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# Alternative Calibration Strategies for the Clinical Laboratory: Application to Nortriptyline Therapeutic Drug Monitoring

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BACKGROUND: The addition of a calibration curve with every run is both time-consuming and expensive for clinical mass spectrometry assays. We present alternative calibration strategies that eliminate the need for a calibration curve except as required by laboratory regulations.

METHODS: We measured serum nortriptyline concentrations prospectively in 68 patients on 16 days over a 2-month period using a method employing calibration schemes that relied on the measurement of the response ratio (RR) corrected by the response factor (RF), i.e., a measurement of the RR for an equimolar mixture of the analyte and internal standard. Measurements were taken with contemporaneous RF (cRF) measurements as well as sporadic RF (sRF) measurements. The measurements with these alternative calibration schemes were compared against the clinical results obtained by interpolation on a calibration curve, and those differences were evaluated for analytical and clinical significance.

RESULTS: The differences between the values measured by cRF and sRF calibration and interpolation on a calibration curve were not significant ($P = 0.088$ and $P = 0.091$, respectively). Both the cRF- and sRF-based calibration results demonstrated a low mean bias against the calibration curve interpolations of 3.69% (95% CI, −15.8% to 23.2%) and 3.11% (95% CI, −16.4% to 22.6%), respectively. When these results were classified as subtherapeutic, therapeutic, or supratherapeutic, there was categorical agreement in 95.6% of the cRF calibration results and 94.1% of the sRF results.

CONCLUSIONS: cRF and sRF calibration in a clinically validated liquid chromatography–tandem mass spectrometry assay yields results that are analytically and clinically commensurate to those produced by interpolation with a calibration curve.



The growing use of liquid chromatography–tandem mass spectrometry (LC-MS/MS)[3] in the clinical laboratory brings with it the ability to perform a broad range of quantitative assays with a single instrument. Many current protocols for the monitoring of therapeutic drug concentrations in serum are particularly reliant on LC-MS/MS. Therapeutic drug monitoring is integral to the standard of care for a growing number of clinical disciplines, including solid organ transplantation *(1)*, oncology *(2, 3)*, infectious disease management *(4–6)*, and psychiatry *(7)*. Although these tests provide valuable information that improves patient care by facilitating the maximization of the therapeutic effect and minimization of untoward side effects, the laboratory costs involved in performing these tests—either by MS or immunoassay—are not trivial *(8, 9)*.

After the initial expenditure involved in purchasing an instrument, one expensive feature of clinical MS as it is usually practiced in the US is the generation of a calibration curve for calculating results each time the assay is run, in contrast to the biannual calibration required by CLIA *(10)*. Contemporaneous calibration curve measurement for MS assays originated when the instruments were less stable than the ones currently in use in the clinical laboratory. Earlier instruments required more correction for day-to-day variability. There is currently no regulation that requires contemporaneous calibration curve measurement for MS assays. The US Food and Drug Administration requires the robust characterization of a relationship between the response ratio (RR) and analyte in the process of validation *(11)* but does not require calibration with

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[3] Nonstandard abbreviations: LC-MS/MS, liquid chromatography tandem mass spectrometry; RR, response ratio; RF, response factor; cRF, contemporaneous response factor; sRF, sporadic response factor; TCA, tricyclic antidepressant; NT, nortriptyline; IS, internal standard; d3-NT, deuterated NT; SA, single analyte; IR, input ratio.

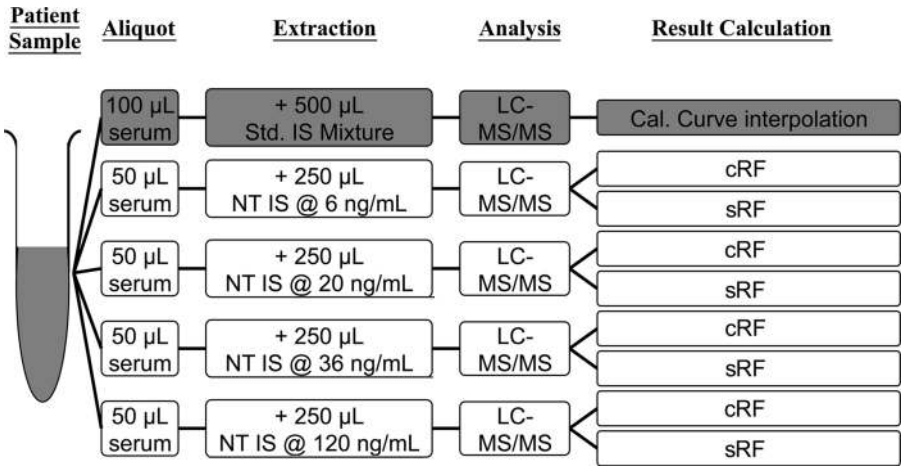


**Fig. 1. Experimental protocol for all the measurements used in this analysis.**
Shaded boxes represent the method used in the clinically reported method. Std., standard; Cal., calibration.

every assay run as part of the standard operating procedure. This relationship is a critical component in assay validation, but there is no requirement that it be repeated with each assay batch. Biannual calibration is sufficient for other kinds of analyzers such as immunoassays and ion-selective electrodes. In those methods, calibration is revisited only during the process of investigating QC failures or after instrument maintenance.

Alternatives to repetitive calibration curve measurement have been demonstrated in one study *(12)*. These strategies rely on the analyte-to–stable isotope RR corrected for the response factor (RF)—the ratio of an equimolar analyte and stable isotope ratio solution. Two variations of this approach are contemporaneous RF (cRF)-based calibration, in which the RF is measured for each assay batch, and sporadic RF (sRF)-based calibration, in which the RF is used over multiple days unless there is QC failure or major instrumental change. cRF- and sRF-based calibrations have been called one-point and no-point calibration, respectively, and no-point calibration has also been called internal calibration *(12)*. However, because all standard MS assays rely on internal calibration, the use of this term is too broad. In addition, the terms one-point and no-point calibration do not fully describe processes used in the calibration scheme. As such, the terms cRF- and sRF-based calibration are used here and recommended to avoid future ambiguities. The usefulness of these approaches has been demonstrated in pharmacokinetics investigations *(13)* and basic science research *(14)*. These laboratories generally analyze large batches of samples over a short period of time, whereas clinical laboratories analyze small numbers of patient samples as they are received. Thus, the impetus to decrease the calibration overhead is even more important in the clinical laboratory than it is in these other environments. Here, we describe the prospective use of these alternative calibration approaches for measuring the serum concentration of the tricyclic antidepressant (TCA) nortriptyline (NT) in a clinical laboratory environment.

## Methods

### PATIENT SAMPLES AND SELECTION

Residual blood samples submitted for routine therapeutic drug monitoring of NT over a 2-month period were used for the analyses. This study was conducted in accordance with a protocol approved by the Johns Hopkins Hospital Institutional Review Board.

### LABORATORY MEASUREMENTS

Clinical results were generated and reported with a previously described and validated method in the hospital-based CLIA certified laboratory. The existing method was a multianalyte assay that involved the addition of a calibration solution containing deuterated internal standards (ISs) for 4 TCAs (amitriptyline, desipramine, imipramine, and NT) *(15)*, but same chromatographic method was also used for a NT-only assay for the purposes of the current study. Briefly, the method was fully automated and included solid-phase extraction followed by a C-18 analytical column online with a TSQ Quantum Access instrument.

Serum remaining after the clinical analysis was divided into aliquots and analyzed with a calibration solution containing only deuterated NT (d3-NT) at differing concentrations, as outlined in Fig. 1. The d3-NT

and NT materials were obtained from Cerilliant (Round Rock) and had certified purities of 99.5% and 99.8%, respectively. The lack of interference between the NT analyte and IS was confirmed (see Table 1 in the Data Supplement that accompanies the online version of this report at http://www.clinchem.org/content/vol59/issue6). Each sample was run in quadruplicate with IS concentrations of 6, 20, 36, and 120 ng/mL. These ISs were prepared by gravimetric weighing and dissolving in methanol to make the 120 ng/mL calibration standard. The lower concentrations were made by dilution of this solution. All calibration solutions were made in a single batch and used for the duration of this study.

Because of sample limitations, the serum volume used for the experimental protocols was half that used in the clinical protocol and previously published method *(15)*. The IS calibrator volume was also halved. The single-analyte (SA) measurements were otherwise analyzed with the same method and on the same instrument and day as the clinical samples. In addition to the patient samples, for each day the RF was measured in quadruplicate.

#### CALIBRATION CURVE INTERPOLATION

Eq. 1 describes the linear relationship between the RR of $A_A$ (the area of the analyte signal) and $A_{IS}$ (the area of the IS signal) with the input ratio of $C_A$ (the concentration of analyte) to $C_{IS}$ (the concentration of IS). The constants *f* and *k* represent the RF and the intercept of the linear fit, respectively:

$$\frac{A_A}{A_{IS}} = f\frac{C_A}{C_{IS}} + k \quad (1)$$

Regressions for the calibration curves were carried out using the native instrumental clinical software with $1/x$ regression weighing *(16)*. Interpolation to yield the clinically reportable value of $C_A$ was achieved with the rearranged formula in Eq. 2:

$$C_A = \frac{C_{IS}}{f}\left(\frac{A_A}{A_{IS}} - k\right) \quad (2)$$

#### cRF- AND sRF-BASED CALIBRATION

For cRF-based calibration, we converted RRs for each of the measurements were converted into serum drug concentrations by using Eq. 2. However, rather than fitting the slope and intercept constants from linear regression of a calibration curve, the intercept was eliminated from this equation, and a single measurement of the RF was used for *f*. Applying these simplifications yields Eq. 3:

$$C_A = \frac{C_{IS}}{f} \times \frac{A_A}{A_{IS}}. \quad (3)$$

In the sRF-based calibration scheme, the value for *f* was not necessarily measured each day. If no instrument maintenance, probe manipulation, mass calibration, or QC failures occurred in between the time of the last RF measurement and the assay run, then the most recent RF was used for *f*. This was the case in 8 of 16 of the assay run days. On days in which these events did occur between the time of the last RF measurement and the assay run, the RF was measured in quadruplicate, and the mean of these values was used for *f* in Eq. 3.

## Results

#### CALIBRATION CURVES

Summaries of the calibration curves for the SA assay are shown in Fig. 2. These were plotted in a unitless RR vs input ratio (IR) graph to facilitate objective comparisons with the unity line. Calibration curves were compared against the unity line because an ideal calibration curve would have an RR vs IR slope of 1 and an intercept of 0. This was a convenient tool for evaluating the assay because it was easy to interpret, well suited to formal statistical analysis, and sensitive to IS impurity and isotopic or chromatographic carryover. When compared against a unity line with a 5% CV, there was no statistical difference between the slope of that line and the calibration curve performed with an IS concentration of 36 ng/mL ($P = 0.064$). The intercept was 0 and not different from that of the line of unity ($P = 0.20$).

Although all of the calibration curves had insignificant constant bias, as evidenced by the fact that the *y*-intercept values had values indistinguishable from 0, none of the other IS concentrations generated a calibration curve that had the same slope as the unity line. This most likely reflected the calibration range. In the method, the IS solution was introduced to the calibrator in 5-fold excess. Thus, it was equimolar with the calibrator when the calibrator was at 150 ng/mL. The calibrators ranged in concentration from 15.625 to 1000 ng/mL or from .011 to 6.7-times the IS concentration. Thus, the assay would be linear in this range because it bracketed 1. In contrast, the 6-ng/mL IS solution would be equimolar with a calibrator at 25 ng/mL, so the calibrators were 0.625- to 64-fold of this IS concentration. With this much broader range, the calibration curve was expected to differ significantly from its expected result *(17)*, and that is what was observed.

#### PATIENT RESULTS WITH cRF-BASED CALIBRATION

The calibration curve experiment demonstrated which IS best reflected the desired linear range of the clinical assay. However, therapeutic targets usually fall in a much narrower therapeutic window. To determine if the ideal IS concentration for reproducing calibration curves was also the ideal IS concentration for patient samples in the alternative calibration schemes, patient samples were also measured with 4 different IS concen-

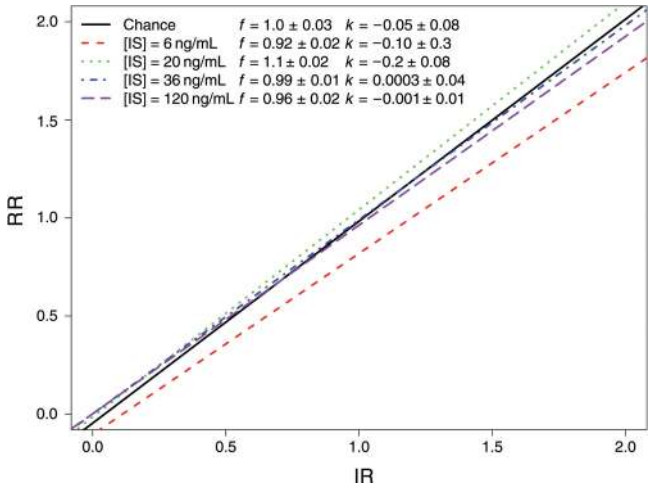


**Fig. 2. RR vs IR curves for calibrators measured with a single IS at different concentrations.**

The dot–dash line representing the calibration curve with 36 ng/mL of IS shows significant overlap with the solid unity line. The highest proportional bias is seen in the dashed line representing the calibration curve with 6 ng/mL of IS.

trations. The cRF calibration results for 68 patients on 16 days of assay operation are plotted against the clinical values in Fig. 3A. The results for all 4 IS concentrations are plotted using different symbols and line styles. This graph shows that the best agreement between the clinical result and the cRF calibration result was achieved with the 36 ng/mL concentration of IS. The summary of the regression statistics for these lines can be seen in online Supplemental Table 2. The difference between the patient samples and the cRF-based calibration showed no significant proportional bias ($P = 0.088$).

A Bland–Altman analysis of the calibration curve and cRF calibration measurements using 36 ng/mL is shown in Fig. 3B. The overall bias and CIs demonstrated reasonable interchangeability with the results obtained from the calibration curve. Because this choice of IS was most suited for drug concentrations close to 150 ng/mL, concentrations closest to this point showed the least bias. Concentrations farther from this one showed greater bias. Specifically, at drug concentrations of >100 ng/mL, cRF calibration had a bias of −1.2% (95% CI, −15% to 12%), but at concentrations ≤100 ng/mL the bias was 6.0% (95% CI, −15% to 27%). Online Supplemental Table 3 shows the full Bland–Altman analyses for the other concentrations of IS. As expected, using the next lower concentration of IS, 20 ng/mL, yielded measurements that were less biased at concentrations <100 ng/mL (−6.4%, 95% CI, −28% to 15%) than measurements of the higher drug concentrations (−14%, 95% CI, −34% to 4.7%).

### PATIENT RESULTS WITH sRF-BASED CALIBRATION

sRF-based calibration results for 68 patients on 16 days of assay operation are plotted against the clinical values in Fig. 4A. The results for all 4 IS concentrations are plotted with different symbols and line styles. This graph demonstrates that the best agreement between the clinical result and the sRF calibration result was again achieved with the 36 ng/mL concentration of IS. The summary of the regression statistics for these lines can be seen in online Supplemental Table 4. As with the cRF calibration measurements with this concentration of IS, the difference between the patient samples and the cRF-based calibration showed no significant proportional bias ($P = 0.091$). A Bland–Altman analysis of the calibration curve and sRF-based calibration measurements using 36 ng/mL IS is shown in Fig. 4B. Interestingly, as was seen in the cRF-based calibration scheme, the higher drug concentrations were least biased from the calibration curve results. Online Supplemental Table 5 shows the full Bland–Altman analyses for the other concentrations of IS. As in the cRF calibration experiments, the bias was smaller for the lower drug concentrations when a lower concentration of IS was used.

### STABILITY OF THE RF

The sRF-based calibration scheme used the same RR measurements as the cRF calibration scheme except for the RF measurements. If the RF were infinitely precise, cRF and sRF would be interchangeable. Fig. 5 shows the values for the RF over the days in which the measurements were made. The red dots show days in which the

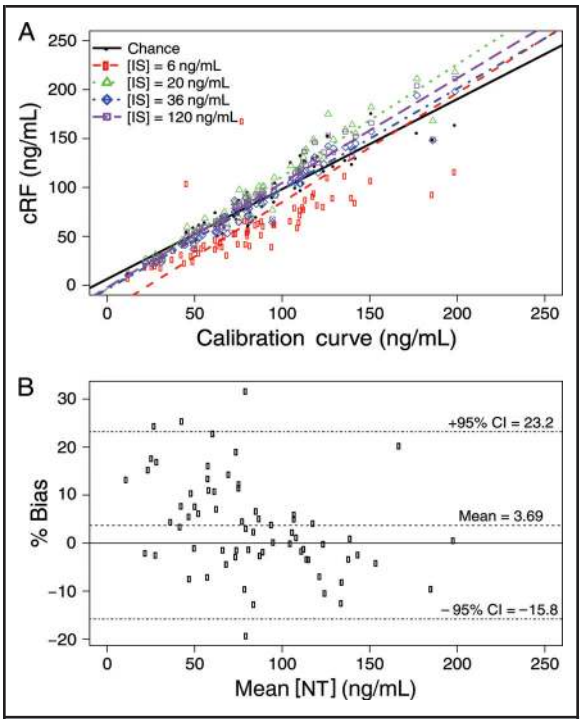


**Fig. 3. Patient results obtained by the cRF-based calibration scheme vs the clinically reported results obtained by interpolation with the calibration curve.**

(A), Patient results measured with 4 different IS concentrations; (B), Bland–Altman analysis of the calibration curve results and the cRF-based calibration scheme with an IS concentration of 36 ng/mL. [NT], nortriptyline concentration.

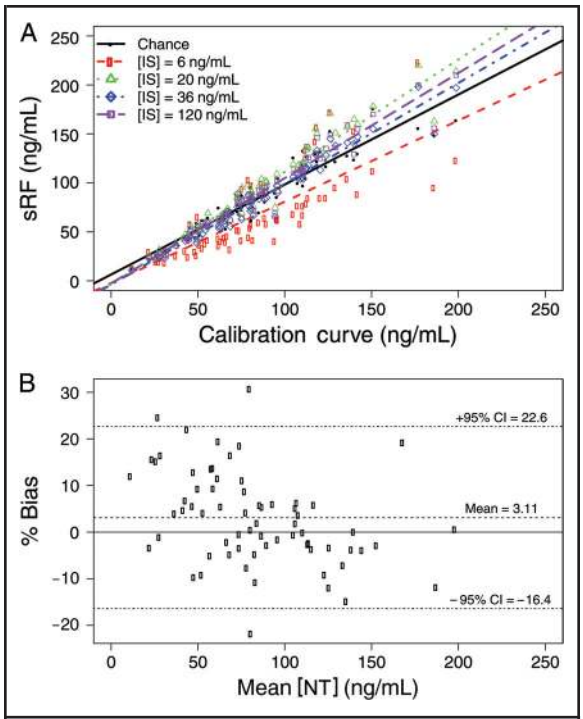


**Fig. 4. Patient results obtained by the sRF-based calibration scheme vs the clinically reported results obtained by interpolation with the calibration curve.**

(A), Patient results measured with 4 different IS concentrations; (B), Bland–Altman analysis of the calibration curve results and the sRF-based calibration scheme with IS concentration of 36 ng/mL. [NT], nortriptyline concentration.

RF was reset for the purposes of sRF calibration because of either instrument manipulation or mass calibration, and the black dots represent days in which the RF was measured and used for cRF-based calibration but not for sRF-based calibration. The RF itself demonstrated significant variance. cRF-based calibration had the potential to correct for sources of variance in the measurement that may have been overlooked in an sRF-based calibration scheme.

### CLINICAL IMPACT OF THE CALIBRATION SCHEMES

To investigate whether the change in calibration scheme would affect the clinical significance of these measurements, the values produced under both schemes and the calibration curve calibration method were divided into clinical decision groups: subtherapeutic (<50 ng/mL), therapeutic (50–150 ng/mL), and supratherapeutic (>150 ng/mL) *(18)*. The distribution of patients is shown in Table 1. Overall, the agreement for cRF calibration and the calibration curve was reasonable, with 95.6% (65 of 68) patients showing no clinically significant difference in results. Of the 3 results that showed a categorical difference, the values for both the calibration curve and the alternative result were close to the dividing line between categories. No results in either the calibration curve or the alternative calibration schemes were close to the critical action value of 500 ng/mL, so the impact of these differences, if any, would be the consideration of dose adjustments rather than rapid intervention in any of the cases. The clinical categorical breakdown of the sRF-based calibration results were similar to those of the cRF-based calibration results. There was categorical agreement in 94.1% (64 of 68) of the cases. The patients with discordant results under the cRF-based calibration scheme also had discordant results under the sRF-based calibration scheme. In addition, the sRF-based calibration resulted in an additional discordance: the upgrade in 1 sample from subthera-

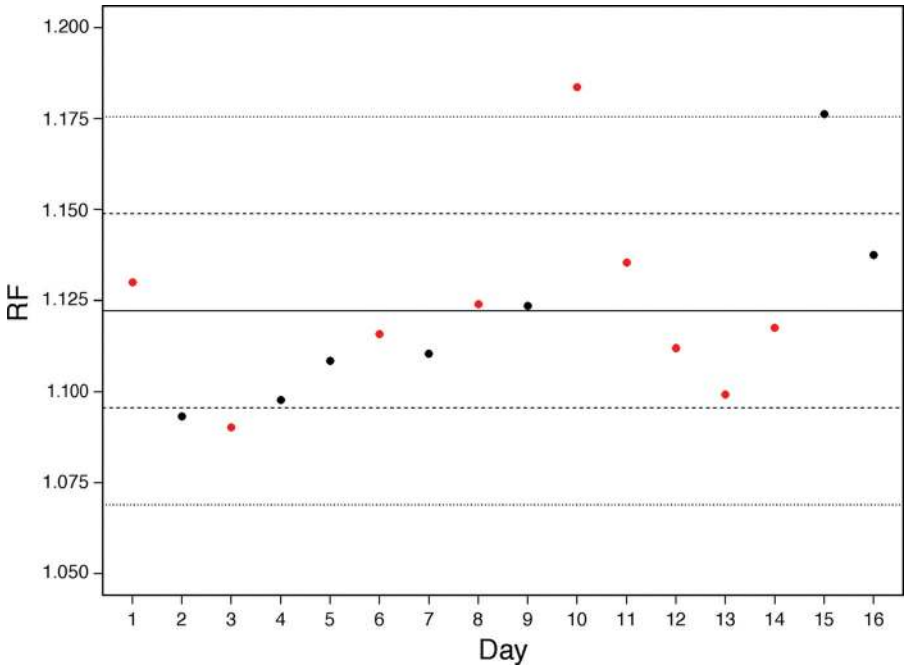


**Fig. 5. Value of the RF over the days on which the measurements were taken.**
The solid line represents the sample mean; the dashed and dotted lines represent 1 and 2 SDs, respectively. Red points represent the days on which the RF was reset for the sporadic calibration scheme.

peutic in the clinical results to therapeutic in the sRF-based calibration results.

## Discussion

The measurement of multiple analytes with a single LC-MS/MS assay is commonplace in clinical MS, even when there is no clinical benefit to bundling all the analytes measured in the assay. The bundling of analytes into single assays is done primarily to minimize the amount of time and resources that a laboratory spends on calibration and quality control; such is the case for TCAs. TCAs are prescription drugs with negligible abuse potential, so the clinician knows which TCA to monitor on the basis of the drug that is prescribed. The majority of patients on a TCA take a single TCA at a time *(19)*, and a second TCA (often a biologically active metabolite) can be monitored separately if clinically appropriate. Thus, SA assays for each TCA are clinically sufficient. Combining every possible drug into a single analysis is not only clinically indefensible, it runs the risk of chromatographic and isotopic carryover, and ion suppression and augmentation for coeluting analytes, as others have shown *(20)*. For both clinical and analytical reasons, SA drug assays are theoretically superior to multiple analyte assays. An alternative calibration strategy such as the one presented here reduces the calibration overhead for SA assays and renders them clinically and financially viable.

MS is unique among the analytical modalities in the clinical laboratory in that it requires an IS to control for matrix effects on ionization in the analytical process. Classically the ideal linear range of the RR is found in the 2 orders of magnitude that bracket unity

**Table 1. Correlation of cases in the clinical decision ranges for the cRF and sRF calibration schemes.[a]**

| | Calibration curve, ng/mL | | |
|---|---|---|---|
| | <50 | 50–150 | >150 |
| cRF calibration, ng/mL | | | |
| <50 | **13** | 2 | 0 |
| 50–150 | 0 | **49** | 1 |
| >150 | 0 | 0 | **3** |
| sRF calibration, ng/mL | | | |
| <50 | **12** | 2 | 0 |
| 50–150 | 1 | **49** | 1 |
| >150 | 0 | 0 | **3** |

[a] Values in bold text cells indicate full agreement between the alternative calibration and the calibration curve strategy. Other values indicate clinically relevant discordance.

*(17, 21)*. Contemporary experimental LC-MS/MS protocols have demonstrated a linear response beyond these confines *(22, 23)*, but these extensions of the dynamic range have yet to be realized in clinical MS and are beyond the dynamic range required for TCA monitoring in live humans. In any case, the choice of the IS concentration should be made considering the desired analytical range as well as the required clinical performance of the assay. The experiments here were performed with different concentrations of IS to reiterate the point that the ideal analytical and clinical performance of the assay occurs when the IS concentration is placed so that ratio of the calibrators and clinical values to the IS concentration brackets 1.

The data presented here do not show dramatic differences between the cRF- and sRF-based calibration schemes. If this observation holds for other assays and in other laboratory settings, then sRF-based calibration could prove to be a clinically useful calibration method. However, the results here are insufficient to endorse the use of sRF-based calibration in a laboratory environment similar to the one used for these experiments. Given that the instrument used in these experiments serves multiple research and clinical purposes other than the assay shown here, it undergoes more manipulations than a dedicated instrument would have to undergo. As a result, the number of days in which the RF was truly sporadic was small. Thus, in practice, the savings of internal calibration would be limited. This feature of our design is a limitation in extrapolating how these calibration schemes would function in a larger laboratory environment. Nevertheless, because clinical MS appeals to an ever-broader range of laboratory practice settings, it is likely that other laboratories also use the same instrument for multiple assays and thus will therefore see the need to perform cRF- rather than sRF-based calibration. Furthermore, although these data suggest that the generation of a calibration curve with every assay run is unnecessary, the inefficiency of doing this would affect low-throughput more than high-throughput laboratories because the ratio of calibrators to billable results goes down with a higher patient volume.

In conclusion, our study demonstrates that cRF- and sRF-based calibration in a clinically validated LC-MS/MS assay yields results that are analytically and clinically commensurate to those produced by interpolation with a calibration curve.

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