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Establishment of detection method for the biological activity of Lumbrokinase

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Lumbrokinase methodology validation research method for the determination of biological .Methods: In pharmacopoeia streptokinase biological activity determination method is improved, the preparation of agarose - fibrin plate, making standard curve. Results: More standard Lumbrokinase biological activity determination methods Conclusions: This method is more easy and accurate determination of Lumbrokinase activity of biology

Keywords: Lumbrokinase; Biological activity; Determination methods ; Validation

INTRODUCTION

Introduction to Earthworm

Shizhen Li, in his book “Compendium of Materia Medica” has documented in detail the morphology, behaviours, and medicinal value of the earthworms, he called it the "Earth Dragon". The earthworms have also been included by the "Shen Nong's Herbal Classic" as one of the collection of 67 animal-based remedies. It can be used for the treatment of urinary obstruction [1], fever, irritability, amenorrhea, convulsions, cough, asthma, physical disability, high blood pressure, nephritis, calculus formation, congenital epilepsy, pneumonia, arthralgia, rheumatism, jaundice, convulsions in children, and many other diseases.

Introduction to Lumbrokinase

Lumbrokinase is one of the biologically active ingredients in the earthworms. It has the ability to directly digest fibrin and activate the plasminogen, thus indirectly causing a fibrinolytic effect. Since Mihara et al. purified a fraction of proteins from the crude extracts of the earthworms with plasmin activity in 1983, lumbrokinase have subsequently been marketed as a thrombolytic drugs [2] in Korea and China. The Biophysics Centre of Chinese Academy of Sciences have developed the lumbrokinase in the form of a capsule, and have used it as a new generation of oral thrombolytic drug for the treatment of various diseases related to thrombosis in clinical trials.

EXPERIMENTAL METHOD

Materials

Plasminogen (bovine serum) (9IU/bottle)	Chinese National Institutes for Food and Drug Control
Thrombin (840IU/bottle)	Chinese National Institutes for Food and Drug Control
Lumbrokinase (26000U/tube)	Chinese Centre for Pharmaceutical and Biological Product Control
Agarose	BIOWEST
Fibrinogen	YINGBIOTECH
Sodium chloride physiological solution	Shandong Qidu Pharmaceutical Co., Ltd.

Principle behind the Determination of Lumbrokinase Biological Activity

The formation of Lumbrokinase-plasminogen complex [3] can activate the plasminogen into the biologically active plasmin, which can degrade the fibrin into water-soluble fibrin protein fragments. The formation of thrombolytic zone on top of the fibrin plate can be used as a quantitative measure of biological activity for the lumbrokinase.

Preliminary Establishment of Biological Activity Determination

A total of 125 mg of agarose was precisely weighed, and dissolved in 23 ml of sodium chloride physiological solution, this is followed by boiling of the solution to fully dissolve the agarose. The solution was incubate at 55-60oC by keeping the tube balanced in a water bath. 14 μl of 100IU/ml thrombin solution, 280 μl of 3IU/ml plasminogen, and 2.2 ml of 6 mg/ml fibrinogen was added to the

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molten agarose, with constant agitation to obtain a homogenised mixture. The molten agarose was immediately poured into an 8-cm diameter petri dish. The petri dish was left on a horizontal surface until fully solidified, stored at 4oC for at least 30 minutes (should be used within two days). In the petri dish containing the fibrin, 2-mm diameter wells were punched out, and 10 µl of the test solution and the standard solution was added to each well. A total of two wells were prepared for each dilution. The petri plates were incubated for 24 hours at 37oC in an incubator. The longitudinal and horizontal diameters of the thrombolytic zone were measured to obtain a mean value. The diameters of the thrombolytic zones were plotted against the concentration of the serially diluted standard solution to form a linear regression curve demonstrating their biological activity [4]. By using the linear regression equations, the biological activity of the test sample can be determined based on the average zone diameters.

Validation of the Lumbrokinase Biological Activity Detection Method

Preparation of the Fibrin Plates

1.Exploration of optimum preparation of fibrinogen solution.

A total of 66mg of fibrinogen and 11ml of sodium chloride physiological solution were incubated at 37oC in a water bath for 15 minutes. The sodium chloride physiological solution was then added to the fibrinogen. To fully dissolve the fibrinogen, the solution was incubated at 37oC in a water bath for a further 30 minutes before use. The condition of the solution was observed and recorded.

After the fibrinogen solution preparation was complete, the solution was removed from water bath and placed on the bench with the room temperature set to about 12oC for the preparation of fibrin plate. The condition of the solution was observed and recorded [5].

2. Configuration of the Timing and Handling of Agarose Swelling.

A total of 125 mg of agarose was added to the 23 ml of sodium chloride physiological solution and boiled. After boiling, the duration of time required for the agarose solution to clear and become fully dissolved was observed and recorded. The agarose solution was then incubated at 55-60oC in a water bath. The condition of the agarose solution was observed and recorded to determine if the volume of the solution is affected by the boiling process.

3. Optimisation of the Amount of Thrombin required.

Three fibrin plates were prepared according to the preliminary method established for the detection of biological activity. The amounts of thrombin added to each plate were 14 µl, 28 µl and 30 µl respectively, while other variables remained unchanged. The plates were incubated at 37oC for 24 hours in an incubator, and the turbidity of the fibrin was observed to determine the optimum amount of thrombin that need to be used.

4. Optimisation of the Amount of Fibrinogen required.

Three fibrin plates was prepared according to the preliminary method established earlier in the study, the amount of fibrinogen added to each plate were 2.2 ml, 4.4 ml and 6.6 ml respectively, while other variables remain unchanged. The plates were incubated at 37oC for 24 hours in an incubator, and the turbidity of the fibrin was observed to determine the optimum amount of fibrinogen that need to be used.

5. Optimisation of the Plasminogen Quantity required.

Three fibrin plates was prepared according to the preliminary method established earlier in the study, the amount of plasminogen added to each plate were 280 µl, 500 µl and 560 µl respectively, while other variables were kept constant, the plates were incubated at 37oC for 24 hours in an incubator, and the turbidity of the fibrin was observed to determine the optimum amount of plasminogen that need to be used.

6. Plating Method (e.g., prevention of the formation of bubbles).

A total of 125 mg of agarose was added to 23 ml of sodium chloride physiological solution and boiled. After boiling, the agarose solution was incubated at 55-60oC in a water bath. The molten agarose was subsequently added with 14 µl of 100IU/ml thrombin solution, 280 µl of 3IU/ml plasminogen, and 2.2 ml of 6 mg/ml fibrinogen with constant agitation. The homogenised agarose solution was immediately poured into the 8-cm diameter of petri dish. The method of how the solution should be poured and the timing of pouring were optimised.

7. Establishment of Detection Method for the Lumbrokinase Biological Activity.

A total of 125 mg of agarose was dissolved in 23 ml of sodium chloride physiological solution, and the solution was boiled to fully dissolve the agarose. It is then incubated at 55-60oC in a water bath. The molten agarose was subsequently added with 42 µl of 100IU/ml thrombin solution, 500 µl of

3IU/ml plasminogen, and 6.6 ml of 6 mg/ml fibrinogen with constant agitation. The homogenised mixture of molten agarose was immediately poured into the 8-cm diameter petri dish. The petri dish was left on a horizontal surface until the solution has fully solidified; followed by incubation at 4oC for 30 minutes.

Validation of Method

1.Correlation Study – Plot Standard Curve from the Standard Solutions

The serially diluted standard solutions: 4000IU/ml, 3000IU/ml, 2000IU/ml, 1000IU/ml and 500IU/ml were added at 10µl per well to the 2-mm diameter wells in the fibrin plate. The plates were incubated at 37oC for 24 hours in an incubator. The vertical and horizontal diameters of the thrombolytic zones were measured [6] to obtain the mean value. The biological activity of the standard solution at various concentrations was plotted against the diameters of the fibrinolytic zones using linear regression to obtain the corresponding linear regression equation.

The procedure of the above-described method was repeated three times to obtain the mean value.

2. Reproducibility and Intermediate Precision Study.

Two technicians were assigned to determine the titre of the given lumbrokinase sample according to the titration method using the test solution (4000IU/ml of lumbrokinase), the test was repeated 6 times by each technician and the results were recorded [7]. The six data sets were compared and the average values were calculated; followed by the calculation of the RSD (relative standard deviation) value of reproducibility. The test results of the two technicians were compared to obtain the mean value from the 12 sets of data, followed by the calculation of RSD values of the intermediate precision.

CONCLUSION

Optimisation of Fibrin Plate Preparation

1. Result of Fibrinogen Solution Preparation.

After the fibrinogen solution preparation was completed [8], the solution was removed from the water bath and placed on the bench with the room temperature set to about 12 °C. Flocculent precipitant was observed in the fibrinogen solution, and resulting fibrin plate was also cloudy and non-homogeneous, with some precipitation. After analysis, the reason for the fibrinogen precipitation observed was a decrease in the solubility of fibrinogen caused by a drop in temperature. Therefore, the fibrinogen should be maintained at 37oC prior to the mixing of solutions to produce a homogeneous fibrin plate.

Fig. 1. Precipitation of fibrinogen.

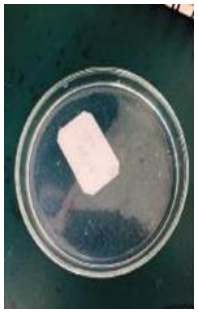

Fig. 2. Uneven cloudy plate.

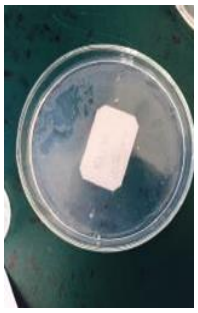

Fig. 3. Homogeneous cloudy plate.

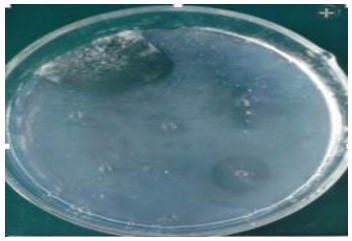

Fig. 4. Molten agarose failed to fully covered the plate due to reduced volume.

2. Result for Timing and Handling of Agarose Swelling Configuration.

A total of 125 mg of agarose was dissolved in 23 ml of sodium chloride physiological solution prior to being boiled. Our observation found that the agarose solution was clear when boiled for 10 minutes [9]. The molten agarose was cooled down to a homogenised temperature by incubating at 55-60˚C in a water bath. Our observations suggest that

the volume of the solution was reduced after the boiling process, which might have been caused by the evaporation of water from the solution during the process of boiling. Therefore, we sealed the flask with cling film to reduce the evaporation of water and maintain the volume of solution.

3. Result for Optimisation of the Amount of Thrombin required.

The amount of thrombin best added to the fibrin plate was 28 µl based on the turbidity of fibrin plate from the optimization process.

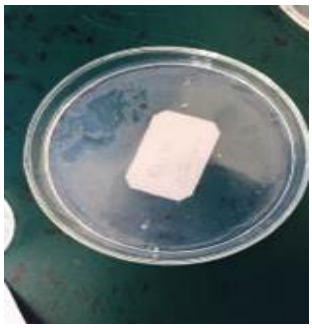

Fig. 5. 28µl of thrombin.

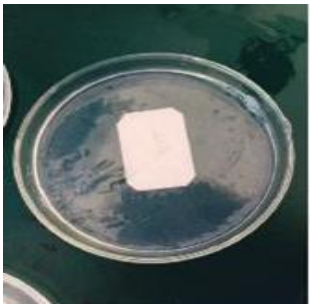

Fig. 6. 14µl of thrombin.

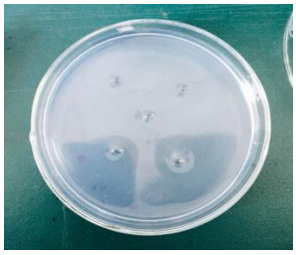

Fig. 7. 4.4ml of fibrinogen.

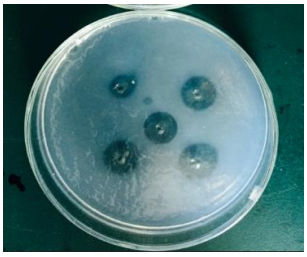

Fig. 8. 6.6ml of fibrinogen.

4. Result for Optimisation of the Amount of Fibrinogen required.

The amount of fibrinogen best added was 6.6ml based on the turbidity of fibrin plate from the optimization process.

5. Result for Optimisation of the Quantity of Plasminogen required.

The amount of Plasminogen [10] best added was 500 µl based on the turbidity of the fibrin plate, as well as the budgetary consideration on efficient usage of Plasminogen.

Fig. 9. 280µl of plasminogen.

Fig. 10. 500µl of plasminogen.

6. Result of the Plating Method (e.g., how to prevent formation of bubbles).

The solution should be adherently poured into the plate, accompanied with shaking to ensure the full coverage of the agarose solution. Adherent pouring can reduce the formation of bubbles, and the timing of the pouring should be fast to prevent the agarose from solidifying before being poured to the plates.

7. Result of Standard Curve from the Standard Solutions.

The equation of linear regression for the biological activity of lumbrokinase was obtained by plotting the diameter of the resolving zone against

the concentration of the serially diluted standard solution [11].

8. Result for the Reproducibility and Intermediate Precision of experiment.

Records of experimental implementation and conclusion (Table 3)

Conclusion: The RSD values of the reproducibility and intermediate precision were less than 2%. Hence, the experiment was valid.

Fig. 11. 560µl of plasminogen.

Table 1. Result for Optimisation of Fibrin Plate Preparation.

Optimised Variables	Results
Fibrinogen solution	Pre-mixed solution of fibrinogen should be maintained at 37ºC
Timing and handling of agarose swelling	Swelling time of 10min, seal the container with cling film
Amount of thrombin	28 µl of thrombin
Amount of fibrinogen	28 µl of thrombin
Amount of plasminogen	6.6 ml of fibrinogen
Plate (e.g., prevent formation of bubbles)	The solution should be poured adherently with shaking

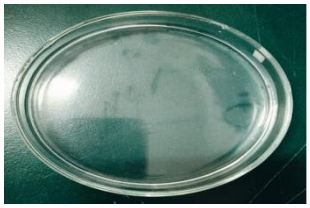

Fig. 12. No bubble formation on the fibrin plate

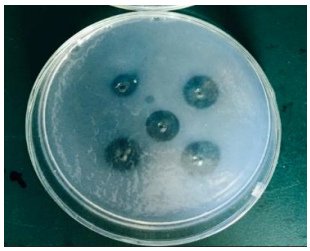

Fig. 13. Determination of lumbrokinase biological activity by measuring thrombolytic zones.

Table 3. Result for the reproducibility and intermediate precision of experiment.

Replicate	Test result 1	Test result 2
1	1.78	1.76
2	1.75	1.75
3	1.77	1.75
4	1.73	1.73
5	1.72	1.76
6	1.76	1.73
Mean value (u)	1.7517	1.7467
RSD value of repeatability (%)	1.3225	0.7822
Overall mean value (u)	1.7492	
RSD value of intermediate precision (%)	1.0473	

Table 2. Data sheet for the standard curve of lumbrokinase biological activity

Replicate	4000 IU/ml	3000 IU/ml	2000 IU/ml	1000 IU/ml	500 IU/ml
1	1.73 cm	1.51 cm	1.30 cm	1.01 cm	0.78 cm
2	1.73 cm	1.51 cm	1.31 cm	0.98 cm	0.80 cm
3	1.76 cm	1.49 cm	1.29 cm	1.01 cm	0.82 cm
Average value	1.74 cm	1.50 cm	1.30 cm	1.00 cm	0.80 cm

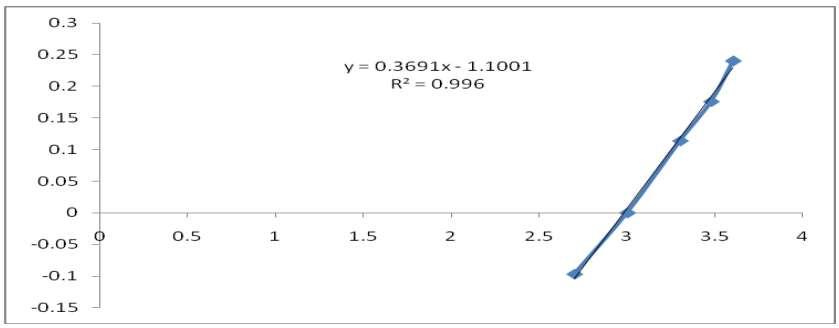

Fig. 14. Standard curve of the lumbrokinase biological activity.

DISCUSSION

1. Due to the limitation of resources in our laboratory and a lack of appropriate well puncher, needle and syringe was used in place of the appropriate well puncher [12]. Thus, extra care had to be taken using the appropriate technique during well punching to ensure removal of excessive gel using the syringe needle and prevent damage to the well.

2. The fibrin plates must be re-stored at -20oC immediately after sample loading [13] to prevent subsequent experiment being affected due to the change in temperature.

3. The fibrin plate must be incubated for 24 hours to avoid results being affected by different duration of incubation [14].

4. The diameter of the thrombolytic zone must be measured immediately after incubation [15] to prevent the results being affected by the change in temperature.

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