Brazilian Journal of Medical and Biological Research (2003) 36: 1561-1569
ISSN 0100-879X

Biological evaluation of recombinant human erythropoietin in pharmaceutical products

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Abstract

The potencies of mammalian cell-derived recombinant human erythropoietin pharmaceutical preparations, from a total of five manufacturers, were assessed by *in vivo* bioassay using standardized protocols. Eight-week-old normocythemic mice received a single subcutaneous injection followed by blood sampling 96 h later or multiple daily injections with blood sampling 24 h after the last injection. Reticulocyte counting by microscopic examination was employed as the endpoint using the brilliant cresyl blue or selective hemolysis methods, together with automated flow cytometry. Different injection schedules were investigated and dose-response curves for the European Pharmacopoeia Biological Reference Preparation of erythropoietin were compared. Manual and automated methods of reticulocyte counting were correlated with respect to assay validity and precision. Using 8 mice per treatment group, intra-assay precision determined for all of the assays in the study showed coefficients of variation of 12.1-28.4% for the brilliant cresyl blue method, 14.1-30.8% for the selective hemolysis method and 8.5-19.7% for the flow cytometry method. Applying the single injection protocol, a combination of at least two independent assays was required to achieve the precision potency and confidence limits indicated by the manufacturers, while the multiple daily injection protocol yielded the same acceptable results within a single assay. Although the latter protocol using flow cytometry for reticulocyte counting gave more precise and reproducible results (intra-assay coefficients of variation: 5.9-14.2%), the well-characterized manual methods provide equally valid alternatives for the quality control of recombinant human erythropoietin therapeutic products.

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Research supported by CNPq and CAPES.

Received February 21, 2003
Accepted July 23, 2003

Key words
- Recombinant human erythropoietin
- Bioassay
- Polycythemia
- Reticulocyte counting
- Normocythemic mice

Introduction

Erythropoietin (EPO) is the main regulator of human erythropoiesis. This sialoglycoprotein hormone consists of 165 amino acids that form a single polypeptide chain containing two intra-chain disulfide bonds (Cys^{7-161} and Cys^{29-33}) and four potential glycosylation sites, three of which are *N*-linked (Asn^{24}, Asn^{38} and Asn^{83}) and one *O*-linked (Ser^{126}) (1,2). The molecular mass of EPO is 30-34 kDa, but the molecular mass of the peptide chain is only about 18 kDa. Thus, about 40% of the glycosylated EPO mole-

cule consists of carbohydrate, mostly in the form of *N*-linked complex-type glycans (3). These play an important role in determining the biological activity of EPO, which appears to be dependent upon the number of sialic acid residues at the termini of the tri- and tetra-antennary sugar chains (4-6).

Recombinant human EPO (rhEPO) is produced commercially by the expression of EPO cDNA clones in eukaryotic cell lines, most commonly in Chinese hamster ovary or baby hamster kidney cells. The recombinant material is homogeneous with respect to the peptide sequence of natural EPO, but heterogeneous with respect to the carbohydrate moieties since the glycosylation profiles appear to differ between preparations (5,7). Such differences may be due to variations in terminal sialylation which can result in different specific activities. However, most studies with rhEPO have shown that its biological effect is equivalent to that of natural EPO (8-10). Therapeutically, rhEPO is used in the treatment of anemia resulting from the reduced production of endogenous EPO in renal failure, and in the treatment of other chronic anemias due, for example, to severe infections.

Several biotechnology laboratories are producing rhEPO for clinical purposes. It is well recognized that the type of host cell line used for its production, the culture conditions employed, and the purification processes applied may all affect the glycosylation profile of the preparations and thus influence the final biopotency. Therefore, it is essential that a robust, well-characterized methodology be applied to ensure batch-to-batch consistency among the different manufacturers in order to guarantee high quality and therapeutic efficacy (11,12).

Early determinations of the potency of rhEPO were carried out in normal rats and mice by following the increase in several parameters such as hematocrit, red blood cell volume and reticulocyte number (3,10). Alternatively, EPO determinations have been carried out using the polycythemic mouse bioassay, which is based on the stimulation of reticulocyte production and ^{59}Fe incorporation into circulating red blood cells of mice made polycythemic by exposure to reduced atmospheric pressure. Although this bioassay is normally sensitive, precise and specific, the procedure has a number of drawbacks in that it requires the use of a radioisotope and elaborate animal preparation. Currently, the normocythemic mouse bioassay is performed in normal animals with single (13-15) or multiple injections (16-18) and the biological activity of rhEPO is measured by the stimulation of reticulocyte production (3,16-18). Bioassays, in particular that involving a single injection into normocythemic mice, have been widely applied in standardization studies and in the potency evaluation of pharmaceutical preparations (13,15,19); however, although they are usually robust, some improvement is needed with regard to response variability and accuracy, most importantly when assessing the potency of commercial rhEPO batches from various sources (13,14).

Recent investigations of rhEPO activity estimates have been carried out using cell culture assays based on different cell lines, including AS-E2, TF1, UT-7 and UT-7/EPO (20-23). However, these *in vitro* assays have a serious disadvantage since they are unable to discriminate between intact EPO and its asialo- or aglycosylated variants, which have a much shorter half-life and, therefore, a greatly reduced bioactivity when administered *in vivo*.

The number of reticulocytes in the circulation is an early indicator of the functional status of erythropoiesis and, since the remaining nuclear reticulum makes the reticulocyte easily recognizable by microscopy, estimation of the percentage of reticulocytes in peripheral blood has been used for the assessment of rhEPO bioactivity (10,18). Manual and automated methods have been applied to the measurement of immature

reticulocytes in rhEPO-treated mice. Visual counting using supravital stains such as brilliant cresyl blue (BCB) is time-consuming, can have wide intra-observer variability and is frequently unreliable (24,25). On the other hand, selective red blood cell hemolysis and reticulocyte counts carried out in a Neubauer chamber have been shown to correlate with, and produce results equivalent to, the automated method (14,25,26). When possible, the manual counting method has been gradually replaced by the automated method in procedures based on either the fluorescence (thiazole orange) or absorbance (new methylene blue) of dyes that interact with reticulocyte RNA. The precision and accuracy of the counting have been markedly improved and have also provided several additional parameters to aid investigation (25,27).

The aims of the present study were i) to optimize the biological assay in normocythemic mice, evaluating those parameters that could contribute to the refinement of the assay for reliable potency assessment, and ii) to compare the visual methods and the automated flow cytometry method for reticulocyte counting and demonstrate the limitations and reliability of the procedures for the quality control of the therapeutic preparations.

Material and Methods

Reagents and pharmaceutical products

The European Pharmacopoeia Biological Reference Preparation of erythropoietin (250 µg/32,500 IU/vial) was obtained from the European Department for the Quality of Medicines, Strasbourg, France. A total of twelve batches of commercial preparations of erythropoietin at 2,000, 4,000 and 10,000 IU/ml were obtained from five manufacturers. Batches were identified by Arabic numbers from 1 to 12; all preparations were within their shelf-life. BCB, methylene blue and sodium EDTA were from Merck (Darmstadt, Germany). Lysis II solution, consisting of the ammonium quaternary salt and potassium cyanide, was from Coulter Electronica (Buenos Aires, Argentina). Reagents for automated counting were from ABX Diagnostics (Montpellier, France), and heparin (5,000 IU/ml) was obtained from Roche Pharmaceuticals (São Paulo, SP, Brazil). All other reagents were of the highest purity available from commercial sources.

Laboratory animals

Male and female CF1 mice were housed under controlled conditions (room temperature, 22 ± 2ºC; artificial illumination, 12 h per day). For the assays the animals were age-matched, usually at 8 weeks, with a body weight range of 27 to 32 g.

Biological assay

The animals were allocated to sample and standard groups in a fully randomized order and identified by a color code for the assay, usually with 8 mice per treatment group. Standard and test samples were diluted to appropriate concentrations with phosphate-buffered saline containing 0.1% bovine serum albumin.

Single injection. A single dose of 10, 30 or 90 IU EPO/0.5 ml per mouse was injected subcutaneously (*sc*) into the respective animal on day 1. On day 5, a blood sample was taken from the orbital venous sinus of each mouse using a glass capillary tube with the appropriate anticoagulant for the counting method being used.

Multiple injections. Multiple doses of 1, 3 or 9 IU EPO/0.2 ml per mouse per day were injected *sc* on days 1, 2, 3 and 4 into the respective animal and blood was collected on day 5. All injections and blood collections were carried out between 9 and 11 am. Reticulocytes were counted by automated flow cytometry.

Manual methods for reticulocytes counting

Brilliant cresyl blue. Blood samples were collected into 5% sodium EDTA. Equal volumes, usually 100 µl, of blood and 1% BCB were mixed and incubated at 37°C in a water bath for 7 min. Blood sample smears were prepared on glass slides with 8 µl of the dilution. The reticulocytes were counted under a microscope (at 1,000X magnification) in ten areas of the stained smears, corresponding to approximately 1,000 red blood cells. The results are reported as percent of the total red blood cells.

Selective red blood cell hemolysis. Blood samples were collected via heparinized glass capillary tubes directly into assay tubes containing 3 µl sodium heparin (0.6 IU/ml). Forty microliters of the mixture was transferred to another series of labeled tubes containing 40 µl 0.9% sodium chloride, to which 70 µl of a 1% methylene blue solution was added. The mixture was incubated in a water bath at 37ºC for 70 min. Under these conditions, reticulocytes containing protoplasmic basophils fix the methylene blue and color can be seen in filaments (immature reticulocytes) or granules (mature reticulocytes). Forty microliters of hemolyzing solution was added and left at room temperature for 7 min in order to induce hemolysis. Then, 40 µl of the hemolyzed mixture was transferred to assay tubes containing 2 ml of a 0.9% sodium chloride solution. Eight-microliter samples of the erythrocyte suspension were transferred to a Neubauer chamber and the reticulocytes were counted under a microscope (400X magnification) and reported as an absolute number.

Automated fluorescence flow cytometry. Blood samples were collected into 5% sodium EDTA with glass capillary tubes and 130-µl samples were aspirated into the automated reticulocyte counter (ABX Diagnostics). In this method, a maximum of 32,000 red cells were analyzed and the instrument, using customized gating for each sample, separated the mature red cells, reticulocytes, white blood cells, and, on the lower threshold setting, the platelets. Results can be reported as the absolute number of reticulocytes and/or percent, the latter being used in the present experiments.

Statistical analysis

Statistical analyses of the bioassay data were carried out according to Finney (28), by parallel line methods (3 x 3), using SAS 6.1 for Windows (SAS Institute Inc., Cary, NC, USA). Analysis of variance was performed for each assay and the assumption of linearity and parallelism of the log dose-log response lines was tested ($P < 0.05$). Statistical weights were computed as the reciprocal of the variance of the log potencies. Estimates of log potency were examined for heterogeneity by the χ^2 test ($P = 0.05$) and were combined as weighted geometric means of homogeneous estimates ($P > 0.05$). The correlation coefficient was determined by the general linear models procedure and the outlier data were calculated by Dixon's test. The intra-assay coefficients of variation (CV) were calculated as the variation in animal response at each dose level and are shown in the Results section as the range values for all assays in the study. The within-run variation was defined as the variation resulting from consecutive counts of the same sample prepared in triplicate from a single animal. The CV were calculated for these replicates and are reported as the range for all assays in the study, as shown in Results.

Results

Assessment of biological potency by single injection

A standard curve for a single injection was prepared using the European Pharmacopoeia preparation of EPO at doses of 2.5, 5, 10, 20, 40, 80 and 160 IU/0.5 ml per mouse,

as shown in Figure 1. For the bioassay protocol, concentrations corresponding to the linear part of the response curve were selected, i.e., 10, 30 and 90 IU/mouse.

Comparison of reticulocyte counting protocols

Using the BCB method, three blood smears were prepared for each animal tested. Microscopic counting was performed by the same experienced technician, and gave within-run CV of 4.9 to 28.3% and intra-assay CV of 12.1 to 28.4% for the whole study. Similarly, using the selective hemolysis protocol, the reticulocyte count for each animal gave within-run CV of 8.3 to 24.3% and intra-assay CV of 14.1 to 30.8%. For the sake of clarity, examples of the data from a single experiment for each protocol are shown in Table 1. Since the confidence intervals of the potency estimates were high, it was necessary to carry out at least two independent assays and to prepare two or three smears from each mouse to achieve acceptable values.

In the flow cytometry method, reticulocytes from each animal were counted in triplicate and gave within-run CV of 0.4 to 3.3%, and intra-assay CV of 8.5 to 19.7%. Examples of data are shown in Table 1. For each method, potency estimates from combined homogeneous assays (N = 3; P > 0.05) were obtained for six commercial rhEPO preparations (Table 2). The highest percent difference between the potencies estimated by the three methods was 9.6% for sample 3, which is highly acceptable for bioassays with different end-point evaluations.

There was a good correlation between the results from the three techniques, with flow cytometry versus BCB giving a correlation coefficient of 0.90 and flow cytometry versus selective hemolysis giving a value of 0.95. Overall, the flow cytometry method appeared to produce the most consistent po-

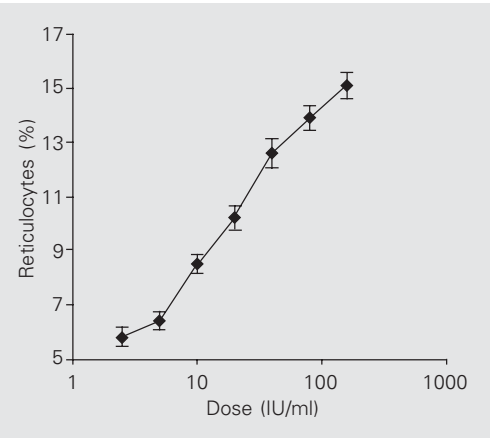

Figure 1. Dose-response curve obtained with the European Pharmacopoeia Biological Reference Preparation of erythropoietin by the subcutaneous injection of a single dose of 2.5, 5, 10, 20, 40, 80 or 160 IU/0.5 ml per mouse (N = 7). Blood was collected after 96 h and the reticulocytes were counted by flow cytometry.

Table 1. Example of the single injection assay showing reticulocyte numbers and coefficient of variation (CV) after the administration of 30 IU/0.5 ml per mouse of the European Pharmacopoeia Biological Reference Preparation of recombinant human erythropoietin using the brilliant cresyl blue (BCB), selective hemolysis (SH) and flow cytometry (FC) counting methods.

Animal No.	BCB method				SH method				FC method			
	Replicates			CV (%)	Replicates			CV (%)	Replicates			CV (%)
	1	2	3		1	2	3		1	2	3	
1	9.2	10.6	10.6	8.0	32	33	37	7.8	12.5	12.1	11.9	2.5
2	18.2	14.6	14.4	13.6	31	38	40	13.0	13.9	13.3	13.6	2.2
3	10.6	7.8	10.7	17.0	40	31	35	12.8	11.4	11.2	11.3	0.9
4	9.8	7.8	13.6	28.3	24	22	26	8.3	12.1	12.5	12.4	1.7
5	9.4	11.2	11.4	10.3	35	25	26	19.2	10.6	11.2	11.1	2.9
6	8.4	9.9	11.6	16.1	47	39	40	10.4	9.5	9.7	10.1	3.1
7	10.2	14.0	10.4	18.5	22	27	28	12.5	9.6	9.7	9.6	0.6
8	10.9	11.0	7.0	23.7	31	38	31	12.1	11.1	10.9	11.0	0.9
Mean	10.8	10.9	11.2	–	32.8	31.6	32.9	–	11.3	11.3	11.4	–
CV (%)	28.4	23.0	20.0	–	24.7	20.6	18.4	–	13.2	11.3	11.1	–

tency estimates, probably due to the larger number of cells counted. This assumption was confirmed by evaluating a sample of known concentration of the European Pharmacopoeia standard for EPO on different days (N = 7), which showed that the flow cytometry method was the most accurate, with the percent difference between potencies estimated at less than 6.3% (data not shown). For all the procedures, nonresponding mice showed reticulocyte counts that were not significantly different from control, and outlying data were excluded.

Other hematological parameters such as erythrocyte number, hemoglobin concentration and hematocrit were evaluated in parallel to the reticulocyte count (data not shown); however, these gave low responses, which might be explained by the low dose of EPO and a test duration that was inadequate for the induction of a significant increase in these parameters.

Table 2. Comparison of combined potencies and assay precision (weight) after bioassay (N = 3) of six commercial recombinant human erythropoietin samples using the brilliant cresyl blue (BCB), selective hemolysis (SH) and flow cytometry (FC) reticulocyte counting methods.

Sample	Method	Potency (IU/vial)			95% Confidence limits	Weight
		Stated	Found	%		
	BCB	2,000	1,804.2	90.21	64.60-125.97	188
1	SH	2,000	1,823.0	91.15	65.95-125.99	200
	FC	2,000	1,967.4	98.37	73.74-131.22	252
	BCB	2,000	2,029.0	101.45	72.28-142.40	182
2	SH	2,000	2,061.0	103.05	74.81-141.93	204
	FC	2,000	2,102.8	105.14	79.45-139.14	267
	BCB	4,000	3,822.0	95.55	68.07-134.13	182
3	SH	4,000	3,713.6	92.84	67.26-128.16	202
	FC	4,000	4,097.6	102.44	77.46-135.48	268
	BCB	4,000	3,836.0	95.90	70.48-130.47	221
4	SH	4,000	3,781.6	94.54	68.33-130.79	199
	FC	4,000	3,964.8	99.12	75.26-130.55	276
	BCB	10,000	10,501.0	105.01	75.51-146.05	192
5	SH	10,000	9,941.0	99.41	71.05-139.08	186
	FC	10,000	10,682.0	106.82	81.12-140.77	276
	BCB	10,000	10,744.0	107.44	75.83-152.23	172
6	SH	10,000	11,292.0	112.92	82.13-155.25	207
	FC	10,000	11,602.0	116.02	88.50-152.10	286

Optimization of other parameters

Time of blood sampling. The biological responses were assessed after the administration of a single *sc* dose of 30 IU EPO/0.5 ml per mouse to 7 animals, followed by collection of blood samples after different times, as shown in Table 3. Protocol 4 was selected because of the significant level of the reticulocyte response and relative CV. The other protocols presented higher variations or a nonsignificant discrimination of the responses.

Animal age and sex. The influence of age on reticulocyte stimulation and the variability of the responses in the normocythemic mouse assay were assessed using single *sc* injections of 10, 30 and 90 IU/0.5 ml per mouse of the European Pharmacopoeia preparation of EPO, administered to 6-, 7-, 8- or 9-week-old-female animals. The 8-week-old animals gave the highest correlation coefficient ($r^2 = 0.9979$). Experiments carried out according to animal sex showed higher variation with a mean intra-assay CV of 20.3% for males and of 13.5% for females.

Number of injections. Multiple injection protocols were evaluated using doses of 10 IU/0.2 ml per mouse followed by collection of blood samples after 72 or 96 h, as shown in Table 4. All of the multi-injection schedules gave higher percent reticulocyte counts relative to the single injection assay protocol. Protocol 6, which showed the highest count and the lowest CV, was selected as the standard protocol.

Assessment of biological potency by multiple daily injections

When the potency of rhEPO pharmaceutical products assessed by the single injection protocol with two independent assays was compared with the results of the single assay, multiple daily injection procedure (Table 5), the procedures gave similar results, but with a higher weight (mean - 258)

for the latter. The intra-assay CV for the multiple injections ranged from 5.9-14.2%, with better precision and lower variation than the single injection protocol.

Discussion

In this study, the biological assay for the potency assessment of rhEPO in pharmaceutical products was standardized using normocythemic mice. The suitability of male or female mice has been investigated previously (3,13,15,17), but the results obtained with females in the present study were more precise and reproducible, with lower variability in the selected linear portion of the dose-response curve.

Reticulocytes were counted using the BCB procedure, a method that is still widely used in clinical chemistry laboratories (10,18,25). Visual reticulocyte counts are often variable (29), although this variation can be minimized by preparing three independent smears from the same animal. In the present study, the CV between replicate counts, expressed as within-run variation, ranged from 4.9 to 28.3%, which is an improvement on values of 25 to 50% described previously (26). The methodology was applied to assess the potency of rhEPO in pharmaceutical products from several manufacturers and gave results in agreement with the specifications of the European Pharmacopoeia (15), with potency values between 80 and 125% of the stated potency and confidence limits between 64 and 156%. The statistical requirements for assay validity, that is, significant regression and no significant deviation from linearity or parallelism of the log dose-log response lines (28), were fully met. The combination of two or three independent assays using a three-fold dose interval and eight animals per dose yielded lower confidence intervals than those reported in previous studies (14,16,17). This provides evidence that manual counts of reticulocytes can generate a valid assay with improved precision, albeit in a labor-intensive format.

Reticulocyte numbers were also estimated by microscopy in a Neubauer cell chamber after treatment with a hemolyzing agent (selective hemolysis technique), by a procedure similar to that used in automated systems (14,17). The results obtained for the potency assessment of several rhEPO pharmaceutical preparations gave combined assay weights (N = 3) between 186 and 207. The values for the independent assays ranged from 50 to 75, similar to those previously described (14). Although the selective hemolysis method was shown to be valid and reproducible, it was also time-consuming and labor-intensive. Two or more independent bioassays were also required to obtain the necessary precision for pharmaceutical preparations.

The automated flow cytometry method counts approximately 32,000 cells compared

Table 3. Percentage of reticulocytes and coefficients of variation (CV) after a single injection of 30 IU/0.5 ml per mouse of the European Pharmacopoeia Biological Reference Preparation of recombinant human erythropoietin using different blood sampling protocols.

Protocol	0 h	24 h	48 h	72 h	96 h	120 h	Reticulocytes (%)	CV (%)
1	C				S		6.19	13.67
2	A		S				7.73	13.33
3	A			S			10.69	15.79
4	A				S		11.20	14.20
5	A					S	9.25	16.42

A = administration; S = blood sample; C = control.

Table 4. Percentage of reticulocytes and coefficients of variation (CV) with different multiple subcutaneous injection protocols using the European Pharmacopoeia Biological Reference Preparation of recombinant human erythropoietin at a dose of 10 IU/0.2 ml per mouse.

Protocol	0 h	24 h	48 h	72 h	96 h	Reticulocytes (%)	CV (%)
1	C				S	5.85	15.50
2	A	A		S		11.93	16.70
3	A	A			S	11.24	18.51
4	A	A	A		S	14.43	14.03
5	A	A	A	S		12.71	13.48
6	A	A	A	A	S	15.75	10.59

A = administration; S = blood sample; C = control.

with the manual methods in which approximately 1,000 cells are counted. The procedure is expensive but less laborious and yields both a more accurate and precise result. The present study has shown that manual counting procedures can give valid results, but the advantages of the automated method for the quality control of rhEPO products for therapy need to be emphasized; moreover, the precision of the potency estimates can vary depending upon the method used for reticulocyte counting. The bioassay parameters were optimized by using the flow cytometry method for automated reticulocyte counting. Injection schedules and blood collection protocols were standardized in preliminary studies and consisted of the administration of a single, *sc* dose of EPO (from a concentration series with three-fold intervals) to mice aged 7 to 9 weeks, followed by blood sample collection after 96 h, or of multiple injections with blood collection after 24 h (14,16,17).

The single injection assay with reticulocyte counting by the flow cytometry method was applied for the determination of the biopotency of six rhEPO pharmaceutical products from different manufacturers (Table 2). The statistical weight of the assays serves as an index of assay precision, which, for the independent assays, ranged from 71 to 145. Combinations of two or three independent bioassays gave potencies and confidence limits for the preparations that satisfied the specifications of the European Pharmacopoeia (15) and gave a precision that agreed with previously published values (13,19). However, by comparing the results and precision of the single injection assay with those of the multiple daily injection assay it can be seen that for the single injection at least two assays were generally necessary to achieve acceptable limits, whereas for the multiple injection assay the confidence intervals were lower, and the precision and reproducibility were higher using results from only one assay. This is an important advantage and the procedure represents a valid alternative to the assay in current use (13,15), thereby contributing to the improvement of the batch-to-batch quality control of rhEPO pharmaceutical preparations. It is also important to point out that the multiple injection protocol, as a consequence of the daily stimulus, resulted in a higher reticulocyte count, albeit with a lower total dose of EPO per animal. This study has highlighted the importance of improving existing methodology for the bioassay of EPO, and of continually seeking ways to implement the criteria of refinement, reduction and replacement in the use of animal models.

Table 5. Comparison of potencies and assay precision after bioassay of six recombinant human erythropoietin pharmaceutical products using single injection or multiple injection protocols, with reticulocytes counted by the flow cytometry method.

Sample	Single injection*				Multiple injection		
	Potency		95% Confidence limits	Weight	Potency found (%)	95% Confidence limits	Weight
	Stated (IU/vial)	Found (%)					
7	2,000	89.3	65-123	201	93.5	70-125	262
8	2,000	104.7	73-150	161	101.3	75-136	244
9	4,000	110.5	80-151	216	104.5	80-137	304
10	4,000	106	77-145	214	103	77-138	259
11	4,000	101.7	73-140	199	99.5	73-136	223
12	4,000	115.3	83-159	203	110.8	83-149	257

*Two independent assays.

Acknowledgments

The authors wish to thank Dr. Maria Elisa Albertengo from the Instituto Nacional de Medicamentos, Argentina, for her helpful suggestions.

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