Application of Micro-kinetic Chromogenic Method in Endotoxin Quantitative Detection of Hydroxypropyl-β-cyclodextrin

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ABSTRACT

The kinetic chromogenic assay with Limulus amoebocyte lysate to detect the bacterial endotoxin in Hydroxypropyl-β-cyclodextrin for injection is investigated in this paper. The test for interfering factors is performed to study the applicability of the kinetic chromogenic assay for detection of bacterial endotoxin in Hydroxypropyl-β-cyclodextrin for injection. The results suggest that r>0.980 while the range is between 50-0.005EU/ml. Besides, when concentration of test solution is 1mg/ml for Hydroxypropyl-β-cyclodextrin, there is no interference between Limulus amebocyte lysate and bacterial endotoxin. The kinetic chromogenic assay can be used to detect the bacterial endotoxin in Hydroxypropyl-β-cyclodextrin.

Introduction

Hydroxypropyl-β-cyclodextrin is the hydrophilic derivatives as a result of condensation between β-cyclodextrin and 1, 2-propylene oxide [1, 2]. In pharmaceutical industry, Hydroxypropyl-β-cyclodextrin is also used as carrier besides dilute. Hydroxypropyl-β-cyclodextrin is amorphous and easily soluble in water, with suitable molecular size and excellent biocompatibility a structure, which make it possible to inclusion drug. Changes will happen in both physics and chemistry characteristics when compounds are covered with Hydroxypropyl-β-cyclodextrin.

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-β-cyclodextrin. The inclusion compounds may show improved water solubility, stability, antioxidant and photolysis resistance. Furthermore, Hydroxypropyl-β-cyclodextrin can also be applied in the preparation of sustained and controlled-release preparations and targeted preparations. Therefore, Hydroxypropyl-β-cyclodextrinis widely used in food, medicine, cosmetics, agriculture and analytical chemistry in recent years [3-6]. Lipopolysaccharide (LPS, endotoxin) is the main component of the cell walls of Gram-negative bacteria and is the very potent initiator of inflammatory reactions in mammals [7]. Bacterial endotoxin has an extensive biological activity, which can generally induce a series of clinical responses when exposed on humans, such as fever, diarrhea, vomiting, septic shock, pyrogenicity, and disseminated intravascular coagulation [8]. In the quality control of Biologicals for human and veterinary use, endotoxin is one of the most important factors. The Limulus Amebocyte Lysate (LAL) assay is the gold standard as of today to detect endotoxin [9], which is contained in the pharmacopeia and is widely used as a convenient method due to its high sensitivity, reliability and simple handling. There are three commonly available methods for endotoxin detection, such as gel-clot assay, kinetic turbidimetric assay, and chromogenic assay. In this paper, chromogenic assay is used to detect the amount of bacterial endotoxin in Hydroxypropyl-β-cyclodextrin.

Materials and methods

1. Material

LAL: Two batches of LAL chromogenic assay from different factories were purchased from Zhanjiang A&C Biological LTD, China (Lot No. 1312260, Sensitivity 50-0.005EU/ml) and Lonza walkerscille, MD, USA (Lot No. NL026-EQKVKS, Sensitivity 50-0.005EU/ml)


Water used in the test: National Institute for Food and Drug Control (5ml each) Lot No. of which is 2014-1, with bacterial endotoxin below 0.005EU/ml.

Hydroxypropyl-β-cyclodextrin: ROQUETTE CO., Ltd. Lot No is E0208, E0193, and E0212.

All glassware is eliminated extraneous endotoxin by heating in a hot-air oven at 250 ºC for 60min. The pipette tips for automatic pipetters are free of detectable endotoxin.

2. Methods

2.1 Test solution: Take a quantity of Hydroxypropyl-β-cyclodextrin to make a solution of 100mg per ml, λ is 0.005EU/ml, L=10EU/g and minimum valid dilution (MVD) is 200 by calculate through the formula MVD = cL / λ, in which, L is the endotoxin limit of the
sample being examined, \( \lambda \) is the labeled sensitivity of LAL reagent (EU/ml) and \( c \) is the concentration of the test solution.

2.2 Assurance of criteria for the standard curve: As is described in the introductions, Assurance of criteria for the standard curve should be carried out for every batch of LAL. Dissolve NSE with water, vertex for 15min on the mini-shaker. Dilute the solution to produce solutions of 50, 5, 0.5, 0.05, 0.005EU/ml with every level in triplicate. Set the temperature of ELISA at 37±1 degree. Take 0.1 ml of solutions above to the plate which is fyrogen-free. Dissolve LAL with 2.6 ml water; add 0.1 ml to every hole of the plate. Put the plate into ELISA immediately. When the reaction finished in nearly one hour, transfer Endotoxin concentration and reaction time to logarithmic respectively, and fit a line.

2.3 Test for Interfering Factors: Developing the test for interfering factor is to find out the concentrate at which the test solution has no interfering to the after-experiment. Dilute the solution of Hydroxypropyl-\( \beta \)-cyclodextrin to produce solutions of 10mg, 5mg, 1mg/ml, do product post control(PPC) at the same time. The concentration of endotoxin added is 5EU/ml. Do make 2 for both test solution and PPC. Calculate the endotoxin concentration with the reaction time of test solution and PPC by corresponding to the standard curve, respectively. Calculate the recovery of every sample. Once the recovery is during 50-200%, calculate the endoxin concentrate and average all the parallel samples.

**Result**

As is calculated above, the results are shown in Table 1. The recovery was within the range of 50%-200%, and the results suggest that \( r>0.980 \) while the range is between 50-0.005EU/ml by chromogenic assay. Test solutions of 10mg/ml and 5 mg/ml showed inhibition in the Test for Interfering Factors. It is 1mg/ml for Hydroxypropyl-\( \beta \)-cyclodextrin at which concentration there is no interference between Limulus amebocyte lysate and bacterial endotoxin. When take EU/g as the unit of endotoxin assay instead of EU/ml, the result should multiply by 1000.

**Discussion**

Test with biological agent may result in variance, compared with chemistry test. Two batches of LAL from different companies should be carried out.

Since it introduces chromogenic group, Micro-kinetic Chromogenic Method shows higher limit of detection (LOD). By contrast, the LOD of Gel Clot is 0.03EU/ml and kinetic nephelometric assay is 0.01EU/ml, conducive to eliminate the interference. The rabbit pyrogen test, which is used in qualitative test rather than quantitive test, is going to
<table>
<thead>
<tr>
<th>Company</th>
<th>Lot No.</th>
<th>Assurance of Criteria for the Standard Curve</th>
<th>Test for Interfering Factors</th>
<th>Recovery**</th>
<th>Concentration of Endotoxin EU/ml</th>
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</thead>
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<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Lonza</td>
<td>E0193</td>
<td>LogT=-0.221logC+6.07 0.999</td>
<td>LogT=-0.229logC+6.07 0.999</td>
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<td>&lt;0.005</td>
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<tr>
<td></td>
<td>E0208</td>
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<td>LogT=-0.234logC+7.32 0.997</td>
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<td>&lt;0.005</td>
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<td></td>
<td>LogT=-0.241logC+6.01 0.999</td>
<td>22%</td>
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<tr>
<td>A&amp;C Biological</td>
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<td>40%</td>
<td>0.006</td>
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<tr>
<td>LTD</td>
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<td>39%</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td></td>
<td>E0193</td>
<td></td>
<td></td>
<td>22%</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td></td>
<td>E0212</td>
<td></td>
<td></td>
<td>11%</td>
<td>0.007</td>
</tr>
</tbody>
</table>

Remarks:
* It should meet the requirement of pharmacopeia when \( r > 0.980 \) \[^{10}\]
** It should be consider as no interference when the recovery of Test for Interfering Factors is during 50-200% \[^{10}\]
be replaced due to its inconformity with 3R rules. The monocyte activation test (MAT), which is contained in European Pharmacopeia, is difficult to gain the material such as human being blood legally in China. As a result, kinetic chromogenic assay is chosen to test the endotoxin in Hydroxypropyl-β-cyclodextrin and it proved to be the best.

References


