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Low Endotoxin Recovery (LER):
A Review

October, 2014

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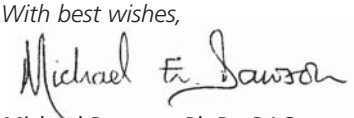
Letter From The Editor

The LAL Update addresses the issue of Low Endotoxin Recovery (LER). This has been a topic of considerable discussion and interest since it was highlighted by Chen and Vinthers in the spring of 2013 at the Annual Meeting of the Parenteral Drug Association in Orlando, FL. The issue concerns the inability to detect some or all of the endotoxin added to certain families of drug products or components of drug product. The FDA has increased their interest in this phenomena as it raises the concern that contaminant endotoxin in drug products might not be detect during release testing. The concern is heightened by reports that samples to which endotoxin has been added but in which it is not detectable in by the bacterial endotoxins test using LAL reagent, were pyrogenic in pyrogen tests performed with rabbits.

The recognition of the LER issue increases the importance of proper method development and validation so that endotoxin in product (whether present as a contaminant or because it has been added) is appropriately detected. The Contract Test Service at Associates of Cape Cod, Inc., has extensive experience in overcoming a wide range of interference problems or masking of endotoxin, including LER, in a wide range of sample matrices. If you are having difficulty with LER, please contact Dr. Barbara Markley at bmarkley@acciusa.com, 888-232-5889 or 508-540-3444.

With best wishes,



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Low Endotoxin Recovery (LER): A Review

Introduction:
The issue of "low endotoxin recovery" (LER) is a relatively new one, having been first introduced in an open forum in April, 2013. This article presents a review of the publications and presentations that have addressed this issue and presents a synthesis of the current situation and the principle themes that relate to LER.

Definition:
At its simplest, LER is the failure to detect all of the known (often added) endotoxin in a product or in-process material, despite the fact that positive product controls (PPCs) show no evidence of inhibition. LER is typically characterized by a decline in the measurable endotoxin concentration over time (such as during sample storage). It has been reported that the sample may maintain pyrogenicity in the USP Pyrogen test, despite a decline in measurable endotoxin. LER may only be evident when an undiluted sample is "spiked" with added endotoxin.

LER was first described by Chen and Vinther[1] who define the phenomenon narrowly, restricting it to masking of endotoxin added to undiluted materials that is attributable to combinations of excipients. These authors specifically distinguished LER from masking of endotoxin caused by APIs, particularly proteins, and focused on polysorbate in conjunction with buffers, particularly citrate and phosphate buffers. Other authors have reported a similar loss of endotoxin from samples that is attributable to proteins and take the view that, because the effect is similar to that described by Chen, such losses should also be described as LER.

LER is not detected by the USP BET Test for Interfering Factors. This test consists of adding a known amount of standard endotoxin (a PPC) to the sample at the dilution at which the test is to be performed and then demonstrating appropriate detection of the added endotoxin. Detection of a PPC does not assure that there is no LER for two reasons. First, the endotoxin added to a diluted test sample may not be masked in the same way as it is when added to an undiluted sample. Second, the Test for Interfering Factors is typically conducted shortly after addition of the endotoxin, whereas the loss of detectable endotoxin that characterizes LER may take considerable time (however, in some cases it occurs rapidly).

This article adopts a pragmatic, effects-based view of LER such that if detectable endotoxin declines in a sample, this is LER. It does not matter to a patient (and thus to a regulator) whether endotoxin is masked by polysorbate and citrate or by the active pharmaceutical ingredient/drug substance (or by a combination of the two). What matters is whether the product contains a pyrogenic concentration of endotoxin that may not be detected in a bacterial endotoxins test.

Mechanism
As LER is observed in a range of different sample matrices it can be expected that there is more than one mechanism by which endotoxin is masked. This is particularly true if the broader definition of LER is adopted as it includes masking by APIs, including proteins. Specific mechanisms of LER have not been elucidated, though a number of hypotheses to explain it have been advanced. In the initial presentation in which LER was first publicly described, Chen and Vinther[1] suggested that, in the presence of chelating agent and polysorbate, endotoxin can form what the authors called an LER complex which does not bind with the lipopolysaccharide (LPS) binding receptor, Factor C, that initiates the LAL reaction. In a more recent presentation Chen[2] refers to the complex as "mixed" micelles. Reich *et al.*[3] have shown that in the presence of low concentrations of

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surfactant (0.0001 % v/v polysorbate 20), endotoxin activity is enhanced to approximately 180 % of the activity of the same endotoxin concentration in the absence of polysorbate. At higher concentrations activity declines and is reduced to almost zero at about 0.0025 % v/v polysorbate 20. The authors hypothesize that, at a surfactant concentration that gives a LPS:surfactant ratio of 1:1, mixed aggregates of LPS and surfactant may be assembled which exhibit enhanced activity. Further

increase of surfactant concentration leads to an abrupt decrease in LPS activity due to re-organization of surfactant-LPS aggregates that do not activate the enzyme. (In the Reich *et al.*study[3] endotoxin concentrations were determined using the EndoLISA endotoxin assay.) In a poster presentation, Reich and Grallert[4] add to this model by suggesting that endotoxin masking is a two-step process. Step one is disaggregation of endotoxin by the depletion of divalent cations due to chelation (e.g by citrate); step two is the inactivation of disaggregated LPS endotoxin by amphiphilic molecules such as surfactants.

Williams[5] discusses the difficulties in recovering endotoxin spikes from protein solutions and suggests that the aggregation states of monoclonal antibodies (mAbs) may affect the degree of endotoxin binding. He cautions that as the protein aggregation states may not be stable, conditions that result in successful recovery of added endotoxin in one study today may not be reproducible in subsequent tests of batches of the product. These considerations would apply to both traditional interference and to LER.

In a more recent presentation on LER Williams[6] focused on the significance of the aggregation state of endotoxin (as opposed to protein). He cites the work of Mueller *et al.*[7] regarding aggregates being the biologically active units of endotoxin and notes that disaggregation of endotoxin micelles by surfactants has been suggested as a cause of LER. In a subsequent article[8] he combines the two possible causes of masking (proteins and excipients) and considers the combined effects.

Regulatory Considerations

Chen[2] describes how, after identifying the LER issue, Genentech reported it to FDA in a Biological Product Deviation Report/Product correspondences and in a Type C meeting. Chen and Vinther[1] presented information on the subject to industry at the 2013 PDA Annual meeting. Since that time FDA has asked other manufacturers to present information on LER, including Lilly[9], Janssen[10] and others. At the PDA Pharmaceutical Microbiology meeting in 2013, Hughes of FDA stated in a discussion that the agency is concerned about LER. FDA is asking a standard set of questions for new submissions for products with a formulation that suggests the possibility of LER, i.e. biological products formulated with polysorbate. She also stated that currently this only applies to new drug applications, not to products that have already been approved. Similarly, Mello[11], a Senior Review Microbiologist at FDA, stated in a presentation that FDA is requesting hold time studies for all formulations containing polysorbate surfactants. These would include the addition of endotoxin to undilute product and then measuring the concentration of detectable endotoxin over time for the material held under appropriate storage conditions. A decline in the concentration of detectable endotoxin is indicative of LER.

An example of the types of study that FDA has requested is that of Janssen Biotech[10], who conducted hold time studies on samples of batches of formulated bulk product, placebo and on finished lyophilized product of Sylvant (siltuximab) formulated with histidine, polysorbate 80 and sucrose. FDA recommended use of control standard endotoxin (CSE). In addition to the data for CSE requested by FDA, data were submitted for unpurified preparations of naturally occurring endotoxin (NOE) derived from *R. pickettii* (an in-house isolate) and *P. aeruginosa* were also used in studies of undiluted drug substance. In these studies added endotoxin was recovered as expected; there was no evidence of LER.

Time and Temperature Effects

In their initial report on the LER issue Chen and Vinther[1] reported that the LER phenomenon occurred more rapidly at room temperature than at 2-8°C. Chen[2] also suggests that LER occurs quite rapidly and that a seven day incubation is sufficient to determine whether a drug product exhibits LER or not. Reich[12] and Reich and Gallert[4] have also shown that loss of detectable endotoxin occurred more rapidly at room temperature than at 4°C. In the case of 10 m*M* phosphate there was no appreciable loss of detectable endotoxin at 4°C while it was evident at room temperature (at least over the period of almost 400 hours studied). LER was evident at both temperatures for polysorbate/citrate. Reich[13] states that the kinetics of LER depend on components and indicates that it is greatest and most rapid with polysorbate+citrate; masking is time and temperature dependent. (It is worth noting that Reich[12] used high endotoxin concentrations (ranging from 100 to 245,000 EU/mL), while Chen[2] used much lower concentrations (e.g. 5 EU/mL).

Platco[14] commented on the rapidity with which LER can occur and reports loss of detectable endotoxin within minutes of addition of polysorbate 80 to a formulation. Under these circumstances testing cannot begin fast enough to obtain 100% recovery of added endotoxin at time zero. By the time samples have been transferred from the production area to the laboratory, masking may already have occurred.

Excipients and LER

As noted in the section on the definition of LER above, Chen and Vinther[1] specifically defined LER as loss of detectable endotoxin due to the effects of excipients. They identified a combination of polysorbate and buffer (particularly citrate and phosphate buffers) as the cause of LER, but not polysorbate or buffer alone. Reich and Grallert[4] corroborate this. These workers report LPS recoveries after 7 days as follows: Water: 100 %; 10 m*M* citrate buffer: 100 %; 0.05 % (w/v) polysorbate 20: 84 %; citrate buffer + polysorbate 20: 0 %. Dubczak[15] has reported LER for RSE in polysorbate and citrate and Associates of Cape Cod, Inc. (ACC) has also repeated the masking of endotoxin by polysorbate/citrate (unpublished data).

Burgenson[16] concluded that the LER phenomenon is real and that it affected all four (RSE, CSE and 2 NOEs) endotoxin types tested. He found that the recovery of endotoxins was reduced by 0.05% polysorbate 20 and that the effect was enhanced when combined with either citrate, phosphate buffered saline or Tris-HCl buffers. Citrate buffers particularly reduced the recovery of endotoxins.

In contrast with the reports of both Chen and Vinther[1] and of Reich and Grallert[4], Bolden[17] found that while LER was not evident in antibodies formulated with combinations of polysorbate and a range of buffers (or in polysorbate 80 and citrate, peptide, mannitol or glycine), it was evident in citrate alone. Reich[12] has also reported LER in a range of organic compounds alone. These include citrate, phosphate, acetate and MES buffers, the benzamidine (protease inhibitor), EDTA and dimethyl sulfoxide.

In contrast with some of the other studies, but like Bolden, Platco[14, 18] showed minimal LER in phosphate buffer with polysorbate 80 with CSE (in the absence of monoclonal antibody). This author reports no LER with CSE or NOE in histidine buffer with polysorbate 80 (i.e. no chelating citrate)[18].

Protein

While Chen and Vinther[1] excluded masking of endotoxin by proteins from their definition of LER, there are reports of LER-like, time dependent decreases in endotoxin recovery caused by proteins, although Bolden[9] reported that there was no LER due to protein drug substance or drug product formulated with citrate at both high and low protein concentrations, other authors

have reported LER type effects with proteins or formulations of proteins with excipients. For example, Dubczak[15] reported LER for a protein preparation when spiked with RSE and to a lesser extent for NOE.

Williams[6] has suggested that protein binding may be more significant than disaggregation of endotoxin. In another work, Williams[5] has stated that LER is not limited to excipients (polysorbate 80 and buffer) and that "… the presence of polysorbate may turn out to be more anecdotal than necessary to the protein component of the LER effect for some biologics." He reports no significant loss of CSE over 7 days in one antibody preparation formulated with polysorbate. For another antibody formulated with polysorbate and citrate there was a marked loss of endotoxin particularly between days 4 and 7. He expresses concern that the unstable aggregation state of mAbs could lead to different degrees of masking.

Platco[14,18] reports marked LER in two mAbs with the same formulation (which includes citrate and polysorbate 80) spiked with CSE (but not for NOE). For one of the mAbs, LER was largely overcome (though not completely) by the addition of $MgSO_4$, but not for the other. As the formulations differed only in the mAbs they contain, this result indicates that the protein has a role in the masking of endotoxin. In another mAb, in histidine with or without polysorbate 80, there was no appreciable LER. In a fourth mAb, in phosphate buffer with polysorbate 80, LER was evident after 4 h for CSE (but not NOE). In one case, inhibition based on the positive product control (PPC) was not evident until endotoxin was added for an LER study, after which the PPC recovery was only 26%. In these studies the relative contributions of protein and excipient to the masking are not identified.

Reich[12] reports LER in mixtures of bovine serum albumin (BSA) and citrate; polysorbate and citrate; and in a combination of all three. In BSA/citrate LER was incomplete, stabilizing at about 25% recovery of the endotoxin. It was most rapid and complete in polysorbate/citrate and was intermediate in the combination of the three components. This result with BSA suggests that the protein moderates the masking of endotoxin.

Williams[8] concludes that LER may be caused by proteins or excipients or by a combination of the two and illustrates this in a Venn diagram. He suggests that protein binding is likely dependent upon both the concentration of the protein and the associated charge. Reich[13] has proposed a similar model of combined effects, suggesting that 70-100 % of masking may be attributable to excipients and that 0 – 30% may be due to API. The masking of endotoxin is clearly highly variable and is a result of the nature and concentration of both excipients and proteins in the product. It is also affected by the nature of the endotoxin.

Naturally Occurring Endotoxin (NOE)

Naturally occurring endotoxins (NOEs) would perhaps better be called unpurified endotoxins as they are often derived from type culture collection organisms that are grown on commercial media. They are not endotoxins obtained directly from nature. Semantics aside, a number of authors (Cooper[19], Platco[14,18], Chen[2], Reich and Gralleert[4], Dubczak[15], Burgenson[16]) have reported that NOEs are much less susceptible to masking than is the purified LPS that comprises RSE and CSEs. Platco[18] states that industry studies are showing that NOEs do not exhibit LER but LPS does and Cooper[19] has suggested that we should "… rename this [LER] phenomena [*sic*] as LLR, low LPS recovery."

Dubczak[15] studied NOEs derived from *E. cloacae, P. aeruginosa, E. coli,* and *S. marcescens* and found variability in the relative detectability between different NOE preparations and between the various assays used (LAL, a recombinant Factor C [rFC] ligand assay and MAT). This author stated that no users of the *E. cloacae* NOE preparation had experienced LER in endotoxin

assays, though he does report limited LER with *E. cloacae* NOE (and for *E. coli* NOE), but only in the ligand assay. Chen[2] found that LER was evident for *E. cloacae* NOE in one drug product, though it was less complete and occurred more slowly than for CSE (he reports 44% recovery of the NOE after 168 h compared with 0% recovery of CSE after 24 h). Burgenson[16] reported LER occurred with both of the two NOEs that were investigated (derived from *P. aeruginosa* and *S. marcescens*) as well as with RSE and CSE. To summarize, masking can occur with NOEs though it generally occurs more slowly and/or to a lesser extent than for purified LPS preparations.

Mello[11] stated that studies using NOE are being reviewed by FDA but are not yet universally accepted. He encouraged firms to contact FDA to discuss LER studies with the reviewers. He noted that FDA does not want to "revisit" the 1970's endotoxin standard issue. (At that time there were multiple different endotoxins in use with results being expressed in units of mass, e.g. ng/mL. The problem was that the activities per unit mass of these endotoxins varied between preparations so results were not comparable between studies. This experience lead FDA to lead the development of a reference standard endotoxin (RSE) with a potency expressed in EU/mL as a measure of activity.)

Williams[8] notes that the use of NOE derived from user-grown organisms, such as those used by Bowers and Tran[20], have been proven to help overcome the dissociation of spike effect. He also cites the work of Reich[13] as showing that masking occurs with NOE just as it does with CSE (though it may be delayed) and that it is formulation-dependent, just as Chen and Vinther[1] first described. In this and in an earlier publication[5] he cautions that NOE is likely to give both variability and conflicting results, depending upon the organism(s) chosen, and states that user-prepared NOE would make standardizing testing very difficult. In a presentation[6] he states that there is a danger of "fixing" LER by use of NOE and notes that adding CSE to undiluted products represents the worst case scenario as the hydrophobic end (i.e. Lipid A) of the LPS is not associated with cellular debris, in contrast with naturally occurring, non-purified endotoxin (NOE). Williams[8] recommends a simple screening approach using a standardized endotoxin (CSE or RSE).

Reich and Gallert[4] show LER with an rFC assay for several NOEs (and for CSE) and report that LER was least pronounced for *E. cloacae* NOE. They found that masking of endotoxins was equally rapid at room temperature for two NOEs, derived from *E. coli* O113 and *E. coli* O55:B5, as for LPS from *E. coli* O55:B5. At 4°C LER for *E. coli* O113 NOE occurred at the same rate as for *E. coli* O55:B5 LPS, but more rapidly than for *E. coli* O55:B5 NOE or *E. cloacae* NOE. These authors also report that growth conditions for the organisms used to prepare NOEs have an influence on the LER response. Thus it is clear that for some NOEs masking can occur as rapidly as for some LPS preparations. There is not a clear separation between NOEs and LPS in terms of their potential for exhibiting LER.

Overcoming LER

It is well known that a wide range of substances interfere with the detection of endotoxins and a corresponding range of strategies has been used to overcome this interference. A number of strategies for overcoming LER have also been described. Burgenson[16] reports that, as with typical interference of endotoxin assays, sample dilution to 1/1000 can significantly improve the recovery of added endotoxin in LER studies. Platco[14] demonstrated that added $MgSO_4$ substantially mitigated LER in one (but not the other) of two mAbs with the same formulation (except for the mAb) that included citrate and polysorbate 80. The added $MgSO_4$ partially overcame LER (but with less than 50 % recovery) in a third mAb with a formulation included sodium phosphate and polysorbate 80. She also states in this presentation that masking may be mitigated by a freeze thaw regime, but does not give specifics. In another report Platco[18] states that no LER was evident when an unspecified endotoxin dispersing agent was added to the sample prior to spiking with CSE. ACC has also demonstrated that masking of endotoxin can be overcome in a similar manner (unpublished data).

Reich[12] reports de-masking of endotoxin but this was only really effective for concentrations of added endotoxin in the range of about 10,000 EU/mL or more. He also notes that different de-masking strategies are required for different matrices. Details of the de-masking strategies are not given, though he refers to a proprietary de-masking agent in another presentation[13]. He reports effective de-masking of endotoxin from *E. coli* O55:B5 (presumably CSE) from a polysorbate 20/citrate solution at three endotoxin concentrations and concludes that de-masking is possible but no general de-masking strategy is available. Different formulations are likely to require different de-masking approaches.

Williams[5] reports recovery of endotoxin added to mAb solutions that contained polysorbate 80 by addition of and continued dilution in an unspecified surfactant. In another report[8] he cites the work of Petsch *et al.*[21] who reported that following protease treatment 100% of the endotoxin spike was detected after days of refrigeration. In common with Platco, Williams notes that Pyrosperse may aid endotoxin recovery and gives examples of normal serum albumin, plasma protein fractions; electrolyte solutions, antihemophilic factor and lipid emulsions. He has reported[6] unmasking endotoxin by digesting IgG with protease followed by heat to denature protease (so that it does not degrade LAL reagent) using both protease K and a proprietary protease (from BioDtech, Inc.). He also reports a marked loss of endotoxin particularly between days 4 and 7 for an antibody formulated with polysorbate and citrate. However recoveries of at least 70 % of the added endotoxin were achieved over 7 days after modifying the "diluent combinations" [sic], despite the fact that the endotoxin spike concentration was halved. Like Reich, Williams[8] has stated that solutions to LER issues are highly formulation-dependent.

Conclusion

A number of points arise from this review of presentations and publications regarding LER, including the following:

1. LER is characterized by a time-dependent failure to adequately recover added endotoxin
2. Masking of added endotoxin may be immediate, particularly at room temperature or higher, but a time course experimental design is required to determine whether endotoxin recovery decreases over time.
3. The phenomenon has been linked to excipients and particularly to formulations that include polysorbate, it has also been reported in the absence of polysorbate. The link between loss of detectable endotoxin and formulations containing polysorbate has been strong enough to lead FDA to routinely request data from LER investigations for new drug approval applications for products with such formulations.
4. LER effects are seen with some proteins or the LER effect may be modified by proteins.
5. NOEs are generally less susceptible to LER than purified endotoxins (LPS) such as RSE and CSE. It has been noted that NOEs vary between preparations and require special preparation.
6. Some authors have recommended the use of LPS preparations such as CSE in LER studies as they represent a worst case. If masking can be overcome for CSE it is considered unlikely to occur with unpurified endotoxins. In none of the studies reported to date has LPS been more easily recovered than NOE. In contrast, in at least one study an NOE was as susceptible to masking as an LPS preparation. One author has even suggested that it might be dangerous to rely on NOE recovery.
7. There are a number of strategies to overcome LER, but these are product specific. Given different interactions with different products and excipients, it is not surprising that treatments are specific to products. This mirrors the normal situation regarding overcoming interference with endotoxin tests.

In conclusion, two general strategies are recommended for addressing the LER issue. The first is to minimize the chance that product will contain endotoxin that might subsequently be masked by the formulation. This necessitates controlling for possible endotoxin contamination in all of the materials that go into a product and in all the processes involved in its manufacture. This is nothing new and should already be a part of the manufacturing process. Williams[5] has stated that "the best assurance of very clean drug solutions (endotoxin-free) is to ensure endotoxin removal at relevant process steps and the prevention of bioburden in manufacturing processes." This has been similarly recommended by Chen[2] who suggests that risk analysis regarding LER should begin at the furthest downstream process step that does not show LER.

The second overall strategy is to test for LER and, if it is evident, conduct test method development studies in order to overcome it. A number of strategies have been presented for recovering and detecting masked endotoxin. Proper method development with appropriate validation of sample treatment will avoid the situation described by Platco[14] (discussed earlier in this article) in which rapid masking of endotoxin could result in loss of detectable endotoxin from a sample between the production area and the laboratory.

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