Journal of Chromatography, 612 (1993) 71–76
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6625

Estimation of the lower limit of quantitation, a method detection performance parameter for biomedical assays, from calibration curves

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(First received August 3rd, 1992; revised manuscript received October 14th, 1992)

ABSTRACT

A model for the lower limit of quantitation for biomedical chromatographic assays is proposed. It is based on the IUPAC definition for the limit of detection and can be estimated from assay calibration data. It has been applied to ten different sets of calibration data from various assays of drugs in biological matrices.

INTRODUCTION

The determination of very low concentrations or very small amounts of analytes in complex matrices of biological, medical or environmental origin poses beyond the separatory difficulties a two-fold challenge to the analyst. The first challenge is to orient himself in the maze of terminology used to describe or define the "limits of detection". However, Massart *et al.* [1] in chapter 7 of the excellent textbook on chemometrics and Currie [2] have discussed this subject and placed it into perspective. The second challenge is to select the most appropriate model for establishing the lowest quantifiable concentration of one's assay so that the reliability of the generated analytical results can be defended based on statistical principles.

The importance of such a lower limit of quantitation (LLQ) in the determination of therapeutic agents and/or their metabolites in biological matrices (plasma, serum, excreta, tissues etc.) cannot be overstated. The absence of a well defined, statistically based model for the LLQ in biomedical assays is clearly evident from published literature although in other fields of trace analysis, such as pesticide residue determinations, various models have been discussed and used [2]. In our experience with drug assay development for basic pharmacokinetic, bioequivalence and patient compliance purposes, the LLQ, or the limit of detection as it is often called, is the most important performance parameter for judging the quality and reliability of data at the lower end of the concentration range of interest. Yet very few published methods have detection limits listed with the exact description of how this parameter was derived; most of them list various multiples of instrumental noise as the limit of the method, when this only reflects the limits of the given instrumentation. Furthermore, detector noise is a complex phenomenon which has various components [3]. As such it is one of the most variable parameters and, therefore, less suitable

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0378-4347/93/$06.00

as a criterion for the limit of quantitation of biomedical assays.

The majority of today's analytical methods in drug level determinations is based on chromatography. Attempts to select a model for the limit of detection applicable to chromatography in general [4,5] and to chromatographic analysis of drugs in biological samples [6] have been made. All of the models require determinations of noise levels or other parameters requiring separate determinations that can make the detection limit estimations in the context of large sample set runs cumbersome.

A simple model is proposed for the estimation of the LLQ for chromatographic methods used in determinations of drugs in biological media. It is based solely on statistical parameters easily obtainable from standard calibration curve data that are needed for calculation of analyte concentrations in actual study samples.

EXPERIMENTAL

Calibration data

Eight calibration data sets of various sizes and concentration ranges obtained from four internal standard high-performance liquid chromatographic (HPLC) assay development studies were used to test-estimate the LLQ.

Method 1: simultaneous determination of drug A and six other comedications in human plasma samples by reversed-phase HPLC with UV detection

Calibration curve 1. Intra-day variability set; three replicate determinations per concentration; eight concentrations (0, 0.0488, 0.1953, 0.6192, 3.124, 12.5, 50 and 200 μg/ml of drug A).

Method 2: determination of drug B in low-volume 0.1-ml dog plasma samples by reversed-phase HPLC with UV detection

All three calibration sets consisted of fourteen concentrations (0, 0.0733, 0.1465, 0.293, 0.5859, 1.1719, 2.3438, 4.6875, 9.375, 18.75, 37.5, 75, 150 and 300 μg/ml of drug B).

Calibration curve 2. Intra-day variability set; three replicate determinations per concentration in one day.

Calibration curve 3. Inter-day variability set; three replicate determinations per concentration each done on a different day.

Calibration curve 4. Three intra-day and three inter-day sets combined.

Method 3: simultaneous determination of drug B and its metabolite C in rat heart tissue homogenate by reversed-phase HPLC with UV detection

Both sets consisted of ten concentrations (0, 0.2, 0.4, 0.8, 1.5, 3.1, 6.125, 12.5, 25 and 50 μg/ml).

Calibration curve 5. Intra-day variability set for drug B; three replicate determinations per concentration in one day.

Calibration curve 6. Intra-day variability set for metabolite C; three replicate determinations per concentration in one day.

Method 4: determination of drug D in guinea pig plasma and in guinea pig lung tissue homogenate by reversed-phase HPLC with fluorescence detection

Calibration curve 7. Intra-day variability full set for drug in plasma; three daily replicate determinations per concentration for three days combined; thirteen concentrations (0, 0.156, 0.312, 0.625, 1.25, 2.5, 5, 10, 20, 40, 80, 160 and 320 ng/ml of drug D).

Calibration curve 8. Intra-day variability full set for drug in lung; three daily replicate determinations per concentration for three days combined; twelve concentrations (0, 20, 39, 78, 156, 313, 625, 1250, 2500, 5000, 10 000 and 20 000 ng/g of drug D).

Short calibration sets

Calibration curve 2A. Method 2; short intra-day calibration set of four concentrations (0, 0.5859, 9.375 and 75 μg/ml). Used for calibration in analyses of unknowns.

Calibration curve 8A. Method 4; short intra-day set of four concentrations (0, 625, 1250 and 10 000 ng/g).

The sets consisted of two columns of values,

the first column was the standard drug concentration X in plasma or tissue homogenate, the second was the measured instrument response Y (= peak-height or peak-area ratio). Each of the above sets was fitted by unweighted least squares to the equation $Y = A + BX$ by the program TableCurve [7] and the resulting parameters and predicted concentrations were saved and further processed by the program SigmaPlot [8] which allowed calculation of the LLQ using a customized set of transform equations in its MATH menu. Manual calculations with the equations below were also done.

Equations

Calibration model

$$Y_{ij} = A + BX_{ij} \quad (1)$$

where Y_{ij} = measured instrument response for the ith calibration concentration and jth replicate, A and B are the intercept and the slope of the line, respectively, and X_{ij} = standard concentration; i = order numbers of concentration points, where $i = 1, 2, ..., m$ and m is the number of concentrations used; j = order number of the replicate measurements at the ith concentration, where $j = 1, 2, ..., r$ and r is the number of replicate measurements at each concentration. The value of r has to be the same for all concentrations if eqn. 2 is used.
The total number of data pairs per calibration set is $n = m \times r$.

Confidence and prediction bands around the regresion line

$$W = t_{n-2}^{0.05}\left[\left(R + \frac{(X_s - \bar{X})^2}{\sum_{i=1}^{m}\sum_{j=1}^{r}(X_{ij} - \bar{X})^2}\right)s^2\right]^{1/2} \quad (2)$$

Confidence band: $R = \frac{1}{n}$, $W = W_c$

Prediction band: $R = \frac{1}{r} + \frac{1}{n}$, $W = W_p$

Variance of Y: $$s^2 = \frac{\sum_{i=1}^{m}\sum_{j=1}^{r}(Y_{ij} - \bar{Y})^2}{n-2}$$

W = half-width of the confidence or prediction band at the 95% probability level for any selected X_s value, $\bar{X}$ or $\bar{Y}$ is the mean value of X or Y for the calibration set, $t_{n-2}^{0.05}$ is the percentile of the t-distribution at the 95% confidence level for $n-2$ degrees of freedom from statistical tables.

Half-width of 95% confidence interval w around the standard concentration X

$$w_s = t_{n-2}^{0.05}\left[\left(\frac{1}{r} + \frac{1}{n} + \frac{(Y_s - \bar{Y})^2}{B^2\sum_{i=1}^{m}\sum_{j=1}^{r}(X_{ij} - \bar{X})^2}\right)\frac{s^2}{B^2}\right]^{1/2} \quad (3)$$

$$w_0 = t_{n-2}^{0.05}\left[\left(\frac{1}{r} + \frac{1}{n} + \frac{(A - \bar{Y})^2}{B^2\sum_{i=1}^{m}\sum_{j=1}^{r}(X_{ij} - \bar{X})^2}\right)\frac{s^2}{B^2}\right]^{1/2} \quad (4)$$

where w_s is the half-width for a selected value X_s and Y_s is calculated from the regression line, w_0 is the half-width of the interval for the blank standard.

RESULTS AND DISCUSION

The definition of the proposed LLQ is derived from the original IUPAC definition [9] and the interpretation of it by Long and Winefordner [10]: "The limit of detection is the lowest concentration level that can be determined to be statistically different from an analytical blank.". The term *statistically different* is according to the above authors ambiguous because of the various interpretations that can be given to it.

In order to avoid the above ambiguity the proposed definition of the LLQ is as follows:
"The lower limit of quantitation is that concentration of the analyte in the matrix of interest for which the confidence interval at the 95% (or any other) probability level does not overlap with the confidence interval of the matrix blank standard.".

A similar concept was suggested by Mitchell *et*

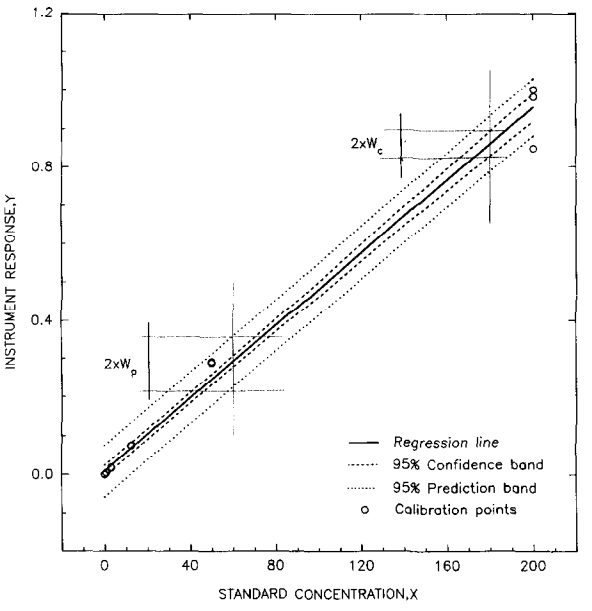

Fig. 1. Example of "real-life" calibration regression line for drug A in human plasma with 95% confidence band and 95% prediction band around the regression line; $n = 24$, $r = 3$, eight standard concentrations.

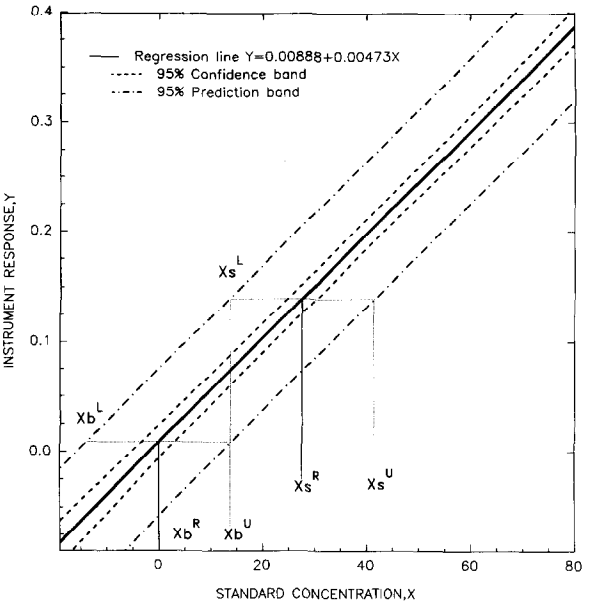

Fig. 2. Estimation of the LLQ by the graphical method based on prediction band around the calibration regression line; X_b and X_s are concentrations for the blank and a sample, R, L and U are superscripts denoting values on the regression line or the lower and upper limits on the prediction band.

al. [11] who defined a "minimum reportable concentration" as such a concentration which includes zero in its confidence interval. However, this definition assumes that the signal measurements for blank samples are zero, which is not always the case.

The confidence and prediction bands around the calibration regression line were found to be the best representation of the overall variability, including all, even the sample preparation steps in the assay. This also involves standard analyte samples preparation, if they are prepared in the matrix of interest. The half-width of the confidence and prediction bands W can be calculated from eqn. 2. These bands, shown in Fig. 1, are very useful in judging assay performance and reproducibility during development. For more details the readers are referred to publications discussing the use of confidence intervals in analytical calibration by Mitchell and co-workers [11,12], Schwartz [13] and Kurtz *et al.* [14] and to specialized monographs [1,15,16].

Mitchell and co-workers [11,12] and Kurtz *et al.* [14] have used confidence intervals derived from confidence bands around the entire regression surface, where the statistical constant in eqn. 2, $t_{n-2}^{0.05}$, is replaced by $(2 \times F_{2,n-2}^{0.05})^{1/2}$, the tabulated percentile value of the F-distribution for two variables. This leads to a more narrow band than the prediction band. In addition, they also applied a confidence band around the measured signal to obtain the confidence interval around the predicted concentration of unknowns.

Application of the confidence band around the entire regression surface to our calibration data showed that more of the actual calibration points were outside the band than when the prediction band was used. This suggests that the use of the prediction band is more appropriate for use in calibrations of analytical methods for drugs in biologic matrices. Because unknown samples are always analyzed simultaneously with calibration sets we feel that the confidence band around the measured signal (response) is not needed for the

estimation of the LLQ, because the statistical variation is included in the prediction band.

The 95% prediction band around the regression line, sometimes also named the dispersion band, is the band within which 95% of future observations at any X are expected to lie. As such it is wider than the 95% confidence band which only covers additional repeated measurements of Y at the fixed X values of the calibration line. In order to estimate the LLQ of an assay based on a specific calibration line for future unknown sample readings at any X, including matrix blank values, we suggest to use the prediction band derived from the corresponding form of eqn. 2. The confidence intervals around any X value obtained by inverse regression from the calibration line are then given by eqn. 3 and in the special case of the matrix blank, $X = 0$, by eqn. 4. To obtain an estimate of the above defined LLQ graphically the prediction band around the regression line is plotted first (some statistical computer software will do this) and then the upper limit of the confidence interval for the blank, X_b^U, is found as indicated in Fig. 2. Extension of this point parallel to the Y-axis onto the upper prediction band line gives the lower limit of the confidence interval, X_s^L, for the concentration X_s^R, equal to the LLQ. As can be seen the confidence intervals of the blank $\{X_b^L < X_b^R < X_b^U\}$ and the LLQ concentration $\{X_s^L < X_s^R < X_s^U\}$ do not overlap but $X_b^U = X_s^L$. The corresponding calculated confidence intervals are for the blank sample $\{-w_0 < 0 < w_0\}$ and for any positive value of X on the regression line, X_s^R, $\{X_s^R - w_s < X_s^R < X_s^R + w_s\}$. The LLQ is that value of X_s^R for which $X_s^R - w_s = w_0$.

In order to test the usefulness of this approach as a sensitivity model for the most commonly used calibration in biomedical chromatographic assays eight different unweighted calibration data sets have been used with the straight line, nonzero intercept eqn. 1 to estimate the LLQ for each set. Processing of each set using TableCurve gave in this case the corresponding line coefficients A and B and the corresponding predicted X and Y values. These values were then imported into SigmaPlot and the prediction band and the LLQ estimate obtained as indicated above. The upper interval limit for the blank was increased by 5% to separate the two intervals.

The estimated LLQ for the eight full calibration sets and the two "short" sets are listed in Table I. Based on the LLQ the eight calibration lines can be arbitrarily divided into three groups, namely the highly satisfactory, satisfactory and

TABLE I

METHOD CALIBRATION SETS OBTAINED DURING VALIDATION OF NEWLY DEVELOPED DRUG ASSAYS AND THEIR ESTIMATED LLQ

Calibration line	Range[a]	Number of			r^2	LLQ	
		Concentrations	Replicates	Points		Concentration[a]	% range
1	0–200	8	3	24	0.9915	16.25	8.1
2	0–300	14	3	42	0.9999	1.37	0.5
2A	0–75	4	3	12	0.9999	1.03	1.4
3	0–300	14	3	42	0.9999	1.46	0.5
4	0–3000	14	18	252	0.9999	0.53	0.2
5	0–50	10	3	30	0.9965	2.41	4.8
6	0–50	10	3	30	0.9843	5.13	10.3
7	0–320	13	9	117	0.9759	19.9	6.2
8	0–20 000	12	9	108	0.9912	781	3.9
8A	0–10 000	4	3	12	0.9991	419	4.2

[a] The units for curves 1–6 are μg/ml or μg/g, for curves 7–8A ng/ml or ng/g.

unsatisfactory, depending on the percentage of the range that lies below the LLQ (last column in table). Calibration lines 2, 2A, 3 and 4 are highly satisfactory and calibration line 6 is unsatisfactory, assuming that the full range of concentrations is needed for the intended purpose of the assay. The value of the LLQ can also be used to compare intra-day and inter-day calibrations, such as sets 2 and 3. In this case very little difference was observed, therefore, most of the variability comes from within-day factors. Calibration set 4, containing all calibration points for this assay, showed an improvement in the LLQ, however, the decrease by 0.9 μg/ml within a range of 300 μg/ml does not warrant the increased effort. The short set 2A, that represents a typical calibration set of four concentrations used during analysis of unknown study samples, gave a very comparable LLQ. This was also true for sets 8 and 8A.

As can be seen from Fig. 2 the LLQ depends on the width of the prediction band around the calibration regression line assuming the calibration model is valid. This in turn is dependent on the total number of calibration points, their position (spread) within the range and their individual variances from the regression line. It also depends on the number of replicate measurements per concentration and the size of the range. For any given assay and concentration range the LLQ can be optimized by the proper selection of the number of standards and the concentrations per calibration set. It is recommended that a matrix blank is always included.

The proposed definition of the LLQ is based on the statistical concept of confidence intervals around the predicted value of X, as discussed by Draper and Smith [15]. These intervals could also have usefulness as possible weighting factors in expressing the relative reliability of predicted analyte concentrations in unknown study samples.

We have only described the estimation of the LLQ in the simplest and most frequently used case of unweighted least-squares fitting. In principle it can be applied to any type of calibration curve, weighted or unweighted, such as second-degree polynomial or transformed equations, whenever a prediction band can be obtained. For biomedical assay calibration unweighted data fitting to a straight line are frequently applicable within a specific range of concentrations in which the "constancy of variance" condition is reasonably fulfilled. If this condition is not true data weighting or transformation is recommended which leads to a narrower prediction band and an improved LLQ. However, it is the analyst's judgement, based on the degree of expected improvement and the computational tools available, which method of calibration data processing he uses.

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