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Heparin Can Be Isolated and Purified From Bovine Intestine by Different Techniques

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ABSTRACT: *Heparin was extracted from intestines by using three different methods: i.e method of Charles and Scott (1933), Max (1957) and Volpi (1999). The percentage yield of heparin extracted by Volpi method was significantly higher (P<0.001) i.e 0.527% with the anticoagulant activity of 22 IU/mg for intestine. The anticoagulant activity of heparin samples was determined by using assay of Mitali et al. (1982). The isolated heparin samples were separated into two species, slow moving and fast moving heparin by agarose gel electrophoresis. Both species of heparins are economically important in the field of medicine and pharmaceuticals. The statistical analysis showed that heparin isolated by the method of Volpi was significantly higher for bovine intestines.*

KEY WORD: *Bovine, Heparins, Anticoagulant, Extraction. Slow moving heparins, Fast moving heparin.*

I. INTRODUCTION

An acidic glycosaminoglycans (GAGs) found especially in lungs, liver and intestinal tissues and their ability to prevent the clotting of blood, is used in the treatment of thrombosis. Heparin is a drug that helps to prevent blood clot formation and belongs to the family of anticoagulants. Heparin is used to decrease clotting of the blood and helps to prevent harmful clots formation in the blood vessels. Heparin does not dissolve blood clots that have already been formed, but it may prevent the clots from becoming larger and causing more serious problems. [1] GAGs are long unbranched polysaccharide chains composed of repeating disaccharide units. One of the two sugar residues in the repeating disaccharide is always an amino sugar (N-acetylglucosamine), which in most cases is sulfated. The second sugar is usually uronic acid (glucuronic or ieduronic). The specific GAGs of physiological significance are hyaluronic acid, dermatan sulfate, chondroitin sulfate, heparin, heparin sulfate and keratin sulfate. These GAGs have several fundamental biological and pharmacological activities, making them important in clinical and pharmaceutical fields. [2] Heparin consists primarily of repeating disaccharide units of glucosamine (GIcN) with L-iduronic (IdUA) respectively. The linkage between the amino sugar and the uronic acid is uniformly 1-4 linkage. Heparin may also contain sulfate on C-2. [1]Heparin is also produced in mast cells. The biosynthesis of heparin, which is believed to take place in the Golgi system, is initiated by the sequential addition of alternating D-glucouronic acid and N-acetyl-D-glucosamine units to the non-reducing termini of nascent acceptor structure. In the 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 accur and thus heparin is formed. [3]

Heparin is an anticoagulant and antithromboitic drug also used as a fat-clearing and anti-inflammatory agent (Lane and Lindah, 1989). Condroitin sulfate is chondroprotective drug in patients with osteoarthrosis and used in the therapy of tibiofibular osteoarthrosis. Volpi (1993a) extracted and purified heparin form beef intestinal mucosa. The two components, fast moving and slow moving heparin, in electrophoresis were purified by selective precipitation as barium salts. The purity of the purified heparin was evaluated by agarose gel and cellulose polyacetate electrophoresis and by specific optical rotation. The molecular mass, sulfate to carboxyl ratio and disaccharides pattern of heparin were estimated by high performance size exclusion chromatography, titrimetric analysis and specific enzymatic cleavage. [5]Sasiekharana, et al. (2000) reported that heparin and heparin sulfate glycosaminoglycans are acidic complex polysaccharides found on the cell surface and in the extracellular matrix. He developed an analytical method for composition and sequence analysis that provided remarkable insights into structure and function relationships of these complexes. [6] Present work was designed to isolate heparin from bovine intestines, as no paper could be traced on heparin isolation and purification from bovine intestine. Three standard methods were evaluated i.e. Charles and Scott (1933), Marx (1957 and Volpi (l999 to identify most effective method for the heparin isolation and purification).

II. MATERIALS AND METHODS

Fresh twenty samples of bovine intestine were collected from the slaughterhouse of Lahore Pakistan and immediately placed in the ice buckets. The samples were stored in a freezer at —25°C in the Institute of Molecular Biology and Biotechnology Lahore.

2.1 Assay For Anticoagulant Activity Of Heparin

The anticoagulant activity was determined by using Mitali et al. (1982) method this method involved the assessment of the quantity of heparin required to prevent 50% clotting of a fixed amount of citrated plasma in the presence of calcium ions. One IU of heparin Keeps 1ml of blood as fluid for 1 hour and 1mg being equivalent to one unit. [7]

2.2 Standard Assay Procedure

To each of the ten meticulously clean-stoppered centrifuge tubes 1 ml of calcium chloride solution was added. The tube No. 1, which served as control, contained 1.8 ml of normal saline and no heparin, was added whereas in the other tubes graded quantities of standard heparin solution were added. The total volume in all the tube was adjusted to 2.8 ml by the addition of the required quantities of normal saline. Thereafter, to each tube 0.2 ml of standard plasma (at 5-minute intervals, for practical consideration) and the tubes was stopper and incubated at 37^0C for 10 minutes. The clot formation varied from tube to tube i.e (full clotting in tube No. 1 to no clotting in tube No. 10), and was squeezed with the help of a thing glass rod and washed 3 times with 5ml of normal saline solution. The washings were discarded. The solid masses of the clot were dissolved by the addition of 1 ml sodium hydroxide solution to each tube and kept for 30 minute at 35^0C for complete dissolution afterward 1 ml biuret reagent was added and the tubes were allowed to stand for 30 mins. to obtain a stable violet color. [8]

2.3 EXTRACTION OF HEPARIN

There are three method used for the extraction heparin from bovine intestine i.e Method of Charles and Scott (1933), Method Marx (1957) & Method of Volpi (1999)

2.4. Method of Charles and Scott (1933)

100gms tissue sample was minced and allowed to stand for autolysis at 25^0C 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 ^{0}C for 30 Minutes in water bath and then heated to 70^0C. The filtrate was acidified to Ph 2.0 with conc. H_2SO_4 mixture and was heated at 65^0C 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. [9]

2.5. Method 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 NH_4 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 37^0C. 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 ^{0}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 temperature and centrifuged at 5000rmp for 15 minutes. The supernatant was precipitated our with 5 volumes of acetone. The precipitates were again centrifuged at 5000 rmp 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. [10]

2.6. Method of Volpi (1999)

100gms of tissue sample was grind in the presence of acetone, centrifuged and treated with chloroform-methanol. After the addition of solvents sample was again centrifuged and dried pallet was obtained. The pallet 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^oC the product was centrifuged.

Two volume of acetone were added to the pool supernatants and stored at 4 ^{0}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 ^{0}C for 24 hours. After centrifugation, the pallet was dried at 60 ^{0}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 ^{0}C for 12 houses. 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 ^{0}C for 24 houses. After centrifugation at 5000 rpm for 15 minutes, the pallets were dried at 60 ^{0}C, weighed and assayed. [11]

2.4 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.

III. RESULTS AND DISCUSSION

3.1 Extraction of heparin

Heparin was extracted from bovine intestine by three different methods: i.e. method of Charles and Scott (1933) Marx (l957) and Volpi (l999).The amount of heparin in intestine 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 1 and table 2. The results showed that 342g/I00g of heparin ware extracted from bovine intestines by using the method Charles and Scott (1933) respectively. The amount of heparin extracted by method of Marx was 408mg/100mg and by Volpi method was 527mg/100g. It was noted that Volpi method had greater %age yield of heparin in bovine intestine that is 0.527%. Our results suggested that Volpi method is the best methods as compared to other methods. These results were the average results of the twenty samples of bovine intestines.Statistical analysis showed that in all of the methods which indicates that our results are highly significant. The value showed that Volpi method yielded significant amount of heparins form bovine intestines. From the results presented in (Table 1 & 3) . It can be concluded that Volpi method had the significantly higher yield of heparins from bovine intestines. The Marx method had second highest yield followed by Charles and Scott. The yield of heparin by all the three method showed significant differences within the results of intestines.

3.2 Anticoagulant activity of Heparin

Anticoagulant activity in IU/mg of heparin samples extracted from bovine intestine was estimated by assay.[12] The result, in (Table 2) showed that anticoagulant effect of heparin extracted from bovine intestine by Volpi method were higher than the other methods.

3.3 Purification of Heparins

Heparin was purified by agarose gel electrophoresis as reported by Volpi (1994) Agarose gel was prepared at a concentration of 0.5% in 0.04 M barium acetate buffer (ph 5.8). The micro litersof each sample was layered on the wells using micropipette. The gel was run in 0.04 M barium acetate buffer for 3 hour at 60 mA. [13] The extracted heparin samples were subjected to agarose gel electrophoresis. Two runs were performed with the barium acetate buffer for 3 hours at 60 mA. After separation, they were fixed in the agarose gel matrix by precipitation cetyluimethylammonium bromide solution, making them visible on a dark background and then gel was subject to staining and destining procedure.

Table 1: Amount of heparin extracted from bovine intestine

No. of obs.	Method for extraction of heparin	Tissue (g)	Amount of Heparin		
			Mg	Mg/g of tissue	% yield
1	Method of Charles and Scott	100	342	3.42	0.342
2	Method of Marx	100	408	4.08	0.408
3	Method of Volpi	100	527	5.27	0.527

Table 2: Anticoagulant activity of heparin extracted from bovine intestine

No. of obs.	Method for extraction of heparin IU/mg	Anticoagulant activity of heparin extracted from bovine intestine IU/mg
1	Method of Charles and Scott	7.2
2	Method of Marx	12
3	Method of Volpi	22

Table. 3 Stat. Analyses for Intestine

Ser.No.	Methods	Mean+S.D	P.Value
1	Charles and Scott	342+2.14	0.001
2	Marx	408+1.67	0.001
3	Volpi	527+1.72	0.001

A B C D E

Fig. I Agarose gel electrophoresis of heparin samples extracted from bovine intestine

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

IV. CONCLUSION

Heparin is one of the most important drugs used for the treatment of thrombosis. It can be obtained from the animal tissues. It can be obtained from the animals tissues. The function and properties of the heparins are extensively reviewed but to obtain higher quantities of heparins from the animal tissues new sources should be explored and this research is an effort to study a new comparative approach of extraction of heparin from Bovine Intestines and pancreas. The present results suggested that the methods of Vopli yielded significantly higher amount of the heparin as compared to the other available methods. It may be concluded that bovine intestines is an important organs and cheaper source, and potential sources for the commercial production of the heparins.

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