (10 CS levels in singlet except the LLOQ, geometric mean, and ULOQ, which were in 4, 4, and 5 replicates, respectively), and 3 × 2 (same as $7 + 6_{gm} + 7$ except all in duplicate). The CS concentrations used for different calibration schemes and different concentration ranges are detailed in Table 1.

For the calibration schemes that used duplicate calibrators, such as 10 × 2 and 3 × 2, the exclusion of a calibrator replicate from regression was based on the biases of back-calculated concentrations (within ±20% at the LLOQ and ±15% for others), with the most deviant one being excluded first and one at a time, if more than one. For other calibration schemes that utilized multiple replicates, e.g. $7 + 6_{gm} + 7$ and 5 × 4, a Grubbs test [26] was performed to remove any outlier first. Then, the individual responses at each concentration level were averaged and their means were used in quadratic regression. In 10 × 1plus regression, the Grubbs test was also performed first for the three concentration levels that had multiple replicates, i.e. the two limits and geometric mean. Then, the corresponding number of replicates with the average response was used in regression with the other concentration levels. For example, if one outlier was detected at the ULOQ level, the responses for the remaining four replicates would be averaged. Then, four