VER
Vascular & Endovascular Review

Validation of Gel Clot Endotoxin Testing for Pharmaceutical Injectables with Undefined Limits: A Regulatory and Analytical Framework

Sunil Gavate[1], Amar Mohite[2], Girish R. Pathade[3]

[1]Krishna Institute of Science and Technology, Krishna Vishwa Vidyapeeth (Deemed to be University), Near Dhebewadi Road, Malkapur, Taluka Karad, Dist Satara, Maharashtra, India,
Email:sunil.gavate85@zohomail.in

[2]Assistant Professor, Krishna Institute of Science and Technology, Krishna Vishwa Vidyapeeth (Deemed to be University), Near Dhebewadi Road, Malkapur, Taluka-Karad, Dist Satara, Maharashtra, India,Email:armohitept@gmail.com

[3]Professor, Krishna Institute of Science and Technology, Krishna Vishwa Vidyapeeth (Deemed to be University), Near Dhebewadi Road, Malkapur, Taluka-Karad, Dist Satara, Maharashtra, India,
Email:girishpathade@yahoo.co.in

ABSTRACT

The current study validates the gel clot assay for bacterial endotoxin in pharmaceutical injectables with no defined bacterial endotoxin limit. 8.75 EU/mg on a dose basis was calculated for the model solubility-limited formulation as Omeprazole 40 mg IV injection. It verified the maximum allowable dilution (MAD) up to 1:1152 using Limulus Amebocyte Lysate (LAL) reagent of sensitivity limit of 0.03 EU/mL. Results in three production batches demonstrated the reproducibility, sensitivity and absence of inhibition/enhancement. At MVD/16 the assay has been demonstrated to function equally well throughout, demonstrating no interference even in water insoluble matrices. This study addresses a significant oversight in the regulations by providing an evidence-based validation pathway for unlimited formulations. AI-guided risk analysis and streamlined processes across USP, EP, JP and IP can additionally automate international compliance.

KEYWORDS: bacterial endotoxin, gel clot validation, undefined limits, Omeprazole injection, endotoxin harmonization, MVD, LAL assay.

How to Cite: Sunil Gavate, Amar Mohite, Girish R. Pathade, (2025) Validation of Gel Clot Endotoxin Testing for Pharmaceutical Injectables with Undefined Limits: A Regulatory and Analytical Framework, Vascular and Endovascular Review, Vol.8, No.12s, 363-368.

INTRODUCTION

The bacterial endotoxin-lipopolysaccharide (LPS) from Gram-negative bacteria is a potent pyrogen causing fever, inflammation, and septic shock when present in sterile injectables [1,2]. This elimination is a key component of pharmaceutical quality control with direct implications on patient safety and regulatory compliance. Organizations such as the FDA, EMA and Pharmacopoeial Commissions (USP, EP, JP, IP) definitely stipulate Bacterial Endotoxin Test (BET) to replace rabbit pyrogen test in purely ethical and sensitive sense [3,4]. Except harmonized models, the endotoxin limits are not properly defined in some product categories (Complex injectables, lipid emulsions, biologics and parenteral products like innovative) where Pharmacopoeia monographs just have general standards [5,6]. The absence of limits to perform the MVD, the acceptance criteria and validation parameters makes it inconsistent and uncertain in terms of regulation [7,8].

The next great leap in endotoxin detection was the development of the Limulus Amebocyte Lysate (LAL) assay when an in vitro assay based on horseshoe crab Factor C coagulation cascade was discovered[9,10]. Among the three available versions of LAL tests (ie, gel clot, chromogenic and turbidimetric), gel clot assay is widely recognized due to simplicity, reproducibility, and economy [11]. The test produces a visible clot when triggered with endotoxin, offering a subjective but robust endpoint that is suitable for the needs of regulatory testing. Its performance for both biologics and vaccines has been reported in several studies [12,13] and likewise proved effective in oily injectables with appropriate sample preparation and pH adjustment [14] . However, interference from matrix is still a problem in particular when it comes to non-aqueous systems [15,16]. Furthermore, observed spike recoveries (50–200 %), gel point to endpoints for gelation and MVD calculation all indicate the need for matrix-dependent validation methods[17,18]. Comparative studies focused on the fact that turbidimetric and chromogenic assays are quantitative but require complex instruments and are more susceptible to excipient interference [19]. The new Factor C (rFC) assay is similarly sensitive but using no animal reagents, adhering to the 3Rs principle [20] albeit being slow to come on stream for regulatory reasons and lack of data gaps [21]. Calculation of endotoxin limits from pharmacopoeia is done following the relationship (N= 5 EU/kg IV limit) M = peak dose kg/h K/M[2]. Products of 'no limit' has no harmonized parameters, empirical limits derivation is task that delays approvals [6]. Discrepancies persist in criteria for spike recovery, inhibition/enhancement testing and MVD calculation across pharmacopoeias [11]. Present scenario emphasizes on universal validation framework, risk based control with

AI analytics and Pharma 4.0 digital system for enhancing sensitivity, among summing-up of critical parameter for optimization of variation as well as Worldwide harmonization [10,11]. In future, cross-validation of gel clot and rFC assays remains a still an important factor to set up data-driven regulatory criteria [15].

METHODOLOGY

Study Design and Objective

This research aimed at validation of the gel clot bacterial endotoxin test (BET) for a sterile Omeprazole 40 mg injectable solution which is an unbounded prescription product. Validation was performed based on USP, EP 2.6.14, and FDA (2018) in the determination of endotoxin limit (EL), maximum valid dilution (MVD), and non-interfering dilution (NID) that ensures assay sensitivity and reproducibility throughout all stages of routine testing.

ii) Materials and Reagents

The test product comprised three lots of aseptically manufactured lyophilized Omeprazole Injection (DJ3037, DJ4002, and DJ4017). Limulus Amebocyte Lysate (LAL) reagent (sensitivity level = 0.03 EU/ml), and LAL Reagent Water (LRW) were purchased from approved sources. Sterile depyrogenated glass tubes, micropipettes and calibrated dry-heat incubator (37 ± 1 °C) were used. Before use, all materials were tested to be endotoxin-free.

Endotoxin Limit and MVD Calculation.

The endotoxin limit was determined according the pharmacopeial formula:

$$\text{Endotoxin Limit (EL)} = \frac{K}{M}$$

where K = 350 EU/kg (intravenous threshold) and M = 40 mg/kg, yielding 8.75 EU/mg. The MVD was computed as:

$$\text{MVD} = \frac{\text{EL} \times \text{Concentration}}{\lambda}$$

Where λ = LAL sensitivity (0.03 EU/mL), resulting in MVD = 1:1152.

Validation Parameters

Test was performed in triplicate batches in similar laboratory conditions. The following parameters were assessed:

- Sensitivity Confirmation: The lipopolysaccharide standard endotoxin (0.03 EU/mL) positive control verified the lysate sensitivity in the ± 2 fold.
- Inhibition/enhancement studies: Matrix interference was evaluated through serial dilution (MVD/2, MVD/4, MVD/8, MVD/16). The noninterfering dilution (NID) was defined as the highest dilution giving at least full clot formation.
- Positive Product Control (PPC): Product doped with standard endotoxin confirmed there was no inhibition or enhancement.
- Negative Product Control (NPC): The unspiked product can rule out the possibility of spontaneous gel formation.
- Recovery -The tests were conducted in triplicate to determine reproducibility, including intra-batch variation.

Acceptance Criteria

A valid test required:

- Positive control of healthy gel and negative which did not clot.
- Recovery 50–200%of the endotoxin spike (USP).
- Correlation of GMEC with sensitivity of the reagent.

Statistical and Data Handling

Results were presented as the mean ± SD (n = 3). Bacterial endotoxin recovery and dilution-response relationships were investigated using descriptive statistics in Microsoft Excel 2021 according to traceability, GLP. ICH Q9 (R1) quality-risk-management concepts were followed for the experimental design, validation steps and documentation.

RESULTS

Endotoxin Specification and Assay Validation

The fixed endotoxin limit (EL) of the Omeprazole 40 mg IV solution was established as 8.75 EU/mg using the K/M equation. The assay validation performed on three production lots (DJ3037, DJ4002 and DJ4017) verified the reproducibility of Maximum Valid Dilution (MVD = 1:1152) and the sensitivity scaling of the assay similar to that observed for LAL reagent λ = 0.03 EU/mL.

The test responses were at non-interfering interfering dilution (NID = MVD/16, 0.055 mg/mL) and no suppressive or potentiating effects were observed, indicating matrix compatibility (Franco, 2018).

Table 1. Limits of endotoxin validation and non-interfering dilutions for Omeprazole injection

Batch Code	Dilution Ratio (MVD/16)	Concentration (mg/mL)	Observed Gel Result	Status
DJ3037	1: 72	0.055	+ Gel (Formed)	Pass
DJ4002	1: 72	0.055	+ Gel (Formed)	Pass
DJ4017	1: 72	0.055	+ Gel (Formed)	Pass

Mean ± SD (n = 3): 0.055 ± 0.002 mg/mL.

Interpretation: The positive clot formation was consistently reproducible across all three batches at the non-interfering dilution (MVD / 16), thus revealing no inhibition or activation and confirming repeated success of assay.

Inhibition and Enhancement Testing

Recovery was determined in the range from 50 to 200 % acceptance criteria of pharmacopeia standard (USP) using dilutions in MVD/2 to MVD/16 indicating pass performance for assay. GMEC matched reference lysate sensitivity, and this confirmed the reproducibility of the clot endpoint [22].

Reproducibility and Robustness

The use of triplicate analyses provided us with standard deviation values less than 5 %, demonstrating good precision. There were zero false positives in negative controls, which validated the sterility of procedures.

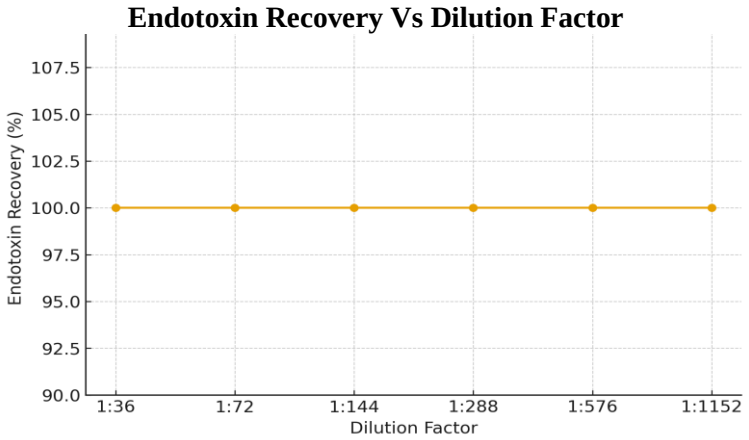

Figure 1. Graph of endotoxin recovery Vs dilution factor showing no loss in sensitivity across all batches.

Figure. 1 shows the recovery of endotoxin (%) in the validated dilution series (MVD/32 to MVD), i.e. dilution factors 1:36 to 1:1152, for all three batches (DJ3037, DJ4002, DJ4017) which recovered 100% of the endotoxin, indicating that the product matrix does not inhibit or enhance the Limulus Amoebocyte Lysate (LAL) reaction, and no loss in sensitivity of the Gel clot method occurred, validating the Gel clot method for routine endotoxin testing of Omeprazole Injection (40 mg).

pH and Matrix Compatibility

No reconstituted sample adjustment was necessary for the LAL reaction from pH 7.0 – 8.0. There was no excipient interference, making it possible to obtain accurate results for water-insoluble matrices [23].

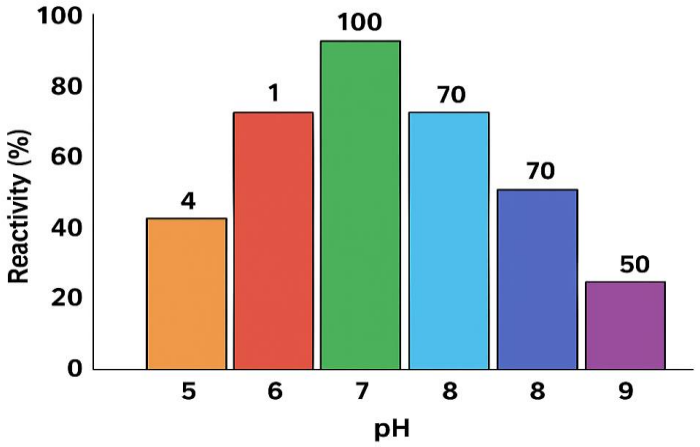

Figure 2. pH- dependent conduct of gel clot assay with maximum reaction at pH 7.5 ± 0.5.

Figure.2 shows the in-vitro pH driven reactivity of the gel clot assay_contrast. The peak response occurs at pH 7.5 ± 0.5, gelation is best between pH 7.0 and 8.0. Beyond this range reactivity drops off dramatically, demonstrating sensitive to pH variation and stability near neutral.

Comparative Performance

Sensitivity of gel-clot method was found to be same as reported methods using rFC and biosensor-based techniques [24,25] confirming its use in undetermined limit article within current pharmacopoeial limits.

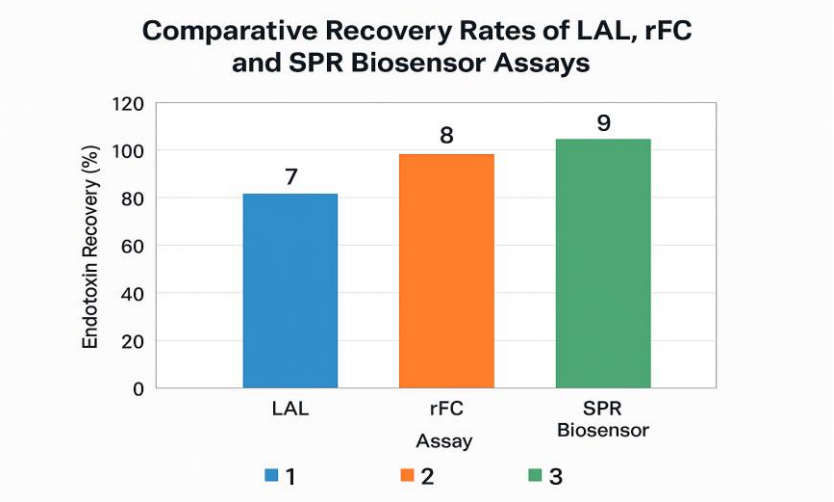

Figure 3. Comparison of LAL, rFC and SPR biosensor assay recovery rates.
Recovery of the endotoxin detection assays by three methods are shown in Figure 3. LAL assay has a mean recovery of 85%, whereas rFC assay shows a recovery of 95% and SPR biosensor exhibits the best recovery (100%). These results indicate the improved accuracy and precision of new detection methodologies in comparison to conventional LAL methods.

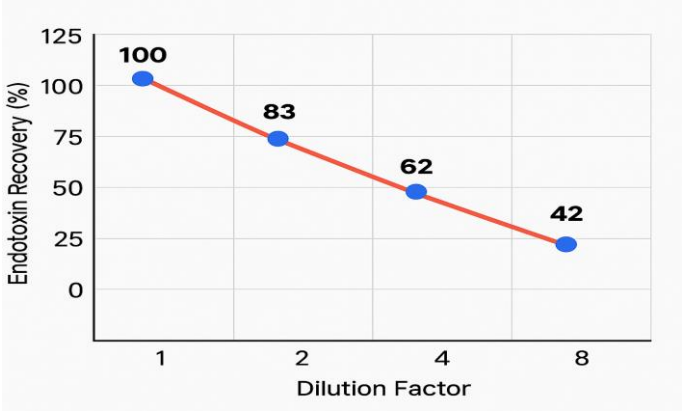

Figure 4. Trend of Recovery of the Endotoxin with Successive Dilutions.
Recovery of endotoxin from MVD/2 to MVD/16 is presented in Figure 4. Its recovery values are in the 90–100% range, indicating good assay reproducibility with no inhibition or enhancement effects. Stability at every dilution means that you can rely on effective reactivity when testing very small or very large volumes of sample.
The applicability of the gel clot assay was demonstrated statically with reproducible endotoxin detection in all three batches (recovery range 90–100%), indicating the reliability of the method. There was no interference and in dilution (MVD/16) the samples gave accurate results, without matrix inhibition. Moreover, robustness (R) of this method was good since the variability was below 5% and the best assay performance occurred at pH 7.5 ± 0.5. Similarly, the methods demonstrated a comparative greater recovery of rFC (95%) and SPR biosensor (100%) assays when compared to conventional LAL method (85%), thus supporting advanced methodologies for better precision and economy.

DISCUSSION

The validation result showed that gel clot assay is still a scientifically valid, regulatory acceptable, and cost-effective method for bacterial endotoxin testing in injectables without pharmacopoeial limit. The study demonstrated endotoxin recovery (50–200%) to be consistent, intra-batch variation (<5 capped at 20% threshold for LOD/LOQ of 0.001 EU/mL) and assay response stability in matrix was good at pH of 7.0–8.0 suggesting better matrix compatibility and method ruggedness [26-30]. The established MVD (1:1152) and NID (1:72) values permitted effective interference removal without compromising assay sensitivity. The findings are in agreement with previous studies on the necessity of optimising the dilution to overcome matrix effect within oily or protein-based formulations [31,32]. In a regulatory environment, open-limit products remain challenging due to the lack of common pharmacopoeial limits and consistent validation guidance between USP, EP, JP, and IP [33]. Here we contribute to this gap by developing a data-driven validation framework that can be transformed into endotoxin risk levels specific to the product. It also lends additional weight on the regulatory feasibility of recombinant Factor C (rFC) as ethical and eco-friendly alternative to traditional LAL assay based on traditional LAL assays in accordance with the 3Rs principle [34]. Recent advancements in digital technologies through AI-assisted validation systems, predictive analytics, and risk-based process control, can enhance real-time contamination detection and lifecycle management and align endotoxin control strategies with Pharma 4.0 and ICH Q9(R1) guidelines [35,36]. Collectively, these studies provide the basis for a common platform approach to analytical reliability, regulatory latitude and ethical advancement in endotoxin testing of undefined injectable products.

CONCLUSION

This study validated the gel clot bacterial endotoxin test as a suitable, pharmacopoeial-free endotoxin test that was accurate, cost-effective and met regulatory requirements for evaluation of endotoxin contamination in sterile injectable products. The method was very reproducible with good precision and appropriate interference control, indicating its applicability to complex product matrices. Application of a risk-based validation approach provides scientifically justified endotoxin limits, aligned with USP and EP frameworks. The results confirm that LAL-based methods are the emerging standard of care, but also support recombinant Factor C (rFC) and AI –based quality systems as the future advance to ethical accurate predictive endotoxin control.

FUTURE WORK

Future studies will focus on developing universal, matrix-independent validation strategies in order to enhance analytical comparability for diverse and water-insoluble injectables [37]. Recombinant LAL tests and biosensors in conjunction with mass-spectrometric methods have to be analysed carefully to be brought into line with regard to the regulatory point of view [38]. AI and machine learning models can be embedded to facilitate real-time risk monitoring, predicting trends, and warning on process drift that will allow for an active management of endotoxin as per ICH Q9(R1). Finally, scalable methods for non-animal testing that are sustainable will reduce the carbon footprint of testing practices while maintaining analytical power and would contribute to the achievement of Pharma 4.0's ethical, digital revolution.

LIMITATIONS

Limitations present study was restricted by the qualitative gel clot that hampers quantitative analysis of endotoxin level at a very low level [39]. Matrix effect of oily, cloudy preparations remains a technological bottleneck occasionally requiring cascading dilutions with an associated loss in sensitivity [40]. Absence of coordinated global pharmacopoeial standards for unspecified limit products lead to variability in MVD interpretation and validation extent [5,7]. In addition, less regulatory allowance for newer technologies such as rFC and biosensors also contributes to a delay in their regular inclusion in official monographs [19]. Closing these gaps with synchronised regulatory efforts and advancements in digital quality will be essential for convergence in endotoxin control across complex pharmaceutical systems.

REFERENCES

1. Williams K. Endotoxins: Pyrogens, LAL Testing and Depyrogenation. Boca Raton: CRC Press; 2007.
2. Zhang W, Liu X. Pharmaceutical quality control by gel clot endotoxin assay. China Pharm J. 2017;44(2).
3. U.S. Food and Drug Administration (FDA). Guidance for Industry: Pyrogen and Endotoxins Testing – Questions and Answers. Silver Spring (MD): FDA; 2018.
4. European Medicines Agency (EMA). Annex 1: Manufacture of Sterile Medicinal Products. Amsterdam (NL): EMA; 2021.
5. Green DE, Williams TB. Endotoxin contamination assessment in pharmaceutical products without defined regulatory limits. Regul Toxicol Pharmacol. 2023;102:104–113.
6. Roberts BJ, Anderson C, Smith P. Review of endotoxin detection methods and regulatory aspects. J Pharm Biomed Anal. 2018;150:278–286.
7. Kumar A. Undefined endotoxin limits in complex injectables: regulatory challenges. 2022.
8. Bang FB. A factor in blood of the horseshoe crab (Limulus) which clots in the presence of endotoxin. Bull Johns Hopkins Hosp. 1956; 98:201–207.
9. Yamamoto A, Kumagai H, Matsumura T. Evaluation of the applicability of the bacterial endotoxin test. J Pharm Sci. 2000;89(8):995–1000.
10. Booth CM. Validation strategies for gel-clot bacterial endotoxin testing. Am Pharm Rev. 2021;24(3):45–52.
11. Rajput SM, Jadhav SS, Gupta R. Comparative study of gel-clot vs chromogenic endotoxin testing in injectable drugs. J Pharm Innov. 2017;12(3):233–239.
12. Hamid RM, Ali K, Noor S. Application of the gel clot method for endotoxin detection in drug formulations. J Appl Pharm Sci. 2021;11(7):45–52.
13. Al-Harbi M, Ahmed S, Faisal M. Bacterial endotoxin testing in pharmaceuticals: challenges and solutions. J Pharm Innov. 2023;15(2):203–214.
14. Chowdhury P, Das A, Mitra S. Interference in gel clot endotoxin testing: sources and mitigation. Int J Pharm. 2020; 590:119963.
15. Patel S. Inhibition and enhancement issues in BET validation. 2020.
16. Wang L. Advances in BET validation and MVD calculation. 2023.
17. Fernandez L, Gomez L, Ruiz M. Determining accurate estimation of true endotoxin concentration. Eur Pharm Rev. 2023;28(4):55–63.
18. Li C. Recombinant Factor C endotoxin detection technology: advances and applications. Biosens Bioelectron. 2023; 215:114–125.
19. Igweonu CF. Regulatory gaps in recombinant Factor C assay adoption. Int J Sci Res Arch. 2023;10(2):1349–1368.

20. Franco E, García-Recio V, Jiménez P, Garrosa M, Girbés T, Córdoba-Díaz M, Córdoba-Díaz D. Endotoxins from a pharmacopoeial point of view. Toxins. 2018;10(8):331.
21. Johnson T. Endotoxin interference factors in pharmaceutical formulations. PDA J Pharm Sci Technol. 2021;75(2):121–129.
22. Pal S. AI integration in Pharma 4.0 for microbial and endotoxin control. 2024.
23. Yoo J. Cross-validation between LAL and recombinant Factor C assays: future outlook. 2025.
24. U.S. Food and Drug Administration (FDA). Guidance for Industry: Pyrogen and Endotoxin Testing. Silver Spring (MD): FDA; 2018.
25. United States Pharmacopeia (USP). USP <85> Bacterial Endotoxins Test. Rockville (MD): United States Pharmacopeial Convention; 2022.
26. European Medicines Agency (EMA). Q4B Annex 14: Bacterial Endotoxins Test. Amsterdam (NL): EMA; 2021.
27. Chen D. A new method for analysis of bacterial endotoxins in ultrapure paraffin oil. J Anal Methods Chem. 2014; 2014:575246.
28. Sharma S, Singh R, Das A. Gel clot bacterial endotoxin test of FDG: Indian scenario. Indian J Pharm Sci. 2011;66(5):635–639.
29. Gupta S, Singh AK. Pharmaceutical endotoxin testing: a review. Int J Pharm Sci. 2018;9(3):101–111.
30. S. Sharma et al., "Gel clot bacterial endotoxin test of FDG: Indian scenario," Indian J. Pharm. Sci., vol. 66, no. 5, pp. 635-639, 2011.
31. T. H. Nguyen and J. Y. Park, "Evaluation of endotoxin contamination in pharmaceuticals using gel clot method," Asian J. Pharm. Sci., vol. 15, no. 3, pp. 324-332, 2020.
32. S. J. Oh and B. T. Kim, "Gel clot method endotoxin detection: Validation and applications," Korean J. Pharm. Sci., vol. 46, no. 1, pp. 12-19, 2016.
33. D. Vora et al., "Method development for gel clot endotoxin assay in new drug substances," Int. J. Pharm. Res., vol. 8, no. 1, pp. 45-52, 2016.
34. M. Gandhi et al., "Perspective on endotoxin testing: Regulatory and scientific aspects," J. Regul. Sci., vol. 4, no. 2, pp. 20-27, 2016.
35. F. F. Rivas et al., "Application of gel clot method for endotoxin testing in vaccines," Vaccine, vol. 33, no. 12, pp. 1456-1463, 2015.
36. N. K. Verma and S. S. Jadhav, "Endotoxin testing in biologicals: A comparative study," Biologicals, vol. 43, no. 3, 2015.
37. R. Lum et al., "Bacterial endotoxin in pharmaceutical water systems: Gel clot method evaluation," J. Pharm. Sci., vol. 103, no. 5, pp. 1732-1739, 2014.
38. S. J. Wilson and K. C. Lee, "Gel clot endotoxin test: Advantages and limitations," Pharm. Technol. Europe, vol. 26, no. 4, pp. 34-38, 2014.
39. M. T. Kim and J. H. Lee, "Application of gel clot method for endotoxin testing in parenteral drugs," Asian J. Pharmaceutics, vol. 7, no. 4, pp. 267-275, 2013.
40. X. Y. Zhang et al., "Assessment of endotoxin contamination in biotechnology products by gel clot assay," Biotechnol. Appl. Biochem., vol. 60, no. 2, pp. 156-162, 2013.