Endotoxin Detection in Pharmaceuticals and Medical Devices with Kinetic-QCL, a Kinetic-Quantitative Chromogenic Limulus Amebocyte Lysate Assay

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Zusammenfassung

Die Beobachtung, daß Endotoxin in Extrakten von Limulus Amöbozyten ein Gel erzeugt, wurde zu einem in vitro Test ausgebaut, einem kinetischen, quantitativen Chromogen-LAL-Assay (Kinetic-QCL), mit dem in wässrigen Lösungen Endotoxine bestimmt werden können. In den letzten 15 Jahren hat der Limulus Amöbozyten Lysat Test (LAL) zur Bestimmung von Endotoxinen in Pharmaka und medizinischen Hilfsmitteln breite internationale Anerkennung gefunden. Sowohl die Pharmakopöen der USA als auch Europas enthalten Beschreibungen und Vorschriften zum Einsatz des LAL-Tests für die Bestimmung bakterieller Endotoxine. In beiden Pharmakopöen wurde damit begonnen, die erforderlichen Kaninchen-Pyrogen-Tests in Arzneimittel-Monographien durch Endotoxin-Grenzwerte zu ersetzen, die mit dem LAL-Test bestimmt werden können. Der Einsatz des LAL-Tests zur Endotoxin-Kontrolle von Endprodukten hat sich als unschätzbar erwiesen. Ebensogut kann auch der Endotoxin-Gehalt in Ausgangsstoffen und Verpackungsmaterialien bestimmt werden. Bei der Kontrolle während kritischer Produktionsabläufe können die Ursachen von Endotoxin-Kontaminationen gefunden und Entpyrogenisierungsprozesse durch die Quantifizierung des Endotoxin-Abbaus validiert werden. Schnelligkeit, Reproduzierbarkeit, Empfindlichkeit und Wirtschaftlichkeit, verbunden mit der entsprechenden Ausrüstung und Software, machen aus dem kinetischen QCL-Assay eine sowohl dem in vivo Kaninchen-Pyrogentest als auch dem traditionellen Limulus-Geliertest überlegene Methode, wenn es um die Kontrolle von Endotoxinen in Pharmazeutika und medizinischen Hilfsmitteln geht.

Summary

The observation that endotoxin caused gelation in extracts of Limulus amebocytes has been expanded to the development of an in vitro kinetic, quantitative chromogenic LAL assay (Kinetic-QCL) for the detection of endotoxin in aqueous fluids. Within the last 15 years, the use of Limulus amebocyte lysate to detect and control the presence of pyrogenic substances in pharmaceuticals and medical devices has gained wide international acceptance. Both the United States and European Pharmacopoeias contain descriptions of and requirements for the LAL Bacterial Endotoxin Test. Both pharmacopoeias have begun to remove the rabbit pyrogen test requirement in a majority of drug monographs and have substituted endotoxin limits to be determined by LAL. The use of LAL has proved invaluable in controlling the level of endotoxin in finished product. The endotoxin contribution of raw materials and packaging material can be monitored as well. In-process testing at critical production steps can identify additional sources of endotoxin contamination, and depyrogenation processes can be validated by quantitating the degradation of endotoxin challenges. The speed, reproducibility, sensitivity, and economics of the Kinetic-QCL assay, in conjunction with the appropriate equipment and software, over both the in vivo rabbit pyrogen test and the more traditional LAL gel-clot assay allow a more in-depth approach to the control of endotoxin in pharmaceuticals and medical devices.

Keywords: Limulus amebocyte lysat test, kinetic quantitative chromogenic LAL assay, endotoxins, pharmacopeias, pharmaceuticals, medical devices

1 Introduction

The association of pyrogenic reactions with the intravenous injection of fluids is over 200 years old. In 1874, Panum postulated the existence of a heat-stable pyrogenic material present in the injected fluid as the causative agent. Subsequently, several investigators, Billroth (1862), Burdon-Sanderson (1876), Jona and Centanni (1916), studying the nature of this fever-inducing substance suggested that it was of bacterial origin and exclusively associated with Gram-negative bacteria. Pfeiffer is reported to have proposed the term „endotoxin" to describe the

membrane-associated toxin of Vibrio (cited in Pearson, 1985).

The concern over the presence of endotoxin in pharmaceuticals was raised by the studies of Wechselmann (1911), Müller (1911), Samelson (1913), and Bendix and Bergman (1913). Hort and Penfold (1912a, 1912b, 1912c) expanded the findings that a Gram-negative bacterial substance was responsible for pyrogenicity. These workers are credited also with designing the first rabbit pyrogen assay.

Subsequently, confirmatory observations by Seibert (1923, 1925), Seibert and Mendel (1923), and Rademaker (1930, 1932) reinforced the concept that bacterial contamination was present in all pyrogenic injectables, and that through careful technique to avoid bacterial contamination, non-pyrogenic injectables could be produced. Their work lead to a collaborative study, which standardized the rabbit pyrogen test, and allowed pyrogen testing to obtain pharmacopeial recognition in 1942.

The rabbit pyrogen test remained the exclusive official pyrogen assay, referenced in the U.S. Pharamcopeia (USP) for over 25 years, and is still referenced exclusively in many of the USP drug monographs. However, events occurring in the late 1950s and early 1960s were to lead to a significant *in vitro* alternative to the rabbit pyrogen test for determining pyrogenicity in pharmaceuticals and medical devices.

2 Limulus Amebocyte Lysate Assay

The basis for using *Limulus* amebocyte lysate in the detection of endotoxin lies entirely in the coagulation reaction inherent in *Limulus* blood. In 1956, Frederic Bang, reported that infecting the horseshoe crab, *Limulus polyphemus* with Gram-negative bacteria resulted in fatal intravascular coagulation. By 1964, Levin and Bang had demonstrated that extract of the circulating amebocytes would gel in the presence of Gram-negative endotoxin. Endotoxin catalyzes the activation of a proenzyme contained within the LAL (Young et al., 1972). The initial rate of activation is determined by the concentration of endotoxin present. The activated enzyme cleaves the clotting protein, also present in the LAL, resulting in the formation of an insoluble clot.

2.1 Gel-Clot Assay

The most commonly employed LAL method is the gel-clot assay. This assay utilizes the entire endotoxin-mediated cascade in addition to the clotting protein to product a gelatinous clot after incubation with endotoxin. Basically equal volumes of sample in LAL (typically 0.1 ml each) are combined in a 10×75 mm glass tube. After an incubation period of 60 minutes at 37°C, the tubes are inverted 180°. A positive result is indicated by a clot, which withstands the inversion. By titrating the lysate with an endotoxin of known potency, the minimum concentration of endotoxin required to yield a positive clot can be determined. This minimum endotoxin concentration, or end-point, is referred to as the lysate sensitivity.

The gel-clot assay can be used as a purely qualitative limits test to rank samples as either positive or negative, i.e. greater than or less than the lysate sensitivity. However, by titrating positive samples one can obtain a semi-quantitative measure of the endotoxin concentration in unknowns by multiplying the last positive sample dilution by the lysate sensitivity. The gel-clot assay suffers from the disadvantage that they are best semi-quantitative and require preparing multiple dilutions of samples to determine a positive/negative end-point.

2.2 Kinetic-QCL Assay

A truly quantitative assay has been developed by substituting a synthetic chromogenic substrate for the endotoxin activated clotting enzyme in place of the natural clotting protein. As with the gel-clot assay, the amount of active clotting enzyme produced is proportional to the amount of endotoxin in the sample. However, instead of resulting in a simple yes/no, qualitative, assay; the use of the chromogenic substrate results in an assay which allows for the endotoxin concentration in unknowns to be quantitated.

The combination of LAL and the chromogenic substrate (Kinetic-QCL Reagent) provides a better understanding of the endotoxin mediated reaction (Figure 1). Solutions containing known amounts of endotoxin ranging in concentration from 50 to 0.005 EU/ml were combined with an equal volume of the Kinetic-QCL Reagent, and incubated at 37°C while the absorbance of the reaction at 405 nm monitored. The graph illustrates the time, on the X-axis, before the creation of the active enzyme as seen by the increase in absorbance, on the Y-axis, at different endotoxin concentrations. The concentration of endotoxin is expressed in Endotoxin Units (EU) based on the current FDA/USP Reference Endotoxin Standard, EC-5. With high concentrations of endotoxin, i.e. 50 EU/ml, there is a short lag time followed by a rapid increase in absorbance and an eventual plateau when all the substrate has been consumed. As the endotoxin concentrations decrease, the corresponding lag time significantly increase and the rates of color generation become slower.

This family of curves is different from what one normally sees when performing a kinetic enzyme analysis, where the reaction begins immediately and different concentrations of analyte only cause different reaction rates. It is important to keep in mind, that unlike „classical" kinetics, in the kinetic-quantitative chromogenic LAL (Kinetic-QCL) assay the enzyme does not exist but is created during the reaction by the activation of endotoxin.

In order to estimate the endotoxin concentration in unknown samples,

it is necessary to develop a standard curve which correlates a unique reaction parameter with a corresponding endotoxin concentration. It was decided to focus on the time required for the creation of the active enzyme. This time, designated the Reaction Time, was arbitrarily defined as the time when the absorbance of the reaction mixture increased by 200 mOD. The horizontal line in Figure 1 represents this 200 mOD threshold. This value was chosen to allow extremely low concentrations of endotoxin to be detected as soon as possible and still provide adequate separation between this lowest endotoxin concentration and the blank. There is an inverse relationship between Reaction Time and endotoxin concentration. The larger concentrations of endotoxin have the shorter Reaction Times and the smaller concentrations of endotoxin have the longer Reaction Times.

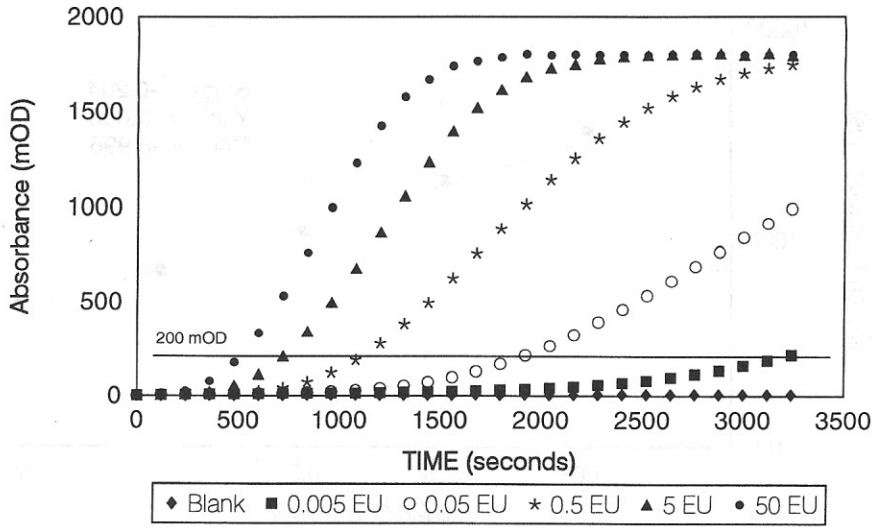

Figure 1: Kinetic-QCL assay, absorbance vs time
Solutions containing endotoxin ranging in concentration from 50 to 0.005 EU/ml were combined Kinetic-QCL reagent, and incubated at 37°C while the absorbance of the reaction was monitored at 405 nm.

A standard curve for Kinetic-QCL assay can be constructed from the data obtained from the kinetic analysis (Figure 2). The log endotoxin concentration, on the X-axis, is plotted against the log Reaction Time, on the Y-axis. Prepared in this manner, the curve is linear and can quantitate endotoxin in unknowns over a concentration range of 50 to 0.005 EU/ml without the need to prepare multiple dilutions of the sample.

Because of the unique method chosen to perform the Kinetic-QCL assay and analyze the data, it was necessary to develop a Kinetic-QCL specific software package combined with the appropriate instrumentation, the Kinetic-QCL Reader. The Kinetic-QCL Reader is an incubating microplate spectrophotometer capable of repeatedly reading a disposable microplate over time while maintaining the reaction at 37°C. The Kinetic-QCL Reader is supplied with a version of the Kinetic-QCL software on-board, or the Kinetic-QCL Reader can be control by an external PC and the Kinetic-QCL PC-based software. The Kinetic-QCL PC based software simplifies data entry and provides for greater storage capacity of assay protocols, sample information and archived data.

Because the endotoxin concentration in an unknown is calculated by comparing its corresponding Reaction Time to that of a series of endotoxin standards, it is essential that the Reaction Times be reproducible across the entire microplate. Of concern is that different portions of the microplate may heat-up to incubating temperature at different rates. Since the assay is monitored kinetically these differences in heating may result in differences in the observed Reaction Time and cause variability in quantitating endotoxin.

In using the Kinetic-QCL Reader, the microplate, filled with just the standards and unknowns, is preincubated in the Reader to 37°C prior to the addition of the Kinetic-QCL Reagent. In this way, independent of the heat-up rate, all positions of the microplate are at the appropriate incubation temperature before the assay is initiated. In addition, the design of the Kinetic-QCL Reader allows for the addition of the Kinetic-QCL Reagent while the microplate remains in the Reader, and is maintained at 37°C. The data presented in Figure 1 was generated by assaying each endotoxin solution in triplicate. The coefficients of variation for the individual Reaction Times obtained for the 50 EU/ml, 5 Eu/ml, 0.5 EU/ml, 0.05 EU/ml, and 0.005 EU/ml solutions were 0.88%, 1.23%, 0.89%, 0.31%, and 1.22%, respectively. To assess the overall reproducibility of the Kinetic-QCL assay performed in this way, 100 æl of a solution containing 0.5 EU/ml of endotoxin was dispensed across the entire microplate, preincubated in the Kinetic-QCL Reader for 10 minutes prior to the addition of 100 µl of the Kinetic-QCL Reagent, and the Reaction Times of each of the 96 wells across the microplate determined. The overall coefficient of variation for the 96 Reaction Times across the entire plate was 0.82%. Comparable CV's were observed both across individual rows or down individual columns.

3 Regulatory Aspects

The United States Pharmacopeia (USP), the United States Food and Drug Administration (FDA), and the European Pharmacopoeia (EP) have published guidelines on the use of LAL for detection of endotoxin in

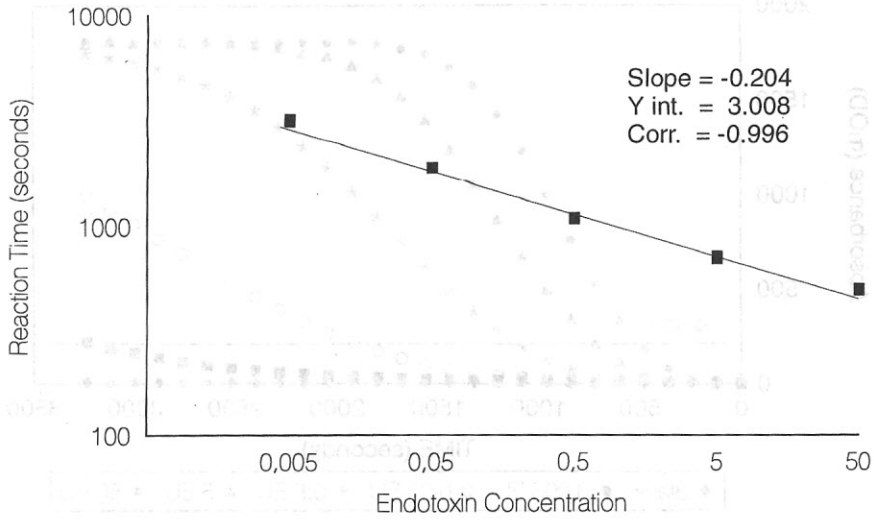

Figure 2: Kinetic-QCL assay, standard curve
Standard curve for Kinetic-QCL assay. The log endotoxin concentration is plotted against the log reaction time. The curve can quantitate endotoxin in unknowns over a concentration range of 50 to 0.005 EU/ml

pharmaceuticals and medical devices. Collectively, these documents outline the acceptable procedures for
1) determining endotoxin limits,
2) validating the LAL tests and
3) developing a routine testing protocol.

3.1 Endotoxin Limits

In the United States, endotoxin potencies are expressed in Endotoxin Units (EU) based on the current FDA/USP Reference Endotoxin Standard, EC-5. In Europe, endotoxin potencies are expressed in International Units (IU) based on the WHO Endotoxin Standard, 84-650. For pharmaceuticals, endotoxin limits are based on the product's human dose. A typical parenteral solution would have an endotoxin limit of 5 units of endotoxin per kilogram body dose of the product. Alternative procedures exist for intrathecals, radiopharmaceuticals and anti-neoplastics. Medical devices are typically tested by extracting the device with 40 mls of an appropriate extraction solution. Each ml of the extraction fluid must contain less than 0.5 units of endotoxin.

3.2 Validation

Validation of the LAL assay is composed of two parts,
1) qualifying the laboratory analyst and
2) qualifying the specific product.

Analyst qualification involves confirming the endotoxin sensitivity of the assay method using an endotoxin preparation of known potency. Product qualification involves spiking the product with known amounts of endotoxin and subsequently detecting an acceptable percentage of the spike. This recovery experiment indicates that the product does not adversely interfere with the LAL assay. Many pharmaceutical products interfere with the performance of LAL test. Guilfoyle and Munson (1982) reported that of 587 pharmaceuticals tested at use concentration, 78% interfered with the LAL assay in some manner. Interference may be defined as the inability to recover an endotoxin spiked within acceptable limits. Enhancement, on the other hand, is characterized by the apparent recovery of more than the known endotoxin spike. However, the authors demonstrated that all of these products could be pre-treated, most notably by dilution alone, and successfully validated using the LAL assay.

The specific validation requirements for analyst qualification using the Kinetic-QCL assay involve confirming the linearity of a standard curve generated using an endotoxin preparation of known potency. The coefficient of correlation, r, determined by linear regression must fall between -0.980 and -1.000. These values are negative because of the inverse relationship between Reaction Time and endotoxin concentration. The product validation requirements using the Kinetic-QCL assay involve spiking a sample of the product with a known amount of endotoxin, typically 0.5 EU/ml, and, after accounting for any background endotoxin contained in the unspiked sample, recovering +/- 50% of the spike

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Richtigstellung:

Die Präsidenten der Schweizerischen Akademie der Medizinischen Wissenschaften (SAMW; Prof. A. F. Muller) und der Schweizerischen Akademie der Naturwissenschaften (SANW, Prof. B. Hauck), weisen uns auf einen Irrtum bei der Besprechung der Ethischen Grundsätze und Richtlinien (EGR) in ALTEX 11/4, 1994, S. 220ff hin.

Da war zu lesen:

„Sie (die EGR) sind für Mitglieder der beiden Akademien freiwillig und unverbindlich. Die Industrie muß diese Empfehlungen nicht berücksichtigen."

Richtig müsse es heißen:

Die SAMW und die SANW haben die EGR bereits 1983 als Kodex für alle in der Schweiz tätigen Wissenschaftler und Wissenschaftlerinnen und deren Mitarbeiter und Mitarbeiterinnen verbindlich erklärt (1). Auch die chemische Industrie der Schweiz steht uneingeschränkt hinter diesen Richtlinien und erachtet sie als Basis für ihre Tätigkeit (2).

(1) Ethische Grundsätze und Richtlinien für wissenschaftliche Tierversuche (1994). *Schweizerische Ärztezeitung 75*, Heft 33, 1255–1263.

(2) Tierversuche sind notwendig (1992). Eine Dokumentation des Arbeitskreises Gesundheit und Forschung, Zürich, Seite 21.