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Statistical Analysis and Quality Control in Radioimmunoassays for Staphylococcal Enterotoxins A, B, and C

D. S. ORTH

The Procter & Gamble Company, Winton Hill Technical Center, Cincinnati, Ohio 45224

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The objective of these studies was to set up a reliable radioimmunoassay (RIA) for staphylococcal enterotoxins A, B, and C (SEA, SEB, and SEC) in a food system. Significant differences (95% confidence limits) were obtained between the 0- and 1-ng/ml enterotoxin standards, so the sensitivity of the RIAs was 1 ng/ml. Polystyrene tubes coated with anti-SEB and stored at 4°C were unstable. The percentage of iodinated SEB bound to these tubes decreased at a rate of 0.33%/day, in contrast to the rate of 0.07%/day obtained with tubes prepared the day before the analyses. Satisfactory precision and maximum sensitivity were obtained by using six replicates for each sample and freshly coated tubes. The antisera used for coating the tubes were reused four times and were frozen between coatings. The process of drum drying mashed potatoes containing 1 μg of SEB per g of mashed potatoes inactivated 83% (wt/wt) of the SEB. Statistical quality control parameters were used to insure that RIAs were performing reliably with a sensitivity of 1 ng/ml. Over 450 samples of potato flakes and granules, which represented different production lots from 12 different manufacturers, were examined for SEA, SEB, and SEC. No enterotoxins were detected.

The detection of *Staphylococcus aureus* and staphylococcal enterotoxins in foods continues to be an important task for microbiologists because staphylococcal food poisoning is one of the most common food-borne diseases. The radioimmunoassay (RIA) is used extensively in clinical laboratories for measuring the levels of hormones and certain blood proteins. The RIA is gaining popularity in food analytical laboratories because of its speed and extreme sensitivity.

The RIA has been used for detecting staphylococcal enterotoxins A, B, and C (SEA, SEB, and SEC). Johnson et al. (9) were the first to report the use of the solid-phase RIA for detecting SEB. The application of RIA for detecting SEA, SEB, or SEC in food systems has been described by several workers (2, 4-7, 9); however, extensive testing of foods with this type of analysis has not been reported.

Before extensive testing is considered, the reliability of an assay must be established. The objectives of the work described in this report were: (i) to determine the precision, sensitivity, and accuracy of the RIA for SEA, SEB, and SEC; (ii) to set up a statistical quality control program to determine if the RIA were performing satisfactorily; and (iii) to examine food samples for the presence of staphylococcal enterotoxins. Dehydrated potato materials were chosen as the food to examine with the RIA, because no data were available on the effect of drum drying on enterotoxins in mashed potatoes and on the presence of enterotoxins in potato flakes and granules.

MATERIALS AND METHODS

Enterotoxins and antisera. Purified SEA and anti-SEA (anti-A) were supplied by M. S. Bergdoll of the Food Research Institute, Madison, Wis. The SEB and anti-SEB (anti-B) were purchased from Makor Chemicals Ltd., Jerusalem, Israel. The SEC used for iodination and for preparing the stock SEC was obtained from R. W. Bennett of the U.S. Food and Drug Administration, Washington, D.C. This SEC preparation was designated "purified C_1."

Partially purified SEB was kindly supplied by J. A. Troller and J. V. Stinson, Procter & Gamble Co. laboratories. The partially purified SEB was prepared in the following manner: *S. aureus* 243 was grown in 3% (wt/vol) N-Z amine NAK (Sheffield Chemical, Norwich, N.J.) + 3% (wt/vol) hydrolysate (Mead Johnson, Evansville, Ind.) protein at 30°C until maximum stationary phase was reached. The cells were removed from the medium by centrifugation at 38,000 × *g*, using a continuous-flow apparatus with the Sorvall centrifuge. The clarified supernatant liquid was acidified to pH 4.5 with 6 N HCl and dialyzed against 40%

Carbowax overnight at 4°C. The concentrated material was washed into one end of the dialysis tubing, using 0.01 M potassium phosphate buffer (pH 7.2). This was dialyzed against 0.005 M potassium phosphate buffer (pH 7.2) overnight at 4°C. The concentrated material was removed from the dialysis tubing, and 10 g of amberlite CG-50 was added per ml. After adjusting the pH to 5.5, the mixture was allowed to stand overnight. The amberlite CG-50 was removed from the liquid by filtration with a Buchner funnel, and the retentate and filter were washed with 0.01 M potassium phosphate buffer (pH 5.5). The amberlite CG-50 and bound SEB were resuspended in 0.2 M potassium phosphate buffer, pH 6.8 to 7.0, and stirred overnight at 4°C. The SEB was separated from the amberlite CG-50 with a Buchner funnel, and the ion exchanger was washed several times with distilled water. The filtrate was dialyzed against several changes of distilled water to remove salts. Then the SEB solution was concentrated by dialysis against 40% Carbowax. The retentate was lyophilized to give partially purified SEB. The concentration of SEB was standardized with Food Reearch Institute SEB in the micro-Ouchterlony slide test of Casman and Bennett (3). This standardized SEB was used in the accuracy studies.

The anti-SEC (anti-C) antiserum used in these studies was prepared by injecting rabbits with purified SEC, obtained from the Food Research Institute. The rabbits were injected with 1 ml of the following mixture: 100 μl of Food Research Institute SEC + 400 μl of 0.85% NaCl + 500 μl of Freund complete adjuvant. Four subcutaneous injections of 0.25 ml were given: one over each shoulder and one in each thigh. The rabbits were bled by marginal ear vein puncture when the precipitin titer reached 1:16. The precipitin reaction was performed by adding 5 μl of antiserum and 5 μl of 5-, 10-, and 100-μg/ml amounts of Food Research Institute SEC to Immuno-Plates (Hyland Laboratories, Costa Mesa, Calif.).

Stock toxin solutions were prepared with 1 μg of purified SEA, Makor SEB, or purified C_1 per ml in 0.15 M potassium phosphate buffer with 0.85% (wt/vol) NaCl (potassium phosphate buffer; PBS) (pH 7.5) + 1% (wt/vol) bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) + 1% (wt/vol) NaN_3. The stock solutions were pipetted into plastic vials and stored frozen until use.

Iodination. SEA and SEC were iodinated by using the lactoperoxidase method of Thorell and Johansson (13). The iodination reaction mixture contained: (i) 10 μl of a 1-mg/ml amount of purified SEA or SEC; (ii) 1 mCi of $Na^{125}I$ in 20 μl of 0.1 N NaOH; (iii) 1 μg of lactoperoxidase (Sigma Chemical Co.) in 10 μl, added at 500 ng at the start of the iodination reaction and 500 ng after 1 min; (iv) 10 μl of 0.3% H_2O_2 added at 5 μl at the start of the iodination reaction and 5 μl after 1 min; and (v) 10 μl of 0.1 M PBS, pH 7.0. The reaction was stopped after 3 min by adding 200 μl of 0.1 M PBS, pH 7.0. Then, 250 mg of bovine serum albumin was added to the reaction vial. After adding 750 μl of 0.15 M PBS, pH 7.5, the unbound $^{125}I^-$ ion was separated from the iodinated SEA or SEC by passage through Sephadex G-15 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.).

SEB was iodinated by using the chloramine-T method of Orth (10). The percentage of $^{125}I^-$ ion bound to the enterotoxins during chloramine-T iodination and after passage through Sephadex G-15 was determined by using a miniature thin-layer chromatography method. This was performed by spotting about 0.5 μl of the reaction mixture or iodinated toxin on a dot made with water-soluble ink about 1 cm from the base of a strip (1 by 5 cm) of chromatography media I.T.L.C., type SG (Gelman Instrument Co., Ann Arbor, Mich.). The thin-layer chromatography strip was placed into a glass scintillation vial containing 1 ml of 15% (wt/vol) trichloroacetic acid. The development of the chromatogram was readily followed because the ink moved with the solvent front. Development of the chromatogram took 1.5 to 2 min. Then, the thin-layer chromatography strip was removed from the vial and cut in half to separate the unbound ^{125}I, which was at the solvent front, from the ^{125}I bound to protein, which was denatured by the trichloroacetic acid at the point of application. The halves of the chromatogram were placed in separate scintillation vials, and the counts per minute in the top and bottom halves were determined with a Packard gamma scintillation spectrometer. The percentage of $^{125}I^-$ ion bound was calculated by the formula: percentage of $^{125}I^-$ ion bound to toxin = counts per minute of bottom half/counts per minute of bottom and top halves. The fraction containing the iodinated toxin was diluted to 50 ml in 0.15 M PBS with 1% bovine serum albumin and 0.1% NaN_3. Portions of the iodinated toxin were pipetted into plastic vials and stored frozen (−15°C) until use.

Coating RIA tubes. The optimal concentrations of whole anti-A, -B, or -C to use for coating the RIA tubes were checked after each iodination. Each antiserum was diluted to 1:50, 1:100, 1:200, 1:500, and 1:1,000 in 0.07 M potassium phosphate buffer + 0.07 M NaCl, pH 7.2 (0.07 M PBS). One milliliter of each antiserum dilution was coated onto LP/3 polystyrene tubes (Luckham Ltd., Sussex, England) by the method of Johnson et al. (8). The tubes were used in the RIA with homologous iodinated toxin. The dilution of antiserum that bound the most iodinated toxin was used for subsequent tube coatings. (The 1:500 dilution generally gave the highest binding.) The diluted antiserum was used to coat tubes a total of five times (i.e., it was reused four times) and was frozen (−15°C) between coatings. The RIA tubes were coated the day before use.

RIA. The RIA was performed using modifications of the basic procedure of Johnson et al. (8). Six RIA tubes were used for each toxin standard concentration and for each sample. A potato slurry was prepared by making a 1:20 dilution of potato flakes or granules in 0.07 M PBS (pH 7.2) and blending for 2 min in a Waring blender. The samples were centrifuged at 1,100 × *g* for 5 min to separate the foam and lipid from the aqueous portion. The lipid and foam were removed by aspiration.

The standard curve was prepared by adding 0, 1, 3, 5, 7, and 10 ng of stock toxin solution to RIA tubes containing sufficient potato slurry to bring the volume to 1 ml. Then, 100 μl of iodinated toxin solution was added to each tube, and the tube contents were mixed with a Labline mixer. The tubes were incubated for 18 h at 37°C. After incubation, the tube contents were removed by aspiration, and the tubes were rinsed with

2 ml of 0.07 M PBS. The amount of iodinated toxin bound to the RIA tubes was determined with a Packard gamma scintillation spectrometer. The mean counts per minute, standard deviation, coefficient of variation (CV), and standard deviation of the mean were calculated for each standard toxin concentration and each potato sample. In all cases, significant differences used 95% confidence limits about the mean, which were determined using ±2 standard deviations of the mean. The ratio of the counts per minute bound to the total counts per minute added to the RIA tubes (i.e., the B/T ratio) was calculated. The B/T ratio in the absence of unlabeled toxin [$(B/T)_0$ ratio] was determined for each assay. The B/T ratios were "normalized" by using $(B/T)_0$ ratio = 100%. The normalized B/T ratios were used in a logit transformation {i.e., logit $B/T = \ln [(B/T)/(1 - B/T)]$}. The slope and 0-intercept were calculated.

The sensitivity was evaluated by determining if the counts per minute for each standard toxin concentration were significantly different. Enterotoxin concentration in samples was calculated by using logit B/T ratios for samples and interpolating between the logit B/T ratios obtained with toxin standards.

Specificity. The $(B/T)_0$ ratios for each toxin type were determined with tubes coated with anti-A, -B, or -C. The $(B/T)_0$ ratio of the homologous antigen-antibody system was set at 100%, and the $(B/T)_0$ ratios of the heterologous systems were calculated relative to this. The extent of cross-reactivity was expressed as a percentage of the $(B/T)_0$ ratio obtained with the homologous system.

Accuracy. Standard curves were obtained with the stock toxin solutions and the standardized toxin preparations. The B/T ratios at 0, 1, 3, 5, 7, and 10 ng/ml were compared for each of the three toxin types.

Analysis of potato samples. A total of 459 samples of potato flakes and granules were obtained from different production lots of 12 potato-dehydrating firms. A 1:20 dilution of a potato flake or granule slurry was prepared as described above. One milliliter of the slurry was pipetted into six anti-A, -B, and -C tubes. Then, 100 μl of the appropriate iodinated toxins was added to each set of tubes. The tubes were mixed, incubated, and handled as described above. Internal standards containing 0 and 10 ng of SEA, SEB, or SEC per ml in the potato slurry were used for each assay. All samples that gave a positive test were reassayed to confirm the presence of enterotoxin.

Drum drying of mashed potatoes containing SEB. Potatoes were peeled and heated for 20 min at 100°C. The water was decanted, and 10% (wt/wt) water was added before mashing with a Hobart mixer. A portion of the mashed potatoes was spiked with SEB to give a concentration of 1 μg of SEB per g of mashed potatoes. The mashed potatoes were dried on a drum drier operating at 60-lb/in^2 steam pressure.

RESULTS AND DISCUSSION

The RIA performed in these studies differed from the basic procedure of Johnson et al. (8). Johnson and co-workers coated polystyrene tubes with the immunoglobulin G fraction obtained from anti-B. We found that tubes coated with whole anti-B bound a greater proportion of iodinated SEB (I-SEB) than tubes coated with about 7 μg of immunoglobulin G; therefore, we used whole anti-A, -B, or -C for coating the RIA tubes. The maximum immunoreactivity of I-SEB was retained when the chloramine-T method was used for iodination (10). The lactoperoxidase/H_2O_2 method of iodinating SEA and SEC was superior to the chloramine-T method, because the former gave iodinated toxins that had $(B/T)_0$ ratios of 17 to 24% and the latter gave iodinated preparations that had $(B/T)_0$ ratios of <10%. According to the procedure of Johnson and co-workers, 10 μl of iodinated toxin is added to each RIA tube after 1 h of incubation at 37°C. No significant difference was obtained when the iodinated toxin was added at the time of adding unlabeled toxin or when the iodinated toxin was added after 1 h of incubation; therefore, the iodinated toxin was added shortly after adding the unlabeled toxin to the RIA tubes.

It was believed that pipetting errors would be reduced by using 100 μl of a 1:10 dilution of the stock solution of the iodinated toxin preparations instead of 10 μl, as described by Johnson et al. (8).

Johnson et al. (8) estimated the amount of SEB from a standard curve in which the log percent uptake of I-SEB was plotted as a function of the log concentration of unlabeled SEB. The studies reported here used the logit transformation (12). A typical standard curve is shown in Fig. 1. The quality control parameters used to determine if the RIA were "in control" were: (i) the $(B/T)_0$ ratio; (ii) the 0-intercept and slope of the plot of logit normalized (B/T) versus ln toxin concentration; and (iii) the CV. The RIA was judged to be performing satisfactorily when these parameters were within the 95% confidence limits of the running average (i.e., mean) of each parameter, provided that the

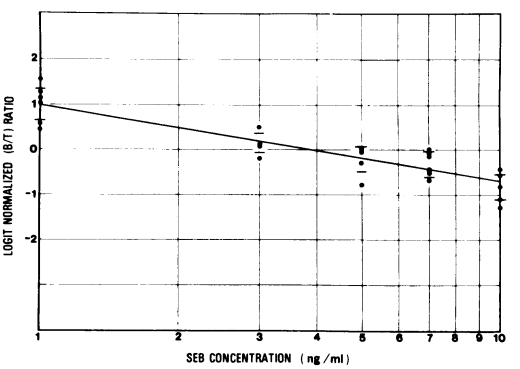

FIG. 1. *Standard curve for the RIA for SEB in a 1:20 dilution of potato flakes in 0.07 M PBS, pH 7.2. Symbol: =, 95% confidence limits.*

$(B/T)_0$ ratio was >10% and the CV was ≤20%.

I reported that the $(B/T)_0$ ratio was the most useful statistical parameter because it provides information about the radioactivity and immunoreactivity of the iodinated toxins (10). The $(B/T)_0$ ratios of the freshly prepared, iodinated toxins were 17 to 24%; however, the $(B/T)_0$ ratios decreased over a period of weeks. The $(B/T)_0$ ratios decreased more rapidly with tubes prepared and stored at 4°C until used than with tubes prepared the day before the analysis. Thus, linear regression analysis of the $(B/T)_0$ ratios with time produced lines with slopes of −0.33%/day for old anti-B-coated tubes (prepared and stored for up to 24 days before use) and −0.07%/day for new anti-B-coated tubes.

These data indicated that the antibody-coated tubes were not stable and should be used shortly after they are prepared. After this was learned, the tubes were coated the day before use. It is believed that the decrease in the $(B/T)_0$ ratios over a period of time represented a loss in immunoreactivity of the iodinated toxins. The $(B/T)_0$ ratios for all RIAs averaged 13 to 19% over a period of months. The mean $(B/T)_0$ ratios for all RIAs were 16% (Table 1). These data were used to determine when to prepare more iodinated toxins. The iodinated enterotoxins could be used for up to 3 months and still give $(B/T)_0$ ratios of >10%.

The antisera could be reused several times for coating the RIA tubes. We wanted to obtain the maximum $(B/T)_0$ ratios; therefore, the proper concentration of antiserum to use for coating tubes was determined after each iodination. Generally, the highest $(B/T)_0$ ratios were obtained with a 1:500 dilution of the antisera. The diluted antisera were stored frozen (−15°C) and were thawed just before coating fresh tubes. The diluted antisera were used to coat tubes a total of five times without a noticeable decrease in $(B/T)_0$ ratios. No attempt was made to see how many times the antisera could be reused.

The use of freshly prepared tubes increased the sensitivity and precision in the RIA. For example, the sensitivity of the RIA for SEB was >1 ng/ml, and the CV ranged from 15 to 18% when the analyses were performed with old tubes. The use of freshly prepared tubes improved the precision, because the CV for the standard SEB concentrations decreased to 6.3 to 11.8%. When the RIAs were performed with tubes coated the day before the analyses, every assay that was in control was able to discriminate between 0, 1, and 3 ng of SEB per ml.

TABLE 1. *Statistical parameters used to determine if RIAs were in control*

Enterotoxin	Statistical parameter[a]			
	$(B/T)_0$[b] (%)	Slope	0-intercept (ng/ml)	CV (%)
SEA	16	−0.8	3.7	10
SEB	16	−0.6	4.7	9
SEC	16	−0.9	5.3	12

[a] Values are the means from 20 assays.

[b] Ratio of the counts bound to the total counts added to the RIA tubes in the absence of unlabeled enterotoxin.

The sensitivity of the three RIAs was 1 ng/ml in 1:20 potato-PBS slurries, or 20 ng of potato flakes or granules per g. Repeated analyses demonstrated that significant differences were always obtained between the 0- and 1-ng/ml standards for the SEA and SEB analyses, and these were obtained on all occasions except one analysis for the SEC assay.

Occasionally, the 3- and 5-, 5- and 7-, and 7- and 10-ng/ml standards gave mean counts per minute that were not significantly different from each other. This occurred more frequently during months 2 and 3 after iodination of the toxins; consequently, it appears that this may have been due to loss in immunoreactivity of the iodinated toxins that occurred with aging.

Although Rodbard et al. (12) recommended using within-assay variance as a measure of precision, the counts per minute obtained in our analyses often gave variances as large as 10^5. Thus, variance was not considered to be a useful statistical quality control parameter. The CV was used as the measure of precision for these RIAs. The CV ranged from 7 to 12%, 6.3 to 11.8%, and 10 to 13% for the assays for SEA, SEB, and SEC, respectively. The mean values for the CV for all assays are shown in Table 1.

According to Rodbard and co-workers (12), the logit transformation produces a curve that does not differ significantly from linearity when the normalized (B/T) values are between 10 and 90%. The slope and 0-intercept were determined by linear regression analysis. A typical standard curve using the logit transformation is shown in Fig. 1. Although six replicates were used for each standard toxin concentration, fewer than six points appear at some concentrations because the B/T values were the same. Reference to Table 1 shows that the mean values for the slope and 0-intercept for numerous assays ranged from −0.6 to −0.9 and from 3.7 to 5.3 ng/ml, respectively.

The accuracy of each RIA was determined by comparing the B/T ratios obtained using our stock toxins with those obtained using standardized toxin preparations. The B/T ratios were within 2% of each other at any toxin concentration for each RIA.

The specificity of each RIA was evaluated by

determining the extent to which iodinated (I-) SEA, SEB, and SEC cross-reacted with heterologous antibodies coated in the RIA tubes. The data in Table 2 show the extent of cross-reaction between the homologous and heterologous antigen-antibody systems. It is apparent that the assay for SEA was the most specific, because ≤4% of the I-SEB and I-SEC were bound to tubes coated with anti-A and ≤2% of the I-SEA was bound to tubes coated with heterologous antisera. The low percentage of binding of I-SEB and I-SEC to tubes coated with anti-A and vice versa is thought to be insignificant. It is possible that the low level of binding here may have been due to nonspecific binding of the iodinated toxins to the RIA tubes.

The data in Table 2 show that there was appreciable cross-reaction between the SEB and SEC systems. Although the 35% binding of I-SEB by tubes coated with anti-C appears to be somewhat high, the 17% binding of I-SEC by tubes coated with anti-B is in agreement with the amount of inhibition of I-SEB uptake by SEC reported by Johnson et al. (8). The cross-reaction studies were performed in potato slurries; however, it is thought that the potato slurry had negligible effect on the cross-reactivity of the SEB and SEC systems, because the level of cross-reactivity of SEA and anti-A with the heterologous antisera and toxins was minimal. The cross-reactivities of the enterotoxins were reviewed by Bergdoll and Robbins (1).

Staphylococci grow and elaborate SEA, SEB, and SEC in many foods; however, the effect of processing on toxin levels has received little attention. In this regard, Read and Bradshaw found that SEB was not inactivated completely during spray drying of milk (11). Troller and Stinson, in our laboratories, reported that *S. aureus* grew and produced enterotoxins in potato doughs (14). Drum drying mashed potatoes spiked with 1 μg of SEB per g inactivated 83% (wt/wt) of the SEB.

TABLE 2. *Cross-reaction test for specificity of the RIA for SEA, SEB, and SEC*

Enterotoxin	Specificity (%) in tubes coated with:		
	Anti-A	Anti-B	Anti-C
SEA	100[a]	2	1
SEB	4	100	35
SEC	1	17	100

[a] $(B/T)_0$ ratio for homologous enterotoxin-antibody system was normalized to 100%, and the $(B/T)_0$ ratios for the heterologous enterotoxin-antibody systems were calculated relative to this.

Over 450 samples of potato flakes and granules were examined for SEA, SEB, and SEC. No confirmed positives (i.e., samples that gave positive results on reanalysis) were found. These data show that the dehydrated potato flakes and granules contain no detectable enterotoxins.

An assay is useful only when its limitations are known. The many steps involved in performing the RIA provide many opportunities for errors; therefore, it is necessary that a statistical quality control program, such as the one described here, be used to monitor the performance of the assay.

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