

Toward Standardization of Insulin Immunoassays

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BACKGROUND: Measurement of circulating insulin may improve the classification and management of diabetes mellitus and assist in treating people with insulin resistance.

METHODS: A work group convened by the American Diabetes Association evaluated results for a panel of 39 single donor sera measured by 10 commercial insulin methods from 9 manufacturers against an isotope dilution–liquid chromatography/tandem mass spectrometry (IDMS) measurement procedure calibrated using purified recombinant insulin. We used a candidate primary (pure substance) reference material, pooled serum, and single donor sera to evaluate approaches to achieve improved agreement of results between the routine and reference measurement procedures.

RESULTS: Four of 10 methods had $\geq 95\%$ of individual serum results within 32% of the IDMS concentrations. However, the bias vs IDMS was more than 15.5% for 7 of 10 methods in 36%–100% of individual samples. A purified recombinant insulin preparation used as a common calibrator did not improve harmonization of results among routine methods but was not used as instructed by all participants. Calibration using serum pools achieved bias $< 15.5\%$ for nearly all results in the concentration range covered by the pools (> 60 pmol/L). Calibration using a panel of individual sera was the most effective to improve harmonization of results over the full measuring range.

CONCLUSIONS: Agreement among methods can be improved by establishing traceability to the IDMS procedure using a panel of native sera. Pooled sera may be useful as trueness control materials. The usefulness of the pure insulin primary reference material [candidate

reference material for insulin (cRMI)] requires clarification of protocols used by manufacturers.

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Measurement of circulating insulin concentrations in serum or plasma may improve the classification and management of diabetes mellitus and assist in treating people with insulin resistance. It is critical that research considered in the formulation of clinical practice guidelines be based on harmonized insulin results. Such harmonization will allow aggregation of information from multiple investigations, enabling evidence-based criteria to incorporate insulin concentrations consistently across assays and laboratories. Robbins et al. (1) reported in 1996 that research and clinical laboratory insulin methods were not adequately standardized to support comparisons among different methods. A work group of the American Diabetes Association in conjunction with the National Institute of Diabetes and Digestive and Kidney Diseases, the European Association for the Study of Diabetes, the CDC, and the IFCC was established to develop guidelines for measurement performance requirements and to develop a standardization program to achieve sufficient agreement between methods.

Recent evaluations (2–4) of routine methods for measuring circulating insulin concentrations concluded that although concordance could be improved, the current harmonization among methods represented an improvement from the earlier report (1). The workgroup previously reported (2) that (a) many routine immunoassays were specific for insulin and had negligible cross-reactivity with proinsulin and cleaved intermediate products of proinsulin conversion to insulin; (b) insulin concentrations should be reported in pmol/L; (c) agreement of results among routine immunoassays needed improvement; and (d) an isotope dilution–liquid chro-

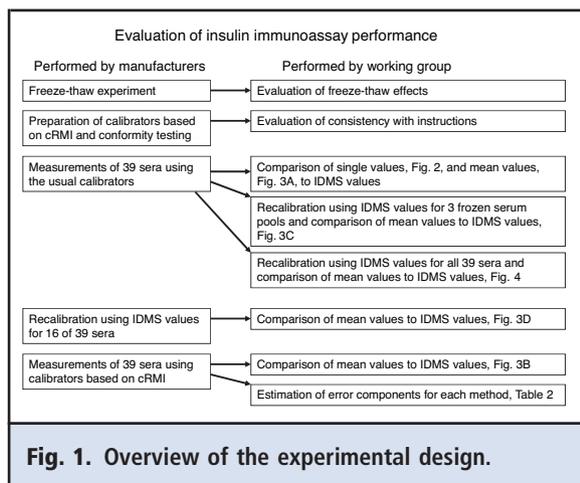
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matography/tandem mass spectrometry (IDMS)⁷ measurement procedure calibrated with recombinant human insulin should be used as the basis for calibration traceability.

We report here an investigation of agreement of results for a panel of 39 single-donor sera measured by 10 commercial insulin methods from 9 manufacturers compared with results from an IDMS measurement procedure calibrated using purified recombinant insulin. We also report the effectiveness of a candidate primary (pure substance) reference material, pooled serum, and a panel of single donor sera to achieve improved agreement of results between the routine and reference measurement procedures.

Materials and Methods

Details of materials and methods, including participating manufacturers, are available in a Supplemental Data file that accompanies the online version of this article at www.clinchem.org/content/vol55/issue5. Fig. 1 provides an overview of the experimental design, and a brief description is provided below.

IDMS MEASUREMENT PROCEDURE

The electrospray ionization IDMS procedure has been reported (3, 5). Highly purified, recombinant human insulin corporate standard provided by Novo Nordisk was used for calibration of IDMS, with verification using corporate standard from Eli Lilly and Company.

SERUM SAMPLES AND CANDIDATE REFERENCE MATERIAL FOR INSULIN (cRMI)

A set of 39 single-donation sera were collected from donors, aliquoted, and frozen at -70°C using a modified Clinical and Laboratory Standards Institute C37-A protocol (6). Three pooled serum samples were prepared from frozen units of the single-donation sera, aliquoted, and frozen at -70°C . The influence on insulin of freezing and thawing was investigated. There was no freeze-thaw effect for 9 of 11 methods; for 2 methods, however, an effect could not be ruled out (see online Supplemental Data file).

Pure recombinant crystalline insulin was used to prepare a lyophilized preparation as a candidate reference material for insulin (cRMI) that, when reconstituted, had an expected concentration of 360 000 pmol/L. Each manufacturer was asked to prepare a set of cRMI-based calibrators, spanning the usual measuring range and mimicking the usual method calibrators in response. The high concentration of the cRMI was chosen to enable a near-complete change of buffer matrix around the insulin using each manufacturer's normal calibrator buffer matrix.

MEASUREMENT PROTOCOL FOR PARTICIPANTS

The manufacturers measured insulin in the set of single-donation sera, serum pools, and diluted cRMI working calibrators using each method's usual procedure and product calibrators with a randomized sequence of samples that was defined for each run by the study protocol. The cRMI-based working calibrators were used to generate a second set of results for the single-donation sera and serum pools. After all results had been submitted to the study organizers, the IDMS results for 16 of the single-donor sera were provided to each manufacturer and used to recalculate results for the remaining 23 single-donor serum samples, thus establishing traceability to IDMS.

ESTIMATION AND INTERPRETATION OF ERROR COMPONENTS

We estimated the contributions from different potential error sources shown in Table 1 using an error model for the differences observed between routine method and IDMS results (7).

Results

COMPARABILITY OF ROUTINE-METHOD SINGLE-MEASUREMENT RESULTS TO IDMS

Fig. 2 shows the first reportable result, of the replicate reportable results from each manufacturer, for each individual serum sample for each routine method compared to the IDMS mean result. Four of 10 methods (Alpco, Mercodia, Roche, Siemens Im-

⁷ Nonstandard abbreviations: IDMS, isotope dilution-liquid chromatography/tandem mass spectrometry; cRMI, candidate reference material for insulin; IRP, international reference preparation; JCTLM, Joint Committee for Traceability in Laboratory Medicine.

Table 1. Error components and their possible sources.		
Error component	Notation	Possible sources
Error component between consecutive measurements within runs	e	1. Variation in performance conditions within runs
Error component between different positions within a run	c	2. Trends in performance conditions within runs (position effects)
Sample-specific error	d	3. Differences in influence quantities between native samples
Error component between runs (may be a function of the concentration μ)	$b(m)$	4. Random error in the estimation of the calibration curve (due to the variation within runs)
Common systematic error component (may be a function of the concentration)	$\delta(m)$	5. Interaction between performance conditions and calibrator properties
		6. Error in the assigned value of the reference material used for preparation of calibrators
		7. Variation between vials of reference material
		8. Errors in the preparation of the calibrators
		9. Unsuitable model for the calibration curve
		10. Unsuitable concentration levels of the calibrators
		11. Noncommutable calibrators (differences in influence quantities between calibrators and native samples)

mulite) had $\geq 95\%$ of results that were within the 32% total error allowance for desirable method performance based on biologic variability criteria (2). Abbott and Beckman-Coulter had acceptable total error over most of the measuring range, with lower concentration results exceeding the criterion. Siemens Centaur and Tosoh had a fairly consistent bias

over the measuring range. Millipore RIA had consistent bias over much of the measuring range that became progressively larger at lower concentrations. Invitron had excessive imprecision at lower concentrations noted as individual results that exceeded both the upper and lower 32% criterion.

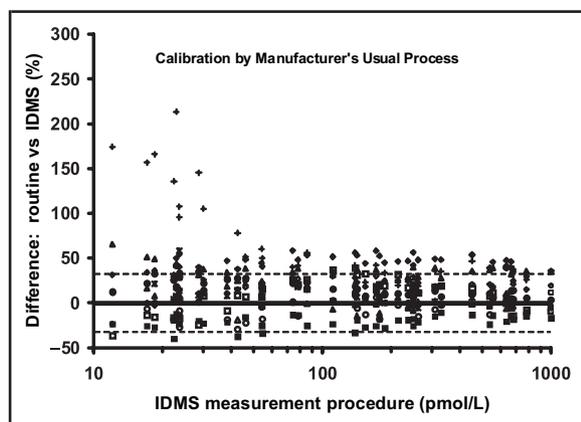


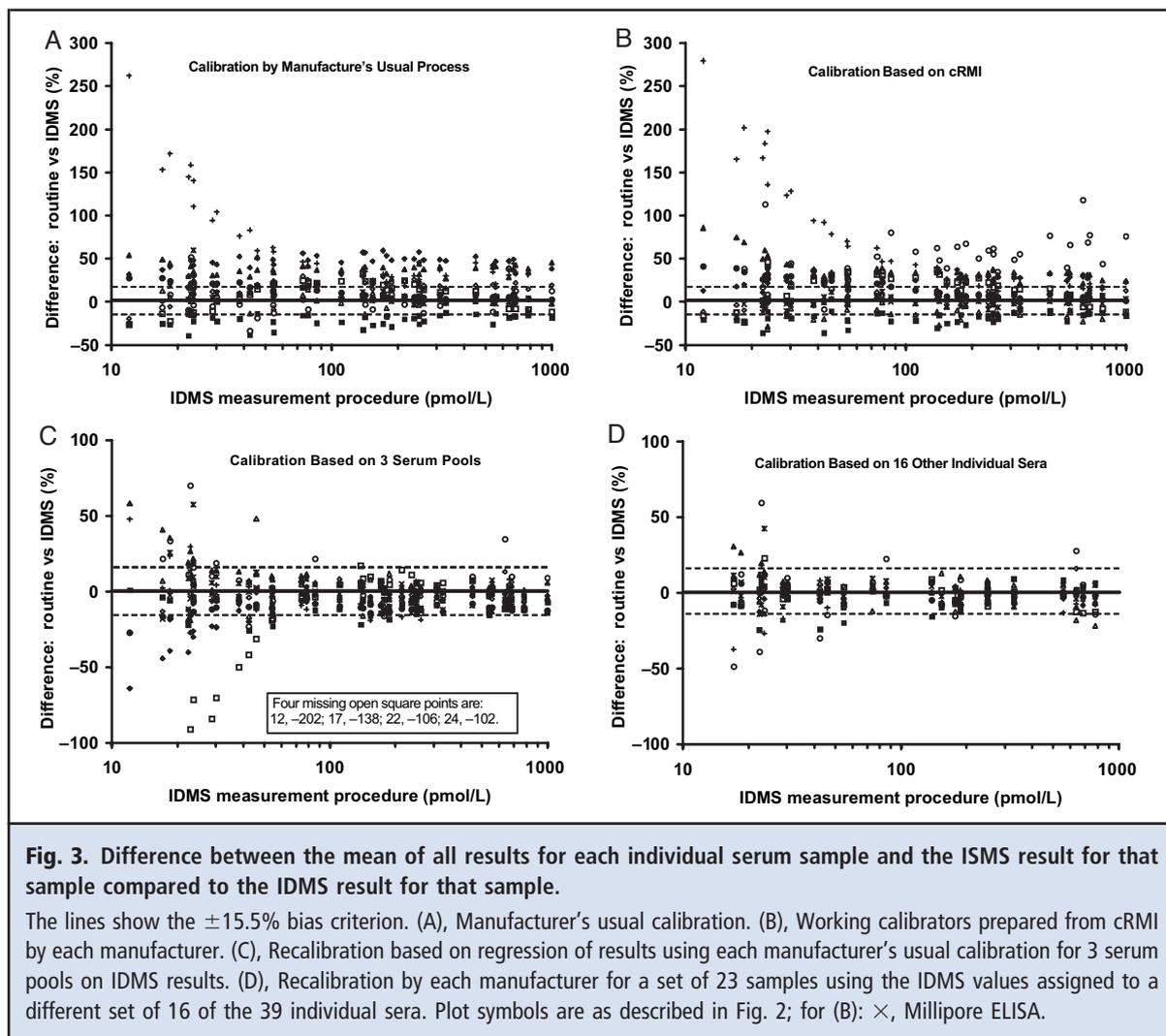
Fig. 2. Differences between the first reported result (singlet measurement) for each individual serum sample and the IDMS result for that sample compared to the IDMS result for that sample.

The lines show the $\pm 32\%$ total error criterion. ●, Abbott; △, Alpc; ■, Beckman-Coulter; □, Invitron; *, Mercodia; +, Millipore RIA; ◇, Roche; ▲, Siemens Advia Centaur; ○, Siemens Immulite; ◆, Tosoh.

BIAS OF ROUTINE METHOD MEAN RESULTS TO IDMS

Fig. 3 shows the bias of the mean of all replicate results for each sample using different calibration procedures for each routine method vs the IDMS mean result. Fig. 3A shows results using the manufacturer's usual calibration. Alpc, Mercodia, and Siemens Immulite methods had 5%, 14%, and 18%, respectively, of individual sample biases (primarily at lower concentrations) that exceeded the 15.5% desirable allowable bias for insulin measurement based on biologic variability criteria (2). Seven of 10 methods (Abbott, Beckman-Coulter, Invitron, Millipore RIA, Roche, Siemens Centaur, Tosoh) had 36%–100% of sample biases that exceeded the criterion. Millipore RIA had an approximately constant bias over most of the measuring range, with a progressively increasing bias at lower concentrations.

Fig. 3B shows that when cRMI was used for calibration, Mercodia, Millipore ELISA, and Roche methods each had 97% of biases within 15.5% of the IDMS values. The remaining 8 methods had 41%–100% of individual samples that exceeded the 15.5% bias criterion. Note that Millipore ELISA results appear only in this figure and the online Supplemental figures, where



calibration was based on cRMI, because the usual product calibrators were reported to be unstable by Millipore.

Fig. 3C shows the results after recalculation of the individual donor results using the linear regression of the results for 3 native serum pools on their IDMS insulin values (50, 217, and 513 pmol/L) to harmonize results. All methods had biases within the 15.5% criterion for nearly all samples with concentrations >60 pmol/L. For concentrations <60 pmol/L, the Invitron method had a pronounced negative bias, and that method also had a poor fit for the linear regression of pool results on the IDMS values. For concentrations <30 pmol/L, 5 methods had progressively increasing negative or positive biases as concentrations decreased.

Fig. 3D shows bias for a set of 23 samples after calibration by each manufacturer using the IDMS

values assigned to a different set of 16 of the 39 individual sera. With this approach, 6 of 10 methods (Abbott, Invitron, Mercodia, Millipore ELISA, Roche, Tosoh) had 0 or 1 result with bias >15.5%, and the remaining 5 of 10 methods had 3–7 results (13%–30%) that exceeded 15.5% bias. Most biased results occurred at concentrations <60 pmol/L, and the percentage bias increased in magnitude as concentrations decreased.

Fig. 4 shows the bias remaining after the mean from each of the 39 individual sera was used to fit either a second- or third-order polynomial to the relationship between results using each manufacturer's usual calibration and the IDMS results. A second-order polynomial was used for the Alpcos, Beckman-Coulter, Siemens-Immulite, and Tosoh methods, and a third-order polynomial was used for the Abbott, Invitron, Mercodia, Millipore RIA, Roche, and Siemens-

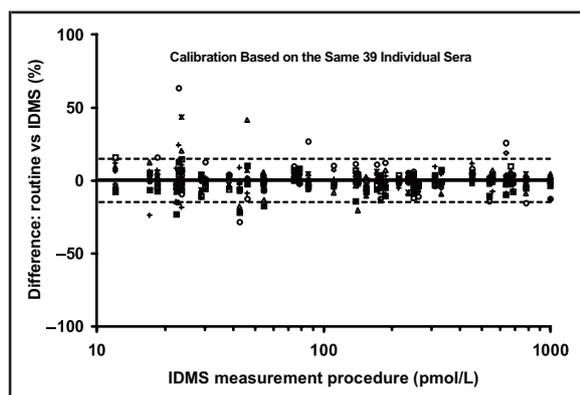


Fig. 4. The difference between the mean of all results for each individual serum sample and the IDMS result for that sample compared to the IDMS result for that sample after recalibration by fitting either a second- or third-order polynomial to the relationship between results using each manufacturer's usual calibration and the IDMS results for each of the 39 individual sera; plot symbols are as described in Fig. 2.

The lines show the $\pm 15.5\%$ bias criterion.

Centaur methods. Seven of 10 methods (Abbott, Invitron, Mercodia, Roche, Siemens Centaur, Tosoh) had 0 or 1 result with bias $>15.5\%$, and the remaining

4 of 11 methods had 3–6 results (13%–26%) that exceeded 15.5% bias. Most of the biased results occurred at concentrations <60 pmol/L, and the percentage bias increased in magnitude as concentrations got lower.

ESTIMATES OF ERROR COMPONENTS

Table 2 presents a summary of the error components for each method. Online Supplemental Figs. S3 to S17 show the difference plots for each routine method. The SDs of the error sources 6–8 in Table 1, $s_{\delta-known}$ were less than or equal to 0.03 for all manufacturers and gave a negligible contribution to the total error.

Based on the error model, both Mercodia and Roche satisfied the total error requirement. For Abbott, Siemens Centaur, and Tosoh, bias was the only meaningful contributor. For Beckman-Coulter and Invitron, the bias and sample specific effects (s_d) should be reduced. For Alpcos, both s_d and SD between runs (s_b) should be reduced, and there may be important contributions from sources 9–11 in Table 1. For Millipore-ELISA, the s_b should be reduced. For Millipore-RIA, there were meaningful contributions from bias, s_d , s_b , and random error within runs (s_e). For Siemens Immulite, bias, s_d , and s_b should be reduced. Alpcos and Millipore RIA also had contributions to total error from inadequate calibrator concentrations or algorithms to fit the calibration relationship over the measuring range.

Table 2. Estimates of error components; all measures are for $\ln(\text{concentration})$.

	Sources ^a (SD ^b)					
	1 (s_e)	2 (s_c)	3 (s_d)	1–3 ($s_{opt-cal}$)	4–5 (s_b)	4–11 (bias function)
Abbott	0.029	0.009	0.016	0.034	0.017	0.0–0.3
Alpcos	0.034	0	0.110	0.115	0.079	≈ -0.2
Beckman-Coulter	0.012	0.019	0.094	0.097	0.046	≈ -0.2
Invitron ^c	0.059	0.042	0.067	0.099	0.045	0.0–0.3
Mercodia	0.024	0.028	0.072	0.081	0.038	≈ 0
Millipore-ELISA	0.051	0.048	0.019	0.073	0.091	≈ 0
Millipore-RIA ^d	0.089	0	0.065	0.110	0.103	0.2–1.2
Roche	0.020	0.022	0.040	0.050	0.020	-0.1–0.1
Siemens Centaur	0.024	0.044	0.019	0.054	0.013	0.2–0.5
Siemens Immulite ^{c,e}	0.066	0	0.145	0.159	0.100	0.1–0.5
Tosoh	0.023	0.007	0.035	0.042	0.030	0.15–0.25

^a See Table 1.

^b s_e , SD between consecutive measurements within runs; s_c , SD between different positions within a run; s_d , SD for sample-specific errors; $s_{opt-cal}$, SD for an optimally calibrated method; s_b , SD between runs (calculated from s_c and s_B , the SD between runs reported by the manufacturer that includes both s_b and s_c); bias function, a systematic error component, $\delta(\mu)$ in Table 1. Refer to the online Supplemental Data file for details.

^c No values of preparation uncertainty for the calibrators were reported by the manufacturer. A value of 0.05 was assumed.

^d No values of s_B were reported by the manufacturer. The same values as for Millipore-ELISA were assumed.

^e No values of s_B were reported by the manufacturer. A value of 0.1 was assumed.

Based on work reported anonymously (2) and manufacturers' instructions for use, 2 of the methods had cross-reactivity with intact proinsulin or its split products that may have influenced the sample-specific effects observed. Total proinsulin, including intact molecule and its split products, was measured in the single donor sera using a Human Proinsulin ELISA (Merckodia AB) procedure. No meaningful correlations with proinsulin concentrations were found for the differences in insulin concentrations measured by a given method and the IDMS procedure (see online Supplemental Data file for details). Consequently, cross-reactivity with proinsulin was not likely to be an important influence on the overall differences between insulin results measured by the immunoassays and the IDMS procedures in this group of samples.

Discussion

A previous report (2) determined that desirable method performance based on biological variability consideration comprised a total error for a single measurement of $\pm 32\%$ and a desirable bias of $\pm 15.5\%$ compared with the reference method value. The biological variability criteria were based on measurement bias and imprecision and assumed there were no sample-specific interferences. It should be noted that small absolute differences at low concentrations will produce relatively large percent differences that may not be clinically meaningful.

However, small differences at low concentrations (<60 pmol/L) may be clinically significant in the investigation of remaining β -cell secretory potential in type 2 diabetes or using mathematical models to assess insulin resistance across a broad spectrum of obese and nonobese patients. Measuring low insulin concentrations to at least 10 pmol/L is clinically important for the investigation of hypoglycemia (8) and derived indices of insulin resistance and β -cell function used in epidemiological studies (9). The higher insulin concentrations of 1000 pmol/L or more found in syndromes of severe insulin resistance are within the measuring range of most insulin immunoassays.

We found that 4 of 10 routine methods using the manufacturer's usual calibration had $\geq 95\%$ of results for the first reportable result of several replicates (simulating typical laboratory practice where a single result is reported) within 32% of the IDMS values (Fig. 2). When the mean of replicate measurements of each sample was used to minimize the influence of imprecision, we found that only 1 of 10 methods had 95% of individual sample biases within 15.5% of the IDMS values (Fig. 3A). For most methods, the consistent bias over most of the measuring range should be correctable by appropriate traceability to a reference system. For

those methods with increasing bias at lower concentrations, the source of the bias may be multifactorial and may include the number of manufacturer's product calibrators, their concentrations, or the algorithm to fit concentrations to responses of calibrators.

We investigated 3 approaches to achieve improved agreement between the routine methods and IDMS results. First, a common calibrator, cRMI, combined with a given protocol for the preparation and conformity validation of the working calibrators prepared by dilution of cRMI was used to calibrate all routine methods. This approach did not improve the harmonization of measured insulin results (Fig. 3B). Some methods had improved agreement with IDMS, and others had poorer agreement. The reason for the failure was investigated from the conformity validation data reported by all participating manufacturers and from responses to follow-up questions (see online Supplemental Data file for details). That review indicated that the instructions and protocol for the establishment of the cRMI-based calibrators were not followed by all participants. Consequently, it was not possible to determine if cRMI could have been successful. Based on our findings, the usefulness of a common cRMI reference material cannot be ruled out.

A second approach to achieve improved agreement between methods used the linear regression of results for 3 native serum pools on the IDMS values to recalculate results for each individual sample (Fig. 3C). This approach was effective in reaching agreement with IDMS within the $\pm 15.5\%$ desirable bias criterion for insulin concentrations higher than approximately 60 pmol/L. At lower concentrations, use of serum pools was not effective, probably owing to inappropriate extrapolation of the regression relationship to concentrations below that of the lowest concentration pool.

In the third approach to achieve improved agreement between methods, manufacturers were provided the IDMS values for a set of 16 of the 39 individual serum samples and asked to use those values to recalibrate the original results for the remaining 23 sera (Fig. 3D). There were limitations in this approach, because manufacturers used different techniques for recalibration based on the native samples. Some used the serum panel as calibrators with the original measurement responses and the usual calibration fitting algorithms. Others used the serum panel to establish a mathematical relationship between the IDMS values and the original results, and used that relationship to recalculate the results for the remaining 23 samples. Five manufacturers used all 16 samples, 5 used 5–12 samples for various reasons, and 1 method with a 96-well format used 10 or 14 samples that were in a specific run. Similarly to recalibration based on the serum pools, this approach produced good agreement with IDMS for in-

sulin concentrations higher than approximately 60 pmol/L. Below 60 pmol/L, use of a panel of 16 sera also failed to achieve the desired agreement, but was generally more effective than serum pools because concentrations were included that extended to the lowest measuring ranges. The results suggest that when designing secondary reference materials, they should have a wide range of concentrations to enable calibration algorithms to fit the complete measuring range.

We used the mean results from all 39 individual sera to establish a mathematical relationship between each routine method and IDMS. Then, using that relationship, we recalculated results for each individual sample (Fig. 4). Either a second- or third-order polynomial was required to obtain the best fit to the nonlinear relationship between results over the measuring range for each method. This recalibration approach should minimize calibration bias and analytical imprecision and show primarily the influence of other sources of variability. At concentrations higher than approximately 60 pmol/L, the 39 sera approach gave a similar improvement in agreement to that observed using only 16 sera or serum pools. In contrast, at lower concentrations, the improvement was better, which can be attributed to the larger number of data used to establish the mathematical relationship. Nevertheless, at low concentrations, residual variation of results remained, mainly owing to the combination of imprecision, sample-related effects, and limit of quantification issues. Note that removing the bias mathematically was not sufficient, and manufacturers need to consider additional investigation of their calibration approach in terms of assignment of the zero calibrator, number and distribution of the calibration points, number of replicates used for measuring the calibrators, and curve-fitting model. Finally, it should be noted that using the same data to both establish a calibration relationship and then examine the effectiveness of the relationship represents an idealized situation. However, the consistency with the 16 donors for recalibration and 23 donors for verification supports the observations based on use of all 39 samples for recalibration.

An error model for $\ln(\text{concentration})$ that allowed isolation and identification of the dominant sources of error (Tables 1 and 2) was applied to the differences in results between each routine method and IDMS for the individual sera. In principle, using cRMI as a common calibrator should reduce the contribution from differences in calibration. The requirement for total error for a single reportable results is $<32\%$ (with a probability of 95%). If, for instance, SD for an optimally calibrated method ($s_{\text{opt-cal}}$) was <0.10 and the maximum bias within a run was <0.15 , the requirement should be satisfied for the worst case (maximum value of both $s_{\text{opt-cal}}$ and bias). A necessary condition to fulfill the

requirement for bias is that the random error between runs, s_b , is <0.07 . Overall, 2 methods had acceptable total error. Improvement was needed by 7 methods for bias, by 5 methods for sample-specific effects, by 4 methods for random error between runs, by 1 method for random error within runs, and by 2 methods in the calibration scheme.

The results in this report and our previous report (2) suggest that improvements are needed in the calibration procedures for routine immunoassays to meet the desirable total error. All of the routine methods evaluated here claim to have calibration traceable to the WHO first international reference preparation (IRP), coded 66/304, a lyophilized impure preparation of human pancreatic insulin (see online Supplemental Data file) (10). Pure recombinant insulin should be more satisfactory as a primary reference material and allow reporting in molar units.

Based on the available results, it appears that improved harmonization of results from routine insulin methods can be accomplished by using secondary reference materials that have been value-assigned by the currently available IDMS procedure calibrated with pure recombinant insulin. We investigated use of 3 serum pools and a panel of individual sera as secondary reference materials. The results suggested that either approach was satisfactory at insulin concentrations >60 pmol/L. The 3 pools were not adequate at lower concentrations, however, most likely because no pool had a concentration <50 pmol/L and the recalibration factors could not adequately correct for nonlinearity in response of some methods at the lower concentrations. The panel of individual sera was the more satisfactory approach because it included samples with low concentrations that approached the limit of quantification of the methods, and the greater number of samples allowed a better mathematical fit over the measuring range. It should be noted that if methods are correctly recalibrated based on a panel of individual sera, subsequent comparisons may show variation related to sample-specific effects, lot-to-lot calibration consistency, and quality control procedures of the manufacturers.

A potential limitation to using serum pools was that an interfering or cross-reacting substance introduced from a donor may yield a different response among routine methods and could contribute to miscalibration for a method. The same limitation also exists with a panel of single-donor sera, but there are more data points to average and the influence of a single aberrant sample may be recognized and discarded as an outlier. Cross-reactivity with proinsulin did not appear to influence the conclusions of this report but is a potential source of error when using single-donor

sera to establish calibration traceability of a nonspecific immunoassay to IDMS.

Conclusions

The harmonization of results among insulin immunoassays can be improved by making several resources available to manufacturers. A panel of single-donation sera (secondary reference material) with concentrations that span the clinically important measuring range of approximately 10–1000 pmol/L and with values assigned by IDMS can be used to establish traceability of a manufacturer's calibration system to IDMS. These panels will need to be made available on a periodic basis as a resource for continued surveillance of calibration procedures and traceability verification.

At this juncture, a complete reference system (primary reference material; reference measurement procedure; secondary reference material) in conformance with International Organization for Standardization requirements (11) has not been established for insulin. The IDMS method used here represents the best available higher-order measurement procedure to determine correct insulin concentrations in different sample matrixes and can be used as a candidate reference measurement procedure. The IDMS procedure needs to be submitted to and accepted by the Joint Committee for Traceability in Laboratory Medicine (JCTLM).

A pure recombinant insulin primary reference material needs to be certified by an internationally recognized standards organization. When available, the primary reference material needs to be accepted by the JCTLM. The primary reference material is essential for calibration of the IDMS procedure and can be used in a manufacturer's system of establishing working method calibrators.

Pooled native serum secondary reference materials value assigned by IDMS may be valuable as trueness controls. Such materials can be used in the manufacturer's internal calibration verification procedures and for monitoring the stability of product calibrator value-assignment procedures.

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