

ISOLATION AND PURIFICATION OF HEPARIN FROM BOVINE PANCREAS BY DIFFERENT METHODS

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ABSTRACT

Background: Different methods of heparin extraction are being used. **Objective:** To compare heparin extraction from bovine pancreas by using three different methods including method of Charles and Scott, Marx and Volpi. **Methodology:** Design: Comparative Study. Setting: The University of Lahore with three month's duration starting from 1st July 2013. Fresh twenty samples of bovine pancreas samples were collected from the slaughterhouse of Lahore Pakistan and immediately placed in the ice buckets. The isolated heparin samples were separated into two species, slow moving and fast moving heparin by agarose gel electrophoresis. The anticoagulant activity of heparin samples was determined by using assay of Mitali et al (1982). **Results:** The percentage yield of heparin extracted by Volpi method was significantly higher ($P < 0.001$) i.e. 0.446% with the anticoagulant activity of 19 IU/ mg for bovine pancreas. **Conclusion:** Heparins are important in the field of medicine and pharmaceuticals. The results of our study showed that heparin isolated by the method of Volpi was significantly higher for bovine pancreas.

Key Word: Bovine, Heparins, Anticoagulant, Extraction.

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INTRODUCTION

Heparin is an anticoagulant agent. Heparin is a highly negatively charged heterogeneous mixture of sulfated mucopolysaccharide, varying in the molecular weights and composed of polymer of alternating derivatives of D-glucosamine and L-iduronic acid or D-glucuronic acid, having the property of prolonging the clotting time of blood. Heparin is the body's natural anticoagulant. Heparin is usually obtained from the lungs, intestinal mucosa or other suitable tissue of domestic mammals used for food by man. Heparin is employed clinically in conditions in which a rapid reduction in the coagulation ability of blood is desired. Heparin calcium and heparin sodium are the calcium and sodium salts of active principles that compose heparin. Heparin calcium and sodium are administered intravenously or subcutaneously.^{1,2}

Heparin and its low-molecular-weight derivatives (e.g., enoxaparin, dalteparin, tinzaparin) are effective at preventing deep vein thromboses and pulmonary emboli in patients at risk, but no evidence indicates any one is more effective than the other in preventing mortality.^{2,3,4,5,6,7}

Heparin binds to the enzyme inhibitor antithrombin III (AT), causing a conformational change that result in its activation through an increase in the flexibility of its reactive site loop.⁵

The activated AT then inactivates thrombin and other proteases involved in blood clotting, most notably factor Xa.^{8,9,10,11} The rate of inactivation of these proteases by AT can increase by up to 1000-fold due to the binding of heparin. A serious side-effect of heparin is heparin-induced thrombocytopenia (HIT), caused by an immunological reaction that makes platelets a target of immunological response, resulting in the degradation of platelets, which causes thrombocytopenia. This condition is usually reversed on discontinuation, and in general can be avoided with the use of synthetic heparins. Also, a benign form of thrombocytopenia is associated with early heparin use, which resolves without stopping heparin.^{12,13,14,15}

It is believed that biosynthesis of heparin take place in the Golgi system which is initiated by the sequential addition of alternating D- acid and N-acetyl-D-glucosamine units to the non-reducing termini of nascent acceptor structure and then in second steps D-glucouronic acid residues are converted in to L-iduronic acid units by C-5 epimerization, at the polymer level. Finally, a number of different O-sulphation reaction occur and thus heparin is formed.^{6,16} This study was designed to isolate and purify heparin from bovine pancreas by three standard methods i.e. Charles and Scott, Marx and Volpi and to compare these methods for the heparin isolation and purification.

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METHODOLOGY

This comparative cross sectional study was conducted in three months from 1st July 2013. All the samples of Bovine Pancreases were collected from the slaughterhouse of Lahore, Pakistan and stored in freezer at -20°C in Molecular Biology and Biotechnology Department the University of Lahore. Ethical approval was sought from Institute. The data was entered and analyzed SPSS version 15.

Isolation of Heparin

Three method used for the extraction of heparin from bovine pancreas (Method of Charles and Scott (1933), Method Marx (1957) & Method of Volpi (1999), were used and compared in form of amount of heparin collected its anticoagulant activity.

1. Method of Charles and Scott (1933)

100gms tissue sample was minced and allowed to stand for autolysis at 25°C for 24 hour. 2.9 g of sodium hydroxide and 12.5 gms of ammonium sulfate dissolved in 150 ml water and autolyzed tissues was added to it with stirring. The mixture was heated at 50 °C for 30 Minutes in water bath and then heated to 70°C. The filtrate was acidified to pH 2.0 with conc. H₂SO₄ mixture and was heated at 65°C and filtered. The precipitates were suspended in ethanol for 20 hour at room temperature. Centrifuged and then dissolved in 15 ml of water. The pH of the solution was adjusted to 8.4, and after decantation of most of the ethanol, precipitates of 200 mg of trypsin and 50 ml toluene were added. The mixture was stirred continuously, maintained at pH 8.4. After 36 hours the pH of the solution was adjusted to 6.0 and then added to 2 Volumes of 95% ethanol. After 24 hour, the solution was decanted and precipitates were centrifuged. After that the precipitates were washed with hot acetone until all the fat was removed. This gave a crude heparin powder.^{1,15}

2. Methods of Max (1957)

100 gms of tissue sample was homogenized with water and added to 5 ml of acetone and centrifuged at 5000 rpm for 15 minutes. The sediment was washed twice with 50 ml of isopropanol-petroleum ether (1:1) and then dried. Dry sediment was suspended in 30 ml of 0.5 M NH₄ Cl buffer of PH 8.5. The mixture was heated to boiling in water for 15 minutes. The samples were then subjected

to dialysis in dialysis tube. After incubation for 48 hours at 37°C. The Sample solution along with trypsin (200 mg in 2 ml of 50% glycerol) was poured into the dialysis tube and closed the other end with a knot. Dialysis bag was placed in a beaker containing 1000ml fresh and contained incubation for 48 hours at 37 °C. After dialysis against running tap water over night, the sample was transferred to centrifuge tube, 2% NaCl was added to tube and heated in a boiling water bathe for 15 minutes. It was cooled and centrifuged at 5000rpm for 15 minutes. The supernatant was precipitated with 5 volumes of acetone. The precipitates were again centrifuged at 5000 rpm for 15 minutes. Then the precipitate was dissolved in 1% NaCl and in 5 volumes of methanol. The precipitates were dissolved in 5ml of water, lyophilized, weighed and assayed.^{5,15,16}

3. Method of Volpi (1999)

100 gms of tissue sample was grinded in the presence of acetone, centrifuged and treated with chloroform-methanol. After the addition of solvents sample was again centrifuged and dried pellet was obtained. The pellet was solublized in distilled water and treated with papain and trypsin in reaction vessel. After heating the mixture was brought to pH 9.0 by adding 2M NaOH. After 24 hours at 40°C the product was centrifuged. Two volume of acetone were added to the pool supernatants and stored at 4 °C for 24 hours. After 24 hours sample was centrifuged again and precipitates were dried and dissolved in distilled water to prolong mixing. After centrifugation at 5000rpm for 15 minutes, the supernatant was applied to column pack with about 40 ml of Q-Sepharose (in original paper equilibrate with 0.05 M NaCl). Two volumes of acetone were added to elute and stored at 4 °C for 24 hours. After centrifugation, the pellet was dried at 60 °C for 6 hours and dry precipitate was dissolved on 10ml distilled water to prolong mixing. 1ml trichloroacetic acid (50%) was added. The preparation was stored at 4 °C for 12 hours. After centrifugation the supernatant was neutralized with 10 M NaOH and 4 volumes of ethanol saturated with sodium acetate were added and the preparation was stored at 4 °C for 24 hours. After centrifugation at 5000 rpm for 15 minutes, the pallets were dried at 60 °C, weighed and assayed.^{12,15}

Purification of Heparin

Agarose gel was prepared at a concentration of 0.5% in 0.04M barium acetate buffers. Single plate of 6.8 x 7.5 cm with a thickness of about 4-5 mm was used.

The gel cassette was set in electrophoresis apparatus. 0.04 M barium acetate buffer was added to the chamber of electrophoresis apparatus in such a way that a thin layer of buffer should be on the gel. Ten micro liters of each working sample was loaded in the wells using micropipette. Electrophoresis was allowed proceeding for 3 hours at constant current of 60 mA using Biometra power pack. The electrophoresis was run in Mini Cold Lab, at 4°C. After migration, the plate was soaked in 0.1% cetyltrimethylammonium bromide solution for 3hours. The gel was then stained with toluidinc blue in 0, 2% ethanol: water: acetic acid 50:49:1 solution over night, and results were retarded (Figure I).¹⁵

Figure I: Agarose gel electrophoresis of heparin samples extracted from bovine pancreas

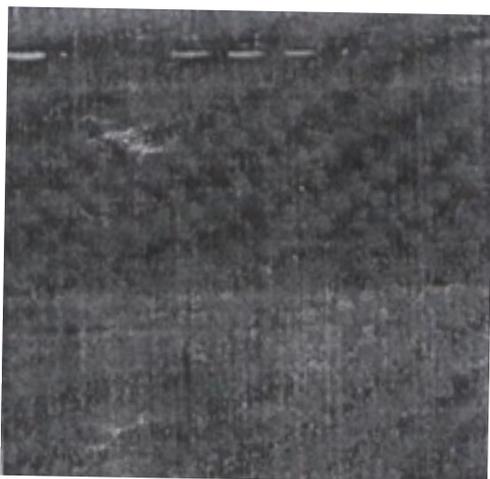
Lane A: Standard heparin

Lane B: Standard heparin

Lane C: Heparin extracted by method of Charles and Scott

Lane D: Heparin extracted by method of Marx

Lane E: Heparin extracted by method of Volpi



A B C D E

RESULTS

Heparin was extracted from bovine pancreas by three different methods: i.e. method of Charles and Scott (1933), Marx (1957) and Volpi (1999). The amount of heparin in pancreas was measured by using Khan and Newman (1990) assay. The recovery of heparin in mg/g of tissue and %age yield is given in Table I.

Table I: Amount of heparin extracted from bovine pancreas

Methods for extraction of heparin	Tissue (g)	Amount of Heparin			P. Value
		mg	mg/g of tissue	% yield	
Method of Charles and Scott	100	250 ± 2.43	2.50	0.250	0.001
Method of Marx	100	341 ± 2.17	3.41	0.341	
Method of Volpi	100	446 ± 3.83	4.46	0.446	

The results shows that 250mg/100g of heparin was isolated from bovine pancreas by using the method of Charles and Scott (1933). 341 mg/100g heparin was isolated by Marx and from Volpi method it was 446mg/100g. Percentage yield of heparin from bovine pancreas by Volpi method was greater (0.446) as compared to others methods. As mentioned in table II that anticoagulant effect of heparin extracted from bovine pancreas by Volpi method was higher (19) than the other methods.

Table II: Anticoagulant activity of heparin extracted from bovine pancreas.

Method for extraction of Heparin IU/mg	Anticoagulant activity of Heparin extracted from Bovine pancrease IU/mg
Method of Charles and Scott	5
Method of Marx	9.5
Method of Volpi	19

Table I shows that in Heparin quantity by Volpi method yielded significantly high (446) amount of heparins form bovine pancreas (p=0.001).

DISCUSSION

Heparin is a naturally occurring anticoagulant. It is found in abundance in liver, lungs and intestinal mucosa. Heparin can be extracted and purified from different animal tissue by using different methods and technique. In this study heparin was extracted from bovine pancreas by using three methods that are method of Charles and Scott (1933), Marx (1957), and Volpi (1999).

In Charles and Scott method bovine pancreas was minced and then autolized before extraction with alkaline solution saturated with ammonium sulfate.

Protein was precipitated by warming the extract and the heparin-protein complex was precipitated from the supernatant liquor or acidification. Extraction of the complex with ethanol removed fatty material and tryptic digestion removed most of protein. Heparin was precipitated with ethanol, redissolved in warm alkaline solution to destroy trypsin and precipitated with ethanol. This gave crude heparin.¹

According to Marx et al,⁹ who demonstrated heparinase activity in several mammalian tissue, in activation of heparinase was essential for quantitative recovery of heparin. In Marx method this was accomplished by treatment of tissue sample with acetone and then with heat (100°C). During these steps, not only heparinase but also the enzymes responsible for autolysis were inactivated, then next step was dialysis, which permitted a continuous removal of dialyzable digestion product. Further advantages of these techniques included to improve maintain of the pH of the digestion of the medium and separation of materials from sample of dialyzable compounds that would otherwise interfere with the assay of heparin.⁹ Heparin was precipitated with acetone. Then heparin was precipitated with methanol to remove that compound that was not acetone soluble.

In Volpi method tissue was mince in acetone and then treated with chloroform and methanol solution for the extraction of lipids. Pellets were solublized in water and then protein was digested by using two enzymes pepsin and collagenase. But in this study collagenase was not available so trypsin was used as replacement. After centrifugation the supernatant was treated with ethanol saturated with sodium acetone to obtain precipitated sodium salt of heparin.¹⁴

Anticoagulant activity of heparin extracted sample from bovine pancreas was estimated by assay Mitali et al.¹⁰ According to this the optical density of the color was dependent on the protein content of the clot which in turn was inversely proportional to the anti coagulant activity of heparin incubation mixture.¹⁷ The percentage yield of heparin extracted by Volpi method was greatest, i.e. 0.446% with the anticoagulant activity of 19 IU/mg for bovine pancreas.

CONCLUSION

The results of present study showed that the methods of Volpi yielded significantly higher amount of the heparin as compared to the other

available methods. It may be concluded that bovine pancreas are important organs and cheaper source, and potential sources for the commercial production of the heparins.

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