Low Endotoxin Recovery

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Abstract

Endotoxin in the bloodstream presents a severe health risk already in small doses. Thus endotoxin tests are extremely important and mandatory for release of parenteral administered drugs. For detection of endotoxin, Limulus-based methods are the gold standard. Many Drug Products however cause interference with such detection methods. Very often this interference can be overcome by dilution, but not in the case of Low Endotoxin Recovery（LER）. Excipients used for drug product formulations like citrate buffer in combination with polysorbates or even the active pharmaceutical ingredient itself are able to cause LER. This effect leads to failure in determination of correct endotoxin contaminations. A controversial discussion about the relevance of LER and the setup of these studies is ongoing. Here we present a review of the molecular mechanism behind LER and the factors that influence this effect. The importance of standardized protocols for LER studies to produce comparable results is summarized and an outlook for dedicated sample treatments that are able to overcome the LER effect is given.

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1. Endotoxin detection

In 1894, Richard Friedrich Pfeiffer reported fever-causing reactions upon intravenous injections. 100 years ago, it was discovered that substances released by dead bacteria were the cause of these fever reactions[1)]. Today it is known that these substances originate from the outer cell membrane of Gram-negative bacteria or cyanobacteria and belong to the family of Lipopolysaccharides（LPS）[2)]. Even low doses of LPS entering the bloodstream cause a systemic inflammatory response that leads to various pathophysiological effects such as endotoxin shock, tissue injury and even death[3)]. In particular, the lipid A part of the endotoxin complex is responsible for the endotoxic effect. Thus, the importance of adequate LPS detection methods became apparent very quickly. In 1912, the Rabbit Pyrogen Test（RPT）was introduced to the British Pharmacopoeia after the discovery that the use of injectables can cause fever.

A major step in the detection of endotoxin was the observation of specific coagulation of the blood of the horseshoe crab（Limulus polyphemus）after exposure to bacterial LPS[4)]. Based on this finding the limulus Amebocyte lysate（LAL）test, also called Bacterial Endotoxin Test（BET）, was developed and found its way into the US Pharmacopeia（USP）and other Pharmacopoeias. The reaction of the horseshoe crab coagulation system is initiated already at very low LPS concentrations of pico to nano gram[4)]. Three kinds of serine proteases（Factor C, Factor B）and a gel-forming protein（Procoagulase）are involved in the LPS mediated cascade. The Factor C activated by LPS activates Factor B. The proclotting enzyme is then converted to clotting enzyme by the activated Factor B[5)]. The clotting enzyme is cleaving specifically two peptide bonds in coagulogen, which leads to an insoluble coagulin gel[6)]. An additional cascade induced by activation of Factor G through β-Glucans activates the clotting enzyme[7)]. Efforts to replace the LAL based assay have led to the development of the Recombinant factor C（rFC）assay. Apart from protecting the natural source, the rFC assay lacks Factor G—and therefore the false positive activation by β-Glucans- leading to a higher specificity than LAL based assays. By the constant use and increasing quantity of horseshoe crabs needed in

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order to produce LAL lysate, and the observation of decreasing horseshoe crab populations and a risk to be one of extinction threatened species the importance of rFC methods is even more important[8].

Endotoxin testing on pharmaceutical products is regulated by pharmacopoeias and the techniques for BET are harmonized in Europe, United States and Japan. They are described in US Pharmacopoeia (USP) Chapter 85, European Pharmacopoeia (Ph. Eur.) Chapter 2.6.14 and Japanese Pharmacopoeia (JP) Chapter 4.01[9~11]. Today Limulus-based methods are the Gold-standard for endotoxin detection but interference can cause inconsistent results.

2. Difference between test and sample interference

In case of test interference, the enzymatic reaction of the Limulus detection system is inhibited or enhanced. The most common interferences are caused by inappropriate pH, unbalanced divalent cation concentration, serine protease, serine protease inhibitors, and non-specific LAL activation. In most of these cases the best attempt to overcome test interference is dilution. Over 90% of these interfering factors are concentration dependent and can be solved by dilution with water. Pretreatment procedures to eliminate the disturbing factor followed by dilution can reduce these interferences further[12]. Nevertheless, not all inabilities of endotoxin detection can be solved by these treatments. Especially Low Endotoxin Recovery (LER) cannot be overcome by dilution of the sample. The term "LER" was introduced by Chen and Vinther in 2013, and met with large interest in the pharmaceutical industry and regulatory institutions[13]. Already before, LER-like phenomena were described in 1988 by Nakamura et al. They described the effects of Triton X-100, altering LPS micelles and thus being able to inhibit the activation of Factor C by LPS strongly[14]. The formulation of large protein molecules often contains substances with similar effects. Citrate or phosphate buffer and polysorbate are often included in the formulation for stability reasons[15]. Exactly these substances in combination were shown to introduce LER effects alone or in combination with the Active Pharmaceutical Ingredient (API)[16]. In some cases the API alone, such as cationic proteins are able to lead to a LER effect[17]. In other words, the endotoxin is masked and not accessible for detection.

LPSs are amphiphilic molecules which form supramolecular structures in aqueous solutions. The structure strongly depends on the chemical structure of the molecules and the aggregate structure has a strong effect on the biological activity[18~21].

LER is not detectable by BET when testing for interfering factors according to current compendial chapters (eg., USP<85>). Therefore, standard endotoxin is added to the diluted sample immediately before the measurement (Positive Product Control (PPC)). However, the detection of PPC is not a proof of nonexistence of a LER effect. The masking of endotoxin depends on the concentrations in the formulation, thus spiking in the diluted sample can attenuate the masking effect. Additionally, spiking of the PPC is done just before the measurement. Because LER is a time-dependent effect, it cannot be detected in this way[16,22~24]. In fact regulatory authorities recommend tests to ensure the ability to detect endotoxins and mentions the importance of storage and handling[25,26].

3. Controversial discussion : LER is not a problem!

A controversial discussion about the importance of LER regarding the validity of measurements of endotoxin contamination in biologicals and patient safety is still ongoing. The combination of chelating buffer and polysorbate has been used for 30 years in biological formulations, thus the LER issue exists already for the same time period. If LER is an issue, an incident with undetected endotoxin should have happened until now, however in literature or among reports on the FDA website, no report of such an incident can be found[27]. However, the traceability of side effects from endotoxin is quite difficult, because typical endotoxin side effects like fever are often observed after injection of biologicals. Consequently, the assignment of fever to potentially masked endotoxin is almost impossible.

Furthermore, there are also controversial discussions about endotoxin spikes used for hold time studies. Very often Endotoxin standards like Control Standard Endotoxin (CSE) or Reference Standard Endotoxin (RSE) are used for hold time studies to examine the LER propensity in Drug Products. These standards are purified endotoxin preparations. Endotoxin contaminations however originate from raw material or occur during the manufacturing process and are therefore not purified. In contrast, naturally occurring endotoxin might be a better choice for such spiking studies. Therefore, so called natural occurring endotoxins

(NOEs) have been created. NOE refers to endotoxin preparations with minimal processing, and should better represent the contaminations as they potentially occur during the manufacturing process. Electron microscopy studies showed structural differences between the NOEs and the purified LPS[28].

In aqueous solutions LPS normally forms aggregates such as micelles, ribbons and other conformations and they tend to disaggregate in the presence of chelator and surfactant. The biological activity of LPS gets reduced by disaggregation[18, 29]. The different aggregation propensity of purified LPS standards and NOEs could explain their different behavior in the presence of LER causing agents. In fact, several recent studies have claimed that LER occurs only with purified LPS standards but not with NOEs[30]. Bolden et al. reported successfully recovered NOE in citrate and phosphate buffer systems containing polysorbate. Based on their findings, the harmonized compendia bacterial endotoxin testing methods are sufficient. They propose to use a NOE liquid stock endotoxin preparation instead of the currently used LPS for LER studies[31]. The results of Schwarz et al. however showed that LER occurs not only for CSEs, but also for NOEs. Both masked endotoxin contaminations, a recombinant protein preparation (NOE) as well as masked LPS (CSE) were not detected in Factor C based Assays. The observation demonstrates not only the masking of LPS by different buffer formulations, but also LER effects on NOE, coming to the conclusion that LER is not exclusively for endotoxin standards (i.e. CSE, RSE). The biologically activity as well as a potent immune response to masked LPS could be shown[32]. Thus, it is suggested that masked LPS is a potent trigger of human immune responses in a TLR4-NF-κB-luciferase reporter gene assay, which raises a warning for the potential danger of masked LPS.

Reich et al. showed NOEs masking, dependend on originating species and growth conditions. Masking characteristics remained unchanged, independent of the purification process, concluding masking characteristics are mainly influenced by growth conditions and molecular structure, not by the purification process[33].

The preparation of the NOEs used for the studies differs from lab to lab and might thus be the cause for the different results. Additionally, endotoxins are heterogeneous and their structure is strongly influenced by the growth conditions and source of the Gram-negative bacteria[34]. Last but not least the sample matrices and hold time conditions need to be considered when different experiments and endotoxin preparations are compared. In order to evaluate the masking susceptibility of an endotoxin preparation, conditions should be chosen which allow the establishment of a new equilibrium. As an example, a matrix containing 0.05 wt % Polysorbate 20 and 10 mM Sodiumcitrate (pH 7.4) with a spike concentration of 100 EU/mL and hold time for 7 days at room temperature can be used.

4. Mechanism

A two-step mechanism of masking was proposed by Reich et al. The LPS equilibrium structure is shifted to an alternated supramolecular structure. The hydrophobic parts of the LPS molecules (lipid A) are the driving force for its self-aggregation. Further, the aggregates are stabilized by ionic interaction formed between the phosphates of the LPS molecules and divalent cations. The addition of complex forming agents is destabilizing the structure by destabilizing the salt bridges (Step Ⅰ). In presence of surfactant, mixed aggregates are formed and the supramolecular structure is changed completely (Step Ⅱ).This structural change leads to a loss in activity and inability to be detected via common endotoxin test methods[16].

A similar mechanism was also reported by Tsuchiya, involving chelating agents to remove divalent cations from the outer layer of the aggregated LPS as well. In this model, LPS molecules are replaced in the next step by detergent molecules. The overall size of the structure stays similar but the number of LPS molecules in these structures are reduced. Less LPS molecule surface is exposed and this is reducing the detectable activity[35]. Wang et al. also studied the aggregation status of masked endotoxin using static and dynamic light scattering methods. These results support the hypothesis that activation of Factor C of limulus-based test methods is dependent on aggregate size, and that the modulating effects of salts and surfactants on activation of Factor C is associated with changes in the LPS aggregation[36]. Taken together, there is a change in LPS aggregation, when it is masked for detection.

The two-step mechanism is kinetically controlled and the time period for these structural changes can range from seconds to several weeks[37]. The kinetic is directly dependent on the energy input and can be changed by the incubation temperature. The energy input at 25℃ is higher than at 4℃ and thus the masking occurs faster. The energy input by mixing is influ-

encing the masking kinetics as well[22]. The first step of the shown mechanism could be identified to be the critical step in endotoxin masking, influencing the masking kinetics very strongly. In general, the setup of hold time studies has a huge impact on the observed masking kinetics. The concentrations of the components involved in the masking of endotoxin are influencing the masking of the formulation very strongly, although it has to be kept in mind that the binding of endotoxin to the API itself is an important factor as well[38,39].

5. Outlook

The phenomenon of LER represents the potential risk of obtaining false negative test results in BET. To overcome the LER effect demasking is a crucial step to secure the correct endotoxin determination in biologicals. If the reduced LAL activity is caused by the binding of endotoxin to the API a digestion of the protein with proteinase K was shown to recover formally masked endotoxin. Especially positively charged peptides and proteins are prone to bind LPS molecules[40]. After treatment, the Endotoxin–Protein Complex was dissociated and the activity of the endotoxin could be determined with the LAL assay[17]. The addition of divalent cations in an excess to the masking components was shown to rectify the LER effect. In fact, the dilution of LER samples in 2 mM magnesium solutions were able to recover endotoxin, while the endotoxin detection after dilution in water was not able to overcome LER[41]. This effect is used in some dispersing agents on the market, which show similar effects. Lowering the pH of the sample and lowering with it the chelating effect can increase the detectability of endotoxin as well.

If dilution in dispersing agent is not sufficient another attempt can be useful. Demasking by sample pretreatment using a combination of dedicated demasking components is recommended. Dedicated kits for demasking include several components that are, in combination, able to disturb the masked endotoxin complex. The components are able to adjust the pH, destabilize the masked endotoxin complex, adsorbs the surfactant and to restore the endotoxin. These components can be combined in different variations and in different ratios in order to optimize the endotoxin recovery. As detection method, a heterogenous test format (e.g., EndoLISA®) is recommended due to its high toleration of test inhibitors, but optimization of the dilution after demasking makes the detection in a compendial LAL test possible.

The LER effect is not completely understood yet, and the importance of the phenomenon for the safety of parenteral drugs is still under discussion. Thus, LER has to be further studied and the testing principles have to be optimized in order to secure correct endotoxin measurements. Additional testing methods like the monocyte activation test may also be able to give new insights in the activity of masked endotoxin.

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