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Second international standard for endotoxin: calibration in an international collaborative study

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photometric tests, especially in the pharmaceutical industry, has increased dramatically and, in the absence of an IS for endotoxin for all applications, the US national standard, EC5, has remained the primary calibrant for *Limulus* lysate, with all the commercially available lysate preparations (which are produced mainly in the US and Japan) calibrated in EU rather than IU.

In 1994, the ECBS of WHO recognised that the increased use of photometric LAL tests for endotoxin had created a need for a new IS for endotoxin that could be used to calibrate all types of LAL test and responded positively to an offer from the USP of 4000 vials from a batch of vials being prepared as a new USP/FDA standard. This positive response reflects the importance attached by WHO to the harmonisation of biological standards between WHO and pharmaceutical and regulatory authorities.

On behalf of WHO, 27 scientists in 13 countries were invited to participate in an international collaborative study to evaluate the candidate international standard (CS) for endotoxin (for all applications). At the request of the WHO, this study was organised in collaboration with the United States Pharmacopoeia (USP) and includes the standards of the European and Japanese Pharmacopoeias.

Aims of the study

1. Calibrate the candidate international standard (CS) in terms of EC5 and assign to it an international unit for endotoxin (for all applications).

2. Compare EC5, the current IS and the CS using data from semi-quantitative (i.e. LAL gelation) and quantitative (i.e. chromogenic and turbidimetric) assays to maintain, as far as possible, continuity of unitage.

3. Examine the relation between the IU and the EU.

4. Compare the CS with the Pharmacopoeial standards of the US, Europe and Japan.

MATERIALS AND METHODS

Preparations for the study

1. The current international standard (IS) of endotoxin, preparation 84/650.[4]

2. EC5. (Although EC5 has been superseded by EC6 [FDA lot EC6, USP RS Lot G], it was judged appropriate that this study use EC5, the primary calibrant for the first IS and the JP standard. EC6 has been calibrated in a study in 6 US laboratories in terms of EC5 and has been assigned a unitage of 10,000 EU per vial. EC6 consists of endotoxin from the same bulk and as nearly as possible identical excipients to EC5 and the CS.)

3. Coded vials from two different sub-lots of the CS: A and its coded duplicate D from one sub-lot and B and its coded duplicate C from another sub-lot. Additional sub-lots were also prepared, one of which is the current USP/FDA standard for endotoxin, EC6.

4. BRP-2 (EP) and JP endotoxin standard.

5. Vials of a 'common' *Limulus* lysate (sensitivity 0.03 EU/ml) provided by Associates of Cape Cod (ACC).

Preparation of the candidate standard

Materials

Escherichia coli endotoxin 0113:H10:K(–)[5] and polyethylene glycol 8000 (PEG), USP/NF CAS 25322-68-3, P0131 Lot No. HG136, were from Spectrum Chemical Manufacturing Corp. (Gardena, CA, USA); α-lactose monohydrate (Eur. Pharm. USP CAS64044-51-5, 12.509.93) Lot No. 68012/1 was from Janssen Chimica (Geel, Belgium); sterile water for irrigation (manufactured to the standard of 'water for injection' Ph. Eur.) Code F7114 Lot No. 94B19B26 was from Baxter Healthcare Ltd (Norfolk, UK); and 0.22 μm disposable filter units (Millipak 60) were from Millipore SA (Molsheim, France). All glassware was depyrogenated by dry heat at 250°C for 1 h. Tubing used in the filtration was depyrogenated by autoclaving for 3 cycles at 120°C and 15 psi for 30 min per cycle. Chemicals were weighed out aseptically in a clean air flow into pre-weighed glass beakers.

Method

The candidate standard was prepared by dissolving 88 mg of endotoxin in 80 l sterile, pyrogen-free water containing 0.1% (w/v) PEG and 1.0% (w/v) lactose. This solution was passed through the 0.22 μm filter and distributed in 1.0 ml aliquots into pyrogen-free vials. The aliquots were frozen at –20°C, freeze-dried and the vials sealed under dry nitrogen and stored at –20°C in the dark. The preparation of the standard was supervised by Dr Peter Dawson in the Standards Division of NIBSC in accordance with the procedures used for international biological standards (Annex 4, 40th ECBS report, 1990).[6]

Design of the study

Participants in the study

As part of the collaborative study, 26 laboratories in 12 countries contributed data. Participating laboratories are

lysate. The assays comprised some 620 tests of individual preparations; of these, 19 tests were omitted as having indeterminate endpoints. Estimates of the activity of the various samples relative to EC5 from the same assay have been compared with one another to give estimates for the various samples relative to one another for each assay in these laboratories. The observed sensitivity of the lysate using EC5 differed from the stated sensitivity by a factor greater than 2.0 (i.e. the assay was not valid) in 28 assays; 21 of these 'invalid' assays used the common lysate. Four repeat assays were carried out using stored samples; these assays did not show any detectable loss of activity. The results have not been included in the analysis and combinations of estimates for the gelation assays.

Photometric assays

133 assays were carried out using chromogenic kinetic (13 laboratories), chromogenic end point (3 laboratories), and turbidimetric kinetic (12 laboratories) methods. 79 assays were carried out using freshly made-up solutions, and 54 assays used solutions which had been stored for a week or longer.

Many assays, after deletion of outlying responses, showed significant heterogeneity of variances. Most assay designs followed a systematic procedure with the sequence of responses to a given dose representing, in some fashion, the sequence in which the responses were obtained. For example, for a particular preparation, at each of the dilutions tested the first response may have come from one column of a microtitre plate, the second response from the next column and so on. The heterogeneity was thus associated with particular 'positions' in many assays and was generally erratic in nature and not suggestive of a relationship with level of response. For 16% of 783 tests for individual preparations, there was a significant ($P < 0.05$) difference among the responses related to their reported order. Subdivision according to assay type gave significant order effects for 11% of 88 CE, 23% of 382 CK and 10% of 313 TK assays.

Parallelism of log dose vs log response lines was numerically assessed relative to the calculated deviations from linearity, and to the difference in slopes between the coded duplicates, either A and D or B and C. For about two-thirds of the quantitative assay systems there was no significant consistent non-parallelism among the preparations compared. For the remaining one-third of assay systems, the log dose vs log response lines for one or more of the different preparations differed consistently and significantly from those of the remaining preparations.

Estimates from laboratory 10 have been excluded from the following analysis because estimates of relative potency obtained by this laboratory (TK assays) had an excessively large inter assay variability, and the estimates relative to EC5 were markedly different from those obtained in any other laboratory. Estimates for the second set of samples of the IS obtained in laboratory 04 with both TK and CK assays were markedly different from other estimates for the IS in this laboratory, and these two estimates have been deleted from the following analysis.

Comparisons within laboratories of fresh and stored samples for preparations A, B and C or D were carried out to examine stability of the reconstituted solutions of the CS. In the majority of cases, there was no significant difference between estimates for the fresh and stored samples relative to the variability between assays. The mean difference between estimates for fresh and stored samples, over all samples examined both fresh and after storage in the same assay system and laboratory, did not differ significantly from 0 (paired *t*-test, $P \sim 0.9$). These data do not suggest any consistent significant loss of activity on storage under the conditions of this study. Consequently, estimates from assays using fresh and stored solutions have been used for the following analysis.

Comparisons between duplicate samples of the CS and between the two sub-lots of CS

LAL gelation assays

Comparisons between estimates for the duplicate samples, namely D with A and C with B, gave estimates which did not differ significantly from one another. The ratio of estimates of activity D:A had a geometric mean over all individual estimates of 1.05 with 95% limits 0.95–1.16, while that for C:B was 1.06 (0.98–1.15). The differences between assays for these estimates were essentially the same as the differences between these samples within the same assay, and were also essentially the same as the differences between estimates made in different laboratories and using different lysates. Comparison of B with A also gave essentially the same results, with the geometric mean of the ratio of activity B:A being 1.00 (0.94–1.06) for all individual comparisons. These comparisons were also made separately for assays using the common lysate and for assays for which the observed sensitivity using EC5 was within 2-fold of the stated sensitivity of the lysate; none of these separate comparisons gave estimates which differed significantly from 1.0 or from one another.

Photometric assays

Comparisons between the duplicates D and A and C and B gave estimates which did not differ significantly from one another, with D:A having a geometric mean of all individual estimates of 1.00 (95% limits 0.97–1.03) and C:B having geometric mean 1.07 (1.00–1.13).

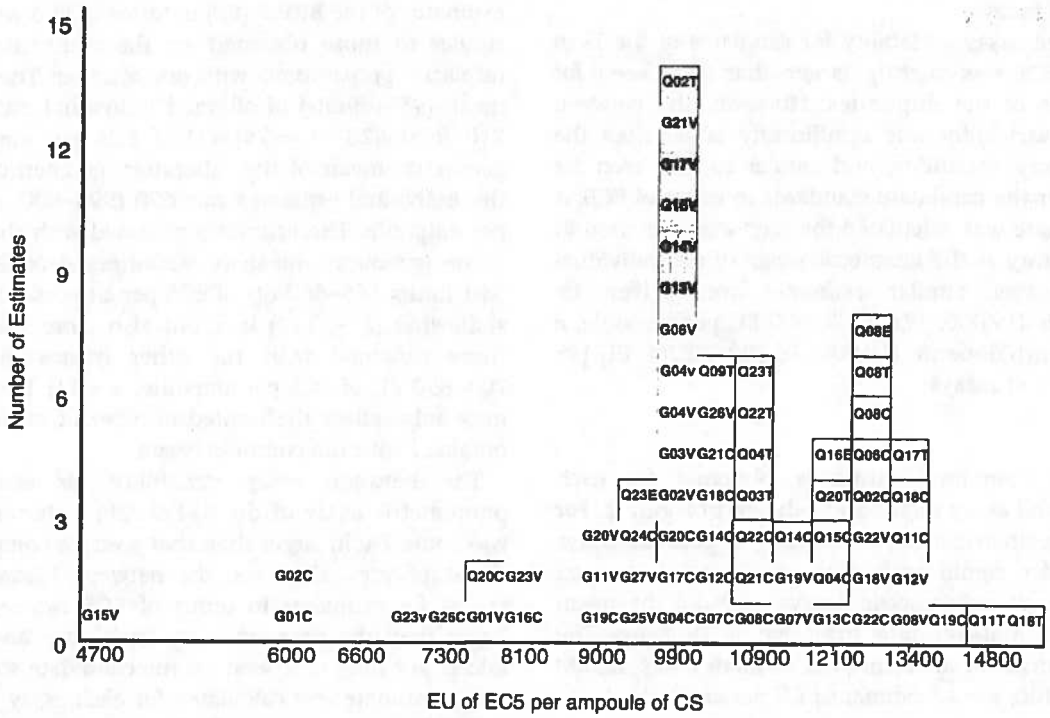

Fig. 1 Laboratory and method geometric mean estimates of the relative potency of the Candidate Standard expressed as EU of EC5 per ampoule. Each square denotes a single combined estimate for each assay method in each laboratory. For LAL gelation assays, use of a different lysate has been considered a separate 'assay method'. The label in the square denotes whether the assay was LAL gelation (G as the first character) or quantitative (Q as the first character), the laboratory code (second and third characters) and, for the gelation assays, whether the lysate was the common supplied lysate (C as the fourth character, clear, unshaded squares), or for photometric assays, whether the method was chromogenic (C as the fourth character for chromogenic kinetic assays, or E as the fourth character for chromogenic endpoint assays, diagonally marked squares) or turbidimetric (T as the fourth character, cross-hatched squares). For the Candidate Standards, the combined estimate was calculated as the unweighted geometric mean over all photometric assays or over all valid gelation assays of assay estimates calculated as the geometric mean of estimates for A, B, C and D within each assay. For other preparations, the single estimate was similarly calculated as the geometric mean of estimates from all photometric assays or from all valid gelation assays.

heterogeneous with highly significant interlaboratory differences. The geometric mean estimate by all quantitative methods (11,700, 11,000–12,400 EU per vial) was significantly larger than the mean by gelation assays. The mean, over both quantitative and gelation assays, of all combined estimates was 10,400 (9900–10,900, $n = 68$) EU per vial.

Comparison of the current IS with EC5

LAL gelation assays

The between assay variability for estimates of the IS in terms of EC5 was similar to that obtained for comparisons of the duplicate preparations with one another. However, the variability between laboratory and lysate was some 2 times larger than the between laboratory variability seen for the comparisons of duplicates. When restricted to valid comparisons of the IS with EC5, the between laboratory variability for estimates made with the common lysate was similar to that for duplicate comparisons, but the between laboratory variability for estimates made with the various lysates was some 3-fold (significantly, $P \sim 0.02$) larger than the between assay variability. The geometric mean of all valid individual estimates was 19,200 (95% limits 17,300–21,200, $n = 48$) EU of EC5 per ampoule of the IS. The mean for valid estimates using the common lysate was 17,600 (15,500–20,100, $n = 18$) EU per ampoule, and for valid estimates using the various other lysates was 20,100 (17,400–23,300, $n = 30$) EU per ampoule. Because of the interlaboratory variability, geometric means of laboratory and lysate geometric means have also been calculated (i.e. a single estimate for each different lysate in each laboratory). The means of these estimates, with 95% limits, were 18,200 (15,900–20,900, $n = 28$) for all lysates, 17,500 (15,100–20,200, $n = 11$) for the common lysate and 18,700 (15,000–23,200, $n = 17$) for the various other lysates.

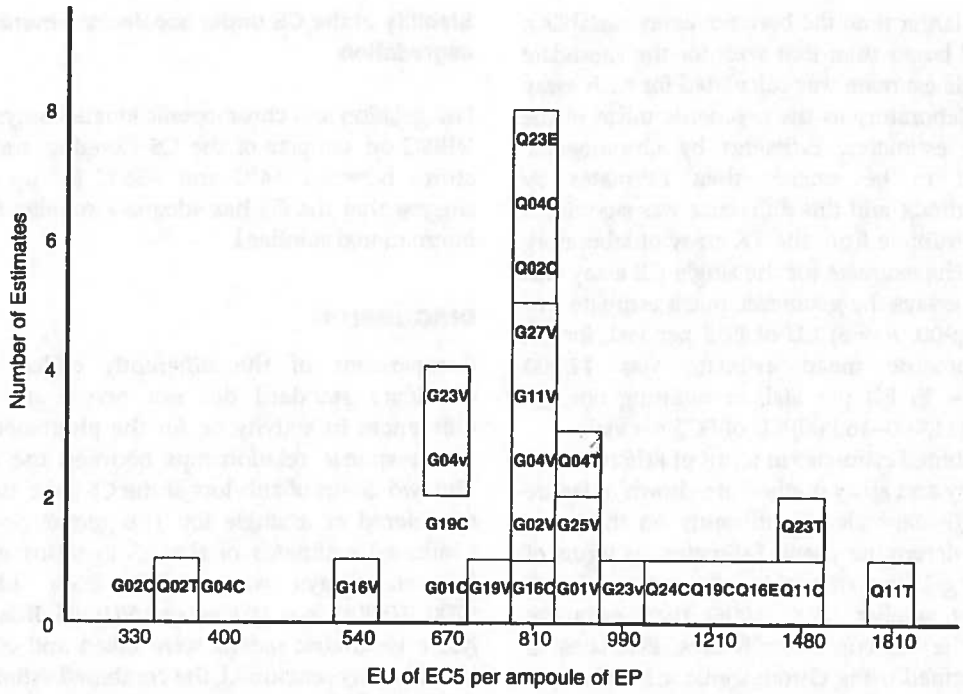

Fig. 3 Laboratory and method geometric mean estimates of the relative potency of the European Pharmacopoeia Standard for Endotoxin expressed as EU of EC5 per ampoule. See caption to Figure 1 for key.

respectively, and were also similar to the observed 1060 (640–1800, $n = 7$) EU of the IS, assuming 18,500 EU per ampoule of the IS.

The single combined estimates in terms of EC5 obtained for each laboratory and assay method are shown in Figure 3. Estimates for BRP-2 (EP) in terms of EC5 are significantly ($P \sim 0.02$) less by gelation assays than by photometric assays, and estimates using the common lysate are both smaller and more variable than estimates obtained using the other lysates; the estimates from the TK assays are more variable between laboratories than estimates from the CK assays or the gelation assays.

Calibration of CS in terms of BRP-2 (EP)

Since BRP-2 (EP) was the only preparation in the study not calibrated directly in terms of EC5, BRP-2 (EP) was also used to calibrate the CS, giving a combined estimate of laboratory and method means of 9700 (8500–11,100, $n = 27$) units of BRP-2 (EP) per ampoule of CS. Estimates separately for gelation assays and photometric assays were, respectively, 10,100 (8500–11,900, $n = 16$) and 9300 (7100–12,000, $n = 11$) units of BRP-2 (EP) per ampoule of CS.

Comparison of JP with EC5

Considered over all estimates by LAL gelation assay, the between assay variabilities for estimates of the JP relative to EC5 were larger than that observed for the duplicate comparisons. These variabilities were reduced to values more similar to that seen for the duplicate comparisons when invalid estimates were excluded. However, the between laboratory and lysate variability was significantly ($P < 0.01$) larger than the variability between assays in the same laboratory using the same lysate. Although much of this variability resulted from an apparent difference in relative activities between the common and the other lysates, the between laboratory variabilities for the various other lysates considered separately were larger than the between assay variability. The geometric mean (95% limits) of laboratory geometric mean estimates over all laboratories and lysates was 10,600 (8300–13,400, $n = 25$) EU of EC5 per vial. However, for common lysate the estimate was 6500 (4900–8600, $n = 10$) EU per vial, significantly less ($P < 0.05$) than the estimate using the various other lysates of 14,600 (11,500–18,500, $n = 15$) EU of EC5 per vial. If the estimates from laboratories 01 (using in house lysates) and 14 (using in house lysates) were excluded, the between laboratory variance of estimates made with the various lysates was reduced by more than 4-fold, and the mean of the remaining estimates was 16,700 (15,300–18,300) EU of EC5 per vial.

The between assay variability for estimates by photometric assay of the JP in terms of EC5 was some 3-fold larger than that seen for comparisons of the duplicates. However, the between laboratory variability

were heterogeneous with highly significant inter laboratory differences. Estimates for the CS from chromogenic assays were generally greater than estimates from turbidimetric assays with the geometric mean estimate by all photometric methods, 11,700 (11,000–12,400) EU/vial, being significantly larger than the mean by gelation assays. However, because of the inter laboratory variability, calibration of the CS by photometric methods may not be reliable: dependence of estimates on method and lysate indicates that the particular methods selected could bias the mean estimate. The mean, over both gelation and photometric assays, of all combined estimates was 10,400 (9900–10,900) EU/vial. This value is in good agreement with the value of 10,000 EU per vial assigned to EC6, which has superseded EC5 as the USP/FDA standard, and which was intended to be as similar as possible to EC5.

When first calibrated, in terms of EC5, the current IS (preparation 84/650) assayed at either 100,00 or 20,000 EU/ampoule in virtually all gelation assays in that study and closer to 20,000 EU/ampoule in photometric assays and in rabbit pyrogen tests.[4] In view of this discrepancy, the IS was designated a standard **for LAL gelation tests** and assigned 14,000 international [gelation] units/ampoule. In the present study, the geometric mean of estimates for the IS in terms of EC5 was 18,200 (15,900–20,900, $n = 28$ estimates) EU/ampoule for gelation assays, compared with 18,500 (17,000–20,100, $n = 47$ estimates) EU/ampoule for all laboratories and methods (gelation and photometric). The estimate of 18,500 EU/ampoule is consistent with the data obtained in photometric assays (and rabbit pyrogen tests) in the earlier study and better reflects the relative mass of endotoxin contained in the IS (2 μg of *E. coli* 0113:H10:K(–) endotoxin) compared with EC5 (1 <μg of *E. coli* 0113:H10:K(–) endotoxin; assigned 10,000 EU) than the unitage assigned.[4] It is likely that the differences between estimates for the IS in terms of EC5 obtained in gelation assays in the two studies is due, at least in part, to the fact that in the earlier study many participants assayed only duplicate 2-fold dilution series whereas in the present study most of the participants assayed four replicate dilution series, permitting more accurate approximations to the end-point to be calculated.

Comparisons among the various endotoxin preparations examined in this study show that the ratio of activity for any pair of preparations may be dependent on the method and/or lysate used since the different methods may distinguish between differences in the endotoxins contained in the standards. Inter laboratory, or inter method variability in this study was very similar to the inter assay variability for the comparison of the two sub-lots of the CS. The comparisons among the CS, EC5 and the IS showed somewhat larger inter laboratory variability, even though each of these standards was prepared from a common bulk preparation (of *E. coli* 0113:H10:K(–) endotoxin). In contrast, comparisons between any one of the CS, EC5 or the IS with either the JP or the BRP-2 (EP) showed substantial inter-laboratory variability, and this method dependence was clearly illustrated by the lysate dependence of estimates by gelation assay for the JP in terms of EC5.

RECOMMENDATIONS

On the basis of the results of this study, and with the agreement of participants, it was recommended to the Expert Committee on Biological Standardization of WHO that the CS is suitable to serve as the international standard for endotoxin for all applications with a unitage of 10,000 IU/vial (on the basis of its calibration in terms of EC5). This value maintains continuity of unitage with EC5, the primary calibrant for the first IS, EC6 (the current FDA/USP standard), the JP standard and commercial preparations of Limulus lysate. This value is also consistent with calibration of the CS in terms of the EP standard, BRP-2 (which was calibrated in terms of the first IS, a standard for gelation assays). With these recommendations carried out, 1 IU = 1 EU, avoiding the need to convert one into the other and achieving the global harmonisation of unitage for standards of endotoxin.

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