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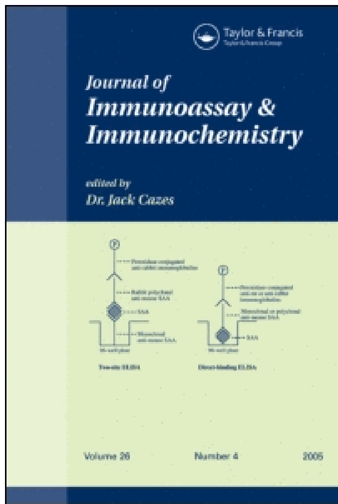

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Intra-assay Total Uncertainty of Results in Immunoassay Techniques

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Intra-assay Total Uncertainty of Results in Immunoassay Techniques

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ABSTRACT

Uncertainty is a parameter associated with the result of a measurement; this parameter characterizes the dispersion of the values that could reasonably be attributed to the sample. Data processing methods do not take into account the influence of the imprecision and deviation of the experimental points of the calibration system and their impact on the final result of a sample analysis. The aim of this work is: (a) to propose, for each run, a simple method to calculate the uncertainty due to the calibration system (Uc); and (b) to present a method to determine the "intra-assay total uncertainty" (Ut) and evaluate its impact on the final result for an analyte. Ten replicates of standards, controls, and two serum-male and female samples were measured in the same run with a manual kit for determination of testosterone. To calculate Ut, random duplicate responses were selected. For controls and samples, Ut was affected by Uc (2.91% to 6.59%) and by the uncertainty of the measurement of the sample (Us) (1.01 to 8.73%); this allowed us to determine that Ut had values from 3.73% to 9.87%. While Us affects the result of a given sample, Uc affects the result of all the samples with a similar response (cpm). In the method proposed, Ut involves Us and Uc, both factors that introduce variations into the result of a sample by random causes. Intra-assay total uncertainty includes the most probable result for the analytical methodology selected.

Key Words: Uncertainty; Immunoassay; Calibration; Curve fitting method; Radioimmunoassay.

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INTRODUCTION

When a magnitude is reported, it is necessary to indicate quantitatively the quality of the result obtained. Therefore, its reliability could be evaluated. Otherwise, the results of this magnitude could not be compared either with one another or with reference values for such magnitude.[1] Uncertainty is a parameter associated with the result of a measurement which characterizes the dispersion of the values that could reasonably be attributed to the sample.[2]

Quantitative immunoassays employing different labels, such as radioactivity, fluorescence, chemiluminescence, enzymatic, etc., have long been used in different bioanalytical applications. No matter what is the label type or the kind of assay (competitive, non-competitive, manual, automated, etc.), they all require a standard curve to determine the concentration of an analyte. Data processing usually analyzes the imprecision of each sample measurement to estimate its impact on the result, expressed as coefficient of variation

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(CV). However, they do not consider the influence of the imprecision and deviation of the experimental points of the calibration system, and their impact on the final result of the patients' samples. Therefore, data processing methods should take into account two important factors responsible for the dispersion of the values that could reasonably be attributed to a sample. These factors are uncertainty due to the calibration of the system (Uc) and uncertainty due to the measurement of the sample (Us).

Since the dispersion of the responses for each standard run is due to the random error in each point, the standard curve is, in fact, a group of calibration curves giving a range of probable results.[3,4] We have called this range "uncertainty due to the calibration system" (Uc).

On the other hand, the uncertainty due to the measurement of a sample (Us) is a consequence of the dispersion of the probable result because of the random error in sample response determination.

Therefore, the "total intra-assay uncertainty of the result" (Ut) of an analyte in a sample will be the statistical addition of Us and Uc.[2]

The aim of this work is:

a. To propose a simple method to calculate Uc *for each run*.
b. To present a method to determine Ut and evaluate its impact on the final result of an analyte.

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EXPERIMENTAL

Materials

Two human serum samples were obtained by venipuncture from one healthy male and one healthy female in order to evaluate results at different levels of the analytical range. Precautions were taken during venipuncture and samples were neither hemolyzed nor lipemic.

Instruments

All reagents and samples were pipetted with micropippettes 10–100 μL (Calibra 822 micropippette, Socorex ISBA SA CH-1020 Renens, Switzerland) and a precision repeating pipette (Eppendorf® Repeater™ 4780, Germany), both calibrated with a coefficient of variation under 3.5%. Tubes were incubated in a temperature-controlled water bath (37 ± 2°C) (Vicking S.R.L., Model Masson N 6090, Argentina), and finally counted in a gamma counter

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(Perkin Elmer Life Sciences Wallac, Wallac 1470, Wizard™ Automatic Gamma Counter Model 1470-020 and Software version 3.3, OY, Finland).

Serum Testosterone Measurements

We used a commercial coated tube radioimmunoassay manual kit for the quantitative measurement of testosterone in human serum (Diagnostic Systems Laboratories, Inc. Webster, Texas, USA). The assay procedure was then followed as described in the technical instructions. Additionally, three commercial controls were assayed: Bio1 (To = 0.38–0.68 ng/mL), Bio2 (To = 4.4–6.4 ng/mL), and Bio3 (To = 8.4–16.4 ng/mL), (Bio-Rad Laboratories, Irvine, CA, USA). Ten replicates of standards, controls, and samples were run in the same assay.

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Statistical Analysis[5]

The average (x), standard deviation (SD), coefficient of variation (CV), and standard error (SE) were calculated, both for the ten replicates and for duplicates of standards, controls, and sample results.

Data Processing

It was performed using Cembal 2.0®. To obtain the standard curve, two methods of calibration were selected: the four logistical parameters method (4P) and the point-to-point method (PP). Both agree with Eqs. (1) and (2).[6,7]

$$\mathrm{B/Bo\%} = \frac{[\mathrm{B} - \mathrm{NSB}]}{[\mathrm{Bo} - \mathrm{NSB}]} * 100 \tag{1}$$

$$\mathrm{B/Bo\%} = \left[\frac{1}{1 + (\mathrm{C}/\mathrm{C}_{50})^{m}}\right] * 100 \tag{2}$$

where Bo is the binding in counts per minute (cpm) in the absence of unlabeled analyte; B, the binding (cpm) in the presence of a given concentration of unlabeled analyte; NSB, the binding (cpm) in the absence of a specific antibody or the background radiation. The slope (m), is the

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slope/−2.3 of the curve in logit−log transformation and C_{50} is the concentration of analyte for which

$$B - NSB = \frac{Bo - NSB}{2}$$

The 4P method calculates the Bo and NSB values in order to use only one slope (m) and C_{50} values along the entire calibration curve. Conversely, the PP method calculates m and C_{50} for each concentration range defined by the standards, considering Bo and NSB experimental values.

RESULTS AND DISCUSSION

Data of the calibration curves are shown in Table 1 and Fig. 1. Data of controls and samples are also shown in Table 1.

Typically, calibration is performed while processing the standards in duplicate. Although it is not possible to perform the determinations by increasing the number of replicates, it would be necessary to express the results with a range, including the true value. The calculation of this range must take into account the principal error and imprecision factors that determine the measured "intra-assay total uncertainty." This range is called "intra-assay uncertainty range."

The true value is not available for this kind of technique due to the lack of international standards which could be included as samples in the assay.[8] As we assayed each standard ten times, including control and sample, we can take the average of those 10 replicates as the most probable value for each of them in the methodology applied. If the system is calibrated with the mean value of each standard and the mean values of controls and samples are interpolated in this calibration curve, we can accept that the interpolated responses obtained are the most probable results (MPR) for the samples and controls.[9] However, as the fitting method chosen for calibration introduces additional differences in the calibration system, it is necessary to consider this choice as another source of doubt on the final result.[10] Table 2 shows the most probable results for samples and controls calculated by 4P and PP fitting methods.

To reproduce the standard assay conditions, we randomly selected two of ten replicates (cpm) of each standard ("Curve A" in Table 3). We then selected one fitting method (4P). There are no strong criteria to select the most appropriate fitting method because they are based on different assumptions. While the PP method accepts that the standards and responses are accurate, the 4P method admits the possibility that the standards could be wrongly determined or that the value indicated may not be correct. Although we have

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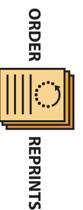

Table 1. Responses (cpm) of testosterone calibrators ($n = 10$), controls, and samples ($n = 10$). Statistical analysis.

	Standard curve					
Number	Bo	0.1 ng/mL	0.5 ng/mL	2.5 ng/mL	10 ng/mL	25 ng/mL
1	26,828	24,031	17,390	10,759	5,853	3,579
2	26,972	23,567	17,307	10,611	5,650	3,594
3	27,021	24,010	17,097	10,649	5,534	3,505
4	27,003	20,751	16,841	10,339	5,837	3,500
5	26,149	23,765	17,224	10,358	5,715	3,693
6	26,179	23,273	17,181	10,534	5,635	3,654
7	26,512	23,663	17,554	10,577	5,597	3,356
8	26,552	23,493	17,805	10,866	5,497	3,505
9	26,255	23,010	17,666	10,529	5,581	3,478
10	26,034	24,369	17,437	10,331	5,600	3,598
Mean	**26,551**	**23,687**	**17,350**	**10,555**	**5,650**	**3,546**
SD[a]	385	1,007	284	179	119	98
CV%[b]	1.5	4.3	1.6	1.7	2.1	2.7
SE $n = 10$[c]	122	318	90	56	38	31
SE $n = 2$[d]	272	712	201	126	84	69
SE% $n = 2$[e]	1.0	3.0	1.2	1.2	1.5	1.9
Upper limit $n = 2$[f]	**26,823**	**24,399**	**17,551**	**10,682**	**5,734**	**3,615**
Lower limit $n = 2$[g]	**26,278**	**22,975**	**17,149**	**10,429**	**5,566**	**3,477**

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Number	Control 1	Control 2	Bio1	Bio2	Bio3	Female sample	Male sample
	Controls and samples						
1	18,462	8,200	15,666	7,351	4,653	18,099	7,845
2	18,484	7,623	15,694	7,299	4,573	18,067	7,294
3	18,780	8,431	14,958	6,949	4,528	19,115	7,605
4	18,414	8,416	15,429	6,751	4,599	18,848	7,747
5	18,571	8,122	15,112	7,332	4,704	18,925	7,827
6	18,413	8,341	15,520	7,319	4,758	17,389	8,093
7	17,809	8,046	15,308	7,005	4,469	18,480	7,917
8	17,798	8,298	15,448	7,450	4,624	18,514	7,866
9	18,274	7,711	15,336	6,906	4,603	18,282	7,567
10	17,755	8,277	15,580	7,366	4,437	18,100	7,780
Mean	**18,284**	**8,147**	**15,405**	**7,173**	**4,595**	**18,382**	**7,754**
SD[a]	350	281	235	244	99	509	220
CV%[b]	1.9	3.4	1.5	3.4	2.2	2.8	2.8
SE $n = 10$[c]	111	89	74	77	31	161	70
SE $n = 2$[d]	248	198	166	172	70	360	156
SE% $n = 2$[e]	1.4	2.4	1.1	2.4	1.5	2.0	2.0
Upper limit $n = 2$[f]	**18,532**	**8,345**	**15,571**	**7,345**	**4,665**	**18,742**	**7,910**
Lower limit $n = 2$[g]	**18,036**	**7,948**	**15,239**	**7,000**	**4,525**	**18,022**	**7,598**

Notes: Bo is the experimental response in cpm in the absence of unlabeled analyte.
[a]Standard deviation.
[b]Coefficient of variation (%).
[c]Standard error (cpm) of the mean determined with 10 replicates.
[d]Standard error (cpm) of the mean determined in duplicate.
[e]Standard error percent of the mean determined in duplicate.
[f]Calculated as Mean + SE, $n = 2$ (cpm), indicates the upper limit of the mean values determined in duplicate.
[g]Calculated as Mean − SE, $n = 2$ (cpm), indicates the lower limit of the mean values determined in duplicate.

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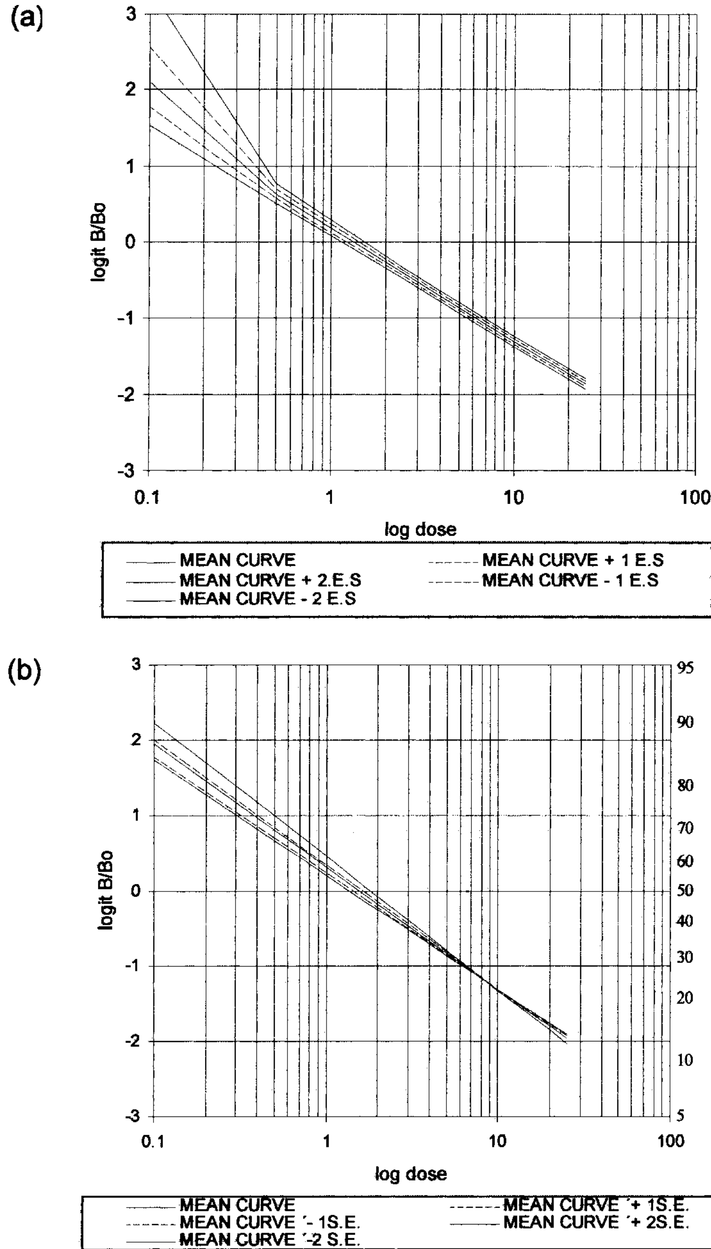

Figure 1. Logit–log of B/B_0 vs. testosterone concentration for all possible combinations of Table 1 values. (a) Point to point calculation; (b) four logistical parameters.

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Table 2. Most probable results for controls and samples of testosterone by two fitting methods: PP and 4P.

Sample	Mean[a] (cpm)	MPR 4P (ng/mL)	MPR PP (ng/mL)
Control 1	18,284	0.437	0.420
Control 2	8,147	4.90	4.64
Bio1	15,405	0.899	0.804
Bio2	7,173	6.40	6.13
Bio3	4,595	15.1	15.1
Female sample	18,382	0.426	0.412
Male sample	7,754	5.44	5.18

[a]Mean value of the individual values of the 10 experimental responses for each control and sample.

chosen the four logistical parameter 4P method to construct Curve A, the calculation of uncertainty takes into account the doubt raised by the choice of the data processing method selected. We also randomly selected two replicates (cpm) of each control and sample (Selection, Table 4).

Table 3. Random selection of the standard duplicates for the calibration Curve A. Calculation of the statistical limits for the means of the experimental responses of each standard according to the imprecision with which they were determined (Curve A).

	Curve A					
Standard (ng/mL)	B[a] (cpm)	B′[a] (cpm)	Mean[b] (cpm)	SE[c] (%)	Mean + SE[c,d] (cpm)	Mean − SE[c,e] (cpm)
0	26,512	26,972	**26,742**	0.86	26,972	26,512
0.1	23,765	23,663	**23,714**	0.21	23,765	23,663
0.5	17,307	17,437	**17,372**	0.37	17,437	17,307
2.5	10,339	10,529	**10,434**	0.91	10,529	10,339
10	5,534	5,837	**5,685**	2.67	5,837	5,534
25	3,589	3,356	**3,472**	3.34	3,589	3,356

[a]B and B′ are the individual values of the experimental responses for each standard, taken randomly from Table 1, as if they were determined in duplicate.
[b]Mean value of B and B′.
[c]Standard error.
[d]Indicates the upper limit of the mean values determined in duplicate.
[e]Indicates the lower limit of the mean values determined in duplicate.

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Table 4. Random selection of duplicates for samples and controls. Calculation of the uncertainty in sample results, due to the calibration (Uc) and to the determination (Us).

	Calculation of uncertainty due to calibration (I)									Calculation of uncertainty due to determination (II)			
				Interpolation									
	Selection of control and sample responses			Mean Curve A	Curve A + SE		Curve A − SE						
Samples	B[a] (cpm)	B′[a] (cpm)	Mean response[b] (cpm)	4P (ng/mL)	4P (ng/mL)	PP (ng/mL)	4P (ng/mL)	PP (ng/mL)	Uc (%)	Dose[c] (ng/mL)	Dose′[c] (ng/mL)	Sample dose[d] (ng/mL)	Us (%)
Control 1	18,462	18,274	**18,368**	0.429	0.435	0.418	0.424	0.41	**2.91**	0.420	0.44	0.429	2.33
Control 2	8,431	8,122	**8,277**	4.65	4.81	4.59	4.50	4.27	**5.81**	4.47	4.85	4.65	4.09
Bio1	15,694	15,520	**15,607**	0.851	0.863	0.775	0.840	0.751	**6.59**	0.834	0.869	0.851	2.06
Bio2	6,949	7,450	**7,200**	6.23	**6.48**	6.33	6.00	**5.81**	**5.38**	6.7	5.81	6.23	7.14
Bio3	4,573	4,624	**4,599**	14.8	15.6	15.8	14.0	14.1	**6.08**	14.9	14.6	14.8	1.01
Female sample	18,067	17,389	**17,728**	0.508	0.515	0.473	0.502	0.463	**4.61**	0.465	0.554	0.508	8.73
Male sample	7,605	8,093	**7,849**	5.21	5.40	5.20	5.03	4.81	**3.74**	5.57	4.88	5.21	6.62

Notes: SE, Standard error; 4P, four logistical parameters; PP, Point to point.

[a]B and B′ Individual values of experimental responses for each sample, taken randomly from Table 1, as if they were determined in duplicate.

[b]Mean of B and B′.

[c]Dose and Dose′ correspond to the concentration obtained by the interpolation of B and B′ in Curve A using 4 logistical parameter (4P) calculation method.

[d]Sample Dose is the concentration obtained by the interpolation of the mean response (Table 3) in Curve A using 4P calculation method.

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Influence of Calibration Factors

In immunoassay results, differences can be observed, from the interpolation of a sample response (cpm) in a calibration curve, that it is different from the most probable one. These differences are aleatory and not systematic, though they are a consequence of a calibration error.[10] Thus, it is necessary to estimate the spread in the dose result as a consequence of Uc.

In this work, we propose a calculation method to estimate this error, which is applicable to the determinations made in duplicate according to the manufacturer's instructions.

The duplicates of the standards give us an idea about the imprecision of their measurement. On the other hand, two fitting methods are used to know the random error observed, resulting from an error in the standard concentration as well as from the dispersion of the response values found for each of them. All these factors introduce an uncertainty in the result. This uncertainty, which we have called Uc, is expressed as the percentage in which the value calculated for a given response (cpm) can be modified.

The first step in the calculation of Uc is knowing the statistical limits for the means of standard responses. We then calculated the mean curve A (Curve A), and the obtained by adding or subtracting the absolute value of SE ("Curve A + SE" and "Curve A − SE") (Table 3).

We took the interpolated values of the "A + SE curve" as the upper limit and those of the "A − SE curve" as the lower limit. Doses in the calibrations are determined by both fitting methods for the different responses. Thus, for the same response in cpm, we obtained four values. We then selected the minimum and maximum value and used that for the response in "Curve A" [Table 4 (I)].

For example, for 7200 cpm (Bio 2), the calculated value for the dose in "Curve A" using the 4P method is 6.23 ng/mL. With the "A + SE curve," the results are 6.48 ng/mL (4P) and 6.33 ng/mL (PP). With the "A − SE curve" the results are 6.00 ng/mL (4P) and 5.81 ng/mL (PP). Thus, the maximum and minimum values are 6.48 ng/mL and 5.81 ng/mL. Uncertainty due to the calibration of the system [shown in Table 4 (I)] was calculated with the following equation:

$$\text{Uncertainty} = \frac{\text{Max value(Curve A} + \text{SE)} - \text{Min value(Curve A} - \text{SE)}}{\text{Mean value(Curve A)} \times 2} \times 100$$

In the example,

$$\text{Uncertainty\%} = \frac{6.48 - 5.81}{6.23 \times 2} \times 100 = 5.38\%$$

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Calculation of the Resultant Uncertainty by Factors Affecting the Sample Response (cpm)

We calculate Us as follows:

1. The values of the individual responses of controls and samples and their mean responses were interpolated in "Curve A."
2. With these values, we calculated Us due to the imprecision in the determination of the sample response. Results are shown in Table 4 (II).

For example, for Bio 2, the mean response is 7200 cpm; the interpolated value in "Curve A" is 6.23 ng/mL. For the individual response values, 6949 cpm and 7450 cpm, the interpolated values in "Curve A" are: 6.70 ng/mL and 5.81 ng/mL, respectively.

Us% was calculated with the following equation:

$$\text{Us\%} = \frac{\text{Max value(Curve A)} - \text{Min value(Curve A)}}{\text{Mean value(Curve A)} \times 2} \times 100$$

In the example,

$$\text{Us\%} = \frac{6.70 - 5.81}{6.23 \times 2} \times 100 = 7.14\%$$

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Calculation of the Total Intra-assay Uncertainty of the Result

Total intra-assay uncertainty is the statistical addition of Uc and Us. Table 5 shows the values of the calculated doses, Uc, Us, and Ut. For our example, Bio 2 control, Ut is 8.94%. With Ut expressed as a percentage and the value of the calculated dose, we calculated the "intra-assay total uncertainty," expressed in ng/mL. This value indicates the limits of the dose (Dose Range) in which the calculated value may be found. The last column shows the most probable result (MPR) for each sample according to the values in Table 2 for the four parameter method.

The purpose of setting limits of the dose, including Uc and Us, is to make sure that the most probable value lies between these limits (1 Ut) with 68% of probability (Table 5).

The magnitude of Ut allows the calculation of the reasonable limits of the sample result and also enables us to express this result with the adequate number of significant digits.[2] Table 5 shows the expression of the results obtained with significant digits. For example, the result of the Bio 2 by the 4P

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Table 5. Calculation of Ut expression of the results with significant digits.

Sample	Curve A 4P[a] ng/mL	Uc[b] %	Us[c] %	Ut[d] %	Ut[d] ng/mL	Dose range[e] ng/mL	MPR[f] ng/mL
Control 1	0.429	2.91	2.33	3.73	0.02	0.41–0.45	0.44
Control 2	4.65	5.81	4.09	7.10	0.3	4.3–5.0	4.9
Bio1	0.851	6.59	2.06	6.90	0.06	0.79–0.91	0.90
Bio2	6.23	5.38	7.14	8.94	0.6	5.6–6.8	6.4
Bio3	14.8	6.08	1.01	6.16	0.9	13.9–15.7	15.1
Female sample	0.508	4.61	8.73	9.87	0.05	0.46–0.56	**0.43**
Male sample	5.21	3.74	6.62	7.61	0.4	4.8–5.6	5.4

[a]Doses obtained by the interpolation of the mean responses of duplicates in Curve A using 4P fitting method (Tables 3 and 4).
[b]Uncertainty due to the calibration.
[c]Uncertainty due to the determination of the sample.
[d]Inter-assay total Uncertainty: statistical sum of Uc and Us.
[e]Dose range where the most probable result is expected to be found.
[f]Mean responses of duplicates interpolated in Curve A, using four logistic parameters (4P) fitting method (Tables 3 and 4).

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method was 6.23 ng/mL (Table 5). This result is affected by 8.94% of Ut; thus, the absolute uncertainty is: $Ut = (6.23\,ng/mL \times 8.94)/100 = 0.557\,ng/mL$, and is expressed as $\pm$ 0.6 ng/mL.

If we apply this interval to 6.23 ng/mL, this number must be expressed as 6.2 ng/mL. The lower and higher limits should be expressed as:

$$\text{Lower limit} = 6.2\,ng/mL - 0.6\,ng/mL = 5.6\,ng/mL$$

and

$$\text{Higher limit} = 6.2\,ng/mL + 0.6\,ng/mL = 6.8\,ng/mL \quad \text{(Table 5)}$$

The calculation method for the “range of uncertainty” proposed in this work fulfills the premise that the most probable value (Table 2) can be found within this range with 68% confidence, and this can be verified in most cases (Table 5). However, the range calculated for the female sample is 0.46–0.56 ng/mL and does not include the most probable value = 0.43 ng/mL. In this case, sample duplicates determine a mean response value of 17,728 cpm [Table 4(I)]. This value is outside the range including 68% of the probable responses determined in duplicate for this sample (18,022–18,742 cpm) (Table 1), but is included in 2 Ut (95% probability) (Table 5).

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CONCLUSIONS

Total intra-assay uncertainty is a suitable parameter to express the range in which the result of a measurement could be found in immunoassays techniques. If Uc is not considered, the range of uncertainty could be underestimated in comparison to the one calculated with Ut. This difference could be very important for the values found near the limits of normal ranges. When the mean response (cpm) of a control or sample determined in duplicate is not included in the range that includes 68% of the probable responses, the most probable value (determined with the analytical methodology utilized) might not be found within the Ut range. The same could have happened if the calibration curve had not been included within the range including 68% of the probable curves. Therefore, for 95% confidence, the 2 Ut range should be used.

The range of Ut allows the definition of the number of significant digits for the correct expression of the result.

This simple and practical method allows us to know the performance of a chosen analytical procedure and if it is adequate for the precision that clinical diagnosis requires in each case.

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