Will a Proposed Reduction in Endotoxin Limits for Drugs and Biologics Improve Patient Safety?

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Any parenteral therapy theoretically carries a risk of bacterial endotoxins contamination which can result in a number of physiological responses in humans, including fever. It is common in modern clinical medicine for a single IV infusion of 250-1000 mL to contain several "piggybacked" therapies. If each component of the therapy (drug product, diluent, infusion fluids, syringes, transfer sets, etc.) were at its allowable endotoxins limit, patients would be at significant risk of a febrile response. However, there are no data in either peer reviewed clinical literature or the compliance literature, including adverse events and product recalls on FDA's website, to support this hypothesis, suggesting that this concern regarding additive endotoxins activity to unsafe levels is not a verifiable clinical issue. Despite the lack of data in the public domain, it is our understanding that a proposal has been advocated by a number of regulators to mitigate this hypothetical problem by reducing the endotoxins limits for drugs and biologicals by at least half.

We take a different view on the proposal to arbitrarily cut the endotoxin limits as we see no published or documented evidence of a problem. We believe that the continuously evolving science of endotoxins chemistry and their variable biological activity, the extensive use of the highly sensitive LAL test as a monitoring tool for manufacturing controls implemented for the mitigation of potential endotoxins contamination and the voluntary imposition of conservative in house limits.

The Problem

Two examples from current therapies serve to illustrate the hypothetical concern of additive endotoxins activity.

Example 1: COVID-19 Vaccine Package Insert

Table 1a. Product dose and Endotoxins Limit

	Adult (70 kg) Total Person Dose = 350 EU	Child (6 months, 7 kg) Total Person Dose = 35 EU
Drug Product Dose	30 mcg	3 mcg
Endotoxins Limit	11.6 EU/mcg	11.6 EU/mcg

Table 1b. Drug Product Administration

ENDOTOXINS CONTRIBUTOR	Adult (70 kg) Total Person Dose = 350 EU	Child (6 months, 7 kg) Total Person Dose = 35 EU
Drug dose	30 mcg/adult x 11.6 EU/mcg = 348 EU/adult	3 mcg/child x 11.6 EU/mcg = 34.8 EU/child
Syringe to withdraw and administer the dose	20 EU	20 EU
Total	368 EU	54.8 EU
Total (DP at half limit)	194 EU	37.4 EU

In Example 1, if the drug product and the syringe used to administer the dose are each at their maximum allowable limits, the "total person" endotoxins limit for both children and adults would be exceeded. If the limit for the drug product is halved, the "total person" endotoxins dose for a 6-month-old child is still exceeded.

Example 2: COVID-19 Combination Antibody Therapy Package Insert

Table 2a. Product dose and Endotoxins Limit

	Adult (70 kg) Total Person Dose = 350 EU	Child (6 months, 7 kg) Total Person Dose = 35 EU
Drug Product Dose	2100 mg/person	270 mg/person
Endotoxins Limit	0.16 EU/mg	0.12 EU/mg

Table 2b. Drug Product Administration

ENDOTOXINS CONTRIBUTOR	Adult (70 kg) Total Person Dose = 350 EU	Child (6 months, 7 kg) Total Person Dose = 35 EU
Drug Products	2100 mg x 0.16 EU/mg = 336 EU/adult	270 mg x 0.12 EU/mg = 32.4 EU/child
2 syringes for transfer of DP for infusion	40 EU	40 EU
1 sterile pre-filled infusion bag containing 0.9% NaCl (sizes 50-250 mL)*	25-125 EU (assuming the maximum LVP endotoxin limit of 0.5 EU/mL)	N/A
1 sterile empty infusion bag*	N/A	20 EU
1 infusion set*	20 EU	20 EU
1 in line or add on filter*	20 EU	20 EU
Total	441-541 EU	132 EU
Total (DP at half limit)	273-373 EU	116 EU

Note: Components with an asterisk (*) are required by the package insert for administration.

In Example 2, calculations indicate that if each of the required components was at its limit, the total body limit for both adults and a 6-month-old child would be exceeded. If the limits for the drug product are halved, the total endotoxins dose for both the adult and child is still exceeded.

Endotoxins and Threshold Pyrogenic Doses

Endotoxins are structural components of the outer membrane of most Gram-negative bacteria. The endotoxin complex affects membrane permeability, resistance to antibiotics, virulence, and recognition by the host immune system. In humans, endotoxins activity can initiate a febrile response that is mediated by the TLR4/MD2 complex (Molinaro, et al, 2015; Simpson and Trent, 2019). Although endotoxins in nature are often associated with outer membrane proteins and other membrane components, it is the lipopolysaccharide (LPS) portion of the endotoxins complex that is biologically active.

The LPS molecule can be divided into three parts: the strain-specific oligosaccharide side chain, the core oligosaccharide, and Lipid A. It is the Lipid A portion that anchors the molecule to the cell membrane and confers its biological activity. The backbone of the typical Lipid A portion of the molecule is an acylated di-phosphorylated diglucosamine, usually with 4-7 acyl chains of varying lengths. However, the specific chemistry of both the backbone and the length and number of acyl chains can differ dramatically among Gram-negative species (Trent, et al, 2006). Additionally, microorganisms can remodel their Lipid A chemistries as they adapt to changes or stresses in their environments (Raetz, et al, 2009; Simpson and Trent, 2019). Therefore, although the general structure of endotoxins is conserved, they can exhibit significant variability at the fine structure or molecular level, notably acyl chain number and length, and phosphorylation.

A foundational area of study during the development of the Bacterial Endotoxins Test (BET) was determination of the Threshold Pyrogenic Dose (TPD) of endotoxins. Greisman and Hornick (1969) were the first to observe that the TPD in rabbit and man were equal for three different purified LPS preparations. However, they found that it took 50-70 times as much *Pseudomonas* LPS (7 acyl chains) as *E. coli* LPS (6 acyl chains) to achieve a pyrogenic response. Several independent studies in rabbits during the 1980s allowed researchers to further define the TPD as 1 ng/kg, calculated as the lower 95% confidence limit of the average pyrogenic dose of purified *E. coli* LPS (Dabbah, et al (HIMA), 1980; Tsuji, et al, 1980; Weary and Pearson, 1982).

Recognizing that the potency (activity per ng) of LPS from a range of microorganisms is highly variable depending on its Lipid A structure, it was proposed that endotoxins be measured in terms of their activity rather than weight. The initial definition of activity in endotoxins units (EU) assigned an activity of 5 EU to 1 ng of the EC-2 *E. coli* standard. Therefore, the empirical TPD of 1 ng/kg for pyrogenicity is equivalent to an activity of 5 EU/kg.

Marlys Weary and co-workers (1982) compared the average pyrogenic dose and LAL test results for several purified LPS preparations. They observed that for purified LPS, the LAL test provides a 2-6X safety factor over the rabbit test for LPS with 6 acyl chains, and a 26-60X safety factor for LPS with 7 acyl chains, confirming the Greisman and Hornick findings. Loppenow and co-workers (1989) indicated that levels of Cytokine IL-1, one of the cytokines in humans released during the fever response, is also dependent on the number of LPS acyl chains, with six acyl chains being most active. These data demonstrate that the potency and therefore the pyrogenicity of and LPS are dependent on its chemical structure.

Environmental Endotoxins

Pearson and co-workers at Travenol (now Baxter) Laboratories and Donald Hochstein working at FDA compared rabbit pyrogenicity and LAL reactivity of "environmental" endotoxins found in raw materials, in process samples, finished biological products, and numerous water sources, including pharmaceutical water systems (Pearson, et al, 1982; Pearson, 1985; Hochstein, 1987). The taxonomic identification of the organisms contributing to this endotoxins activity were unknown, however the endotoxins clearly originated from Gram-negative bacteria autochthonous to the manufacturing environments, including manufacturing materials or source water

used during drug product manufacturing.

- Pearson, et al (1982) performed 8-rabbit tests on a total of 644 manufacturing samples where the endotoxins activity as measured by LAL exceeded 0.25 EU/mL. The researchers found that 99% of the samples exceeding an LAL result of 10 EU/kg samples passed the Rabbit Pyrogen Test (RPT), suggesting that the TPD may offer a "safety factor" particularly when measuring environmental endotoxins.
- In his 1985 study, Pearson looked at RPT results relative to titration of endotoxins detected by LAL in a bulk lot of the product Piromen, a preparation of *P. aeruginosa* polysaccharide. They found that the rabbit test passed at doses that measured 250 EU/kg and under. Notably, *Pseudomonas* LPS has 7 acyl chains.
- Hochstein published data in 1987 comparing the LAL and RPT on 333 lots representing four different finished biological products containing various levels of "environmental" endotoxins measured by the LAL test. He found that in final product the LAL test was on average significantly more sensitive than the RPT.

Taken together, these studies indicate that the utilization of the highly sensitive LAL test can provide a "safety margin" of between 10X and 50X over the RPT using "real world" products and materials containing endotoxins from autochthonous Gram-negative microorganisms.

Endotoxins Limits

The maximum human endotoxins exposure limit for a dose of drug product is calculated by multiplying the TPD (5 EU/kg for all administrations except for intrathecal) by the weight of the patient. The average weight of an adult in the US has been historically assumed to be 70 kg, making the adult "whole person" endotoxins limit equal to 350 EU (5 EU/kg x 70 kg). According to CDC growth charts, an average 6-month-old child weighs about 7 kg (CDC 2022), making the "whole person" endotoxins limit for a 6-month-old equal to 35 EU.

To assure patient safety relative to the empirically derived TPD, endotoxin limits are calculated for every parenteral drug or biologic administered. Per USP <85>, a chapter that is harmonized with the European and Japanese Pharmacopeia, a product-specific endotoxin limit is calculated using the formula:

$$K \div M$$

Where: K is a constant, which is the TPD of 5 EU/kg for all parenteral administrations other than intrathecal, which was assigned a limit of 0.2 EU/kg

M is the maximum dose of the product/kg/hr as defined in the package insert.

There are some caveats to this formula that relate to patient safety:

- If the pediatric dose of the product is higher on a per kilogram basis than the adult dose, the pediatric dose must be used as the denominator when calculating the endotoxin limit.
- If a product is administered for more than one hour, then M is adjusted to dose/hour.

Medical devices with product or patient contact, which include empty infusion bags, syringes, tubing sets, IV needles, and filters associated with the administration of parenteral drugs, were assigned an endotoxins limit of 20 EU/device regardless of where or how they're used. This assignment was based on the way in which transfusion and infusion devices were prepared for testing in rabbits.

Likewise, Large Volume Parenteral (LVP) preparations routinely used as infusion fluids such as saline, Ringer's lactate solution, or dextrose, were assigned a limit of 0.5 EU/mL, also based on the RPT.

The BET is an *in vitro* enzymatic assay, which has a level of analytical variability typical of biological assays. The original gel clot test was constrained by two parameters: test results were binary (either positive or negative) and the "standard" was a series of twofold dilutions. Given these constraints the resolution of the assay could be accurate only within a single two-fold dilution range (½x-2x or 50-200%). For photometric assays, this range is not indicative of assay resolution, expected assay variability or normal error in the assay, but rather represents limits on the range of interference that might arise in any one test sample due to the test sample matrix. It is possible that the proposal to arbitrarily cut endotoxins limits in half is based on misinterpretation of the 50-200% recovery of the Positive Product Control (PPC).

Is There a Problem?

The concern about potential risk to patients during therapy if the drug product and each of the medical devices used for administration are at their allowable endotoxins limit is clearly appropriate. However, arbitrarily changing the limit, as some have proposed, should require a verifiable or documented clinical risk.

- The TPD was originally determined based on a highly purified and highly potent purified LPS derived from the enteric microorganism, *E. coli*. Endotoxins activity in mammals is related to endotoxins chemistry. Enteric Gram-negative bacteria generally exhibit an LPS structure that is different in acyl chain number and length than the less potent non-fermenting Gram-negative bacteria that are more commonly observed in pharmaceutical manufacturing. Enteric or coliform bacteria are extremely rare process or product contaminants.
- "Environmental Endotoxins" are less potent per unit weight than purified LPS. One gram of Gram-negative cell walls has less LPS than one gram of purified LPS.
- While the aggregate "safety margin" afforded by the LAL test may not be easily quantified, the empirical evidence regarding the sensitivity of the test *vis a vis* the RPT coupled with 40 years of clinical use of products released using LAL suggest that there is little or no risk of all products being at their limits for any one administration of therapy, no matter how complex.

- It must be noted that the proposal requiring a 50% reduction in endotoxins activity also reduces the Maximum Valid Dilution by 50%, which may invalidate some existing method suitability studies requiring firms to re-execute costly and time-consuming experiments.

Since the advent of the LAL test in the 1970s and 1980s, manufacturers of parenteral drugs, biologicals and medical devices have taken advantage of a relatively inexpensive yet reliable and sensitive test to monitor the effectiveness of production and process controls intended to reduce or eliminate the risk of endotoxins contamination. Water systems have historically been a source of endotoxins in parenteral products (Seibert, 1923; Seibert, 1925). Although design and engineering of water systems have improved dramatically, the generation and distribution of this ubiquitous raw material are still monitored extensively using the LAL test. Manufacturing materials, particularly those derived from natural sources, are assigned endotoxin limits based on their use (e.g. API, excipient, etc.) and are tested for endotoxins activity prior to use. The identification and routine monitoring of critical control points that can either introduce or reduce endotoxins contamination have provided assurance that processes are consistently "cleaner" than the calculated endotoxins limit would allow. In addition, the voluntary imposition of in-house action/alert/release limits that are generally much lower than the calculated endotoxins limit has assured that products are safe as defined by the TPD.

Current limit-setting strategies based on dose have served patient safety well since first published by FDA in 1983 (Federal Register, 1983). To adjust a product's endotoxins limit to account for endotoxins contribution from co-administered products and delivery devices requires an estimation of the type, number, dosing, time of administration and the endotoxins content of the co-administered products. That number cannot be known by a manufacturer *a priori*. Because of the implementation of prudent risk mitigation measures by manufacturers, the risk of each component of an infinite numbers of combinations of drugs and administration devices being at their allowable endotoxins limits is virtually non-existent. The only estimate of endotoxins contribution would be the acceptance limit, as found in the product monograph or as calculated according to USP <85> or <161>.

The authors maintain that endotoxins science and proactive control of endotoxins in the pharmaceutical and medical device industries have resulted in substantial risk abatement relative to endotoxins contamination. The concern of increased patient risk due to multiple components being at their limits, however well intentioned, is not a documented problem making the arbitrary reduction of endotoxin limits unnecessary.

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