Original article

DETERMINATION OF HEPARIN AND HEPARIN-LIKE SUBSTANCES IN THALASSEMIAS patients WITH AND WITHOUT EPISTAXIS USING A NOVEL MONOCLONAL ANTIBODY

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Abstract A monoclonal antibody to polysulphated polysaccharides was prepared and used in a plasma assay for heparin. The antibody reacted with heparin and semisynthetic heparin-like substances including dextran sulphate, pentosan polysulphate, and glycosaminoglycan polysulphate. The analytical method used a biotin-labeled monoclonal antibody in a competitive enzyme linked immunosorbent assay (ELISA). The level of heparin and heparin-like substances obtained by the assay correlated with those of a method based on the inhibition of Factor Xa, but were numerically different ($y = -0.1747 + 1.0848x$; $r^2 = 0.844$). The assay could detect heparin and heparin-like substances in solution at a submicrogram or milliunit level, which suggested that it was suitable for pharmacokinetic studies of heparin and polysulfated polysaccharides. The heparin concentrations in plasma from thalassemic patients, either with or without epistaxis, were not statistically different from those of normal subjects. Chiang Mai Med Bull 2000; (3-4): 57-66.

Heparin is an alternating copolymer of a uronic acid and an amino sugar, and its structure is commonly represented by its prevalent disaccharide sequences of $\alpha$-1,4-linked L-iduronic acid 2-sulphate $\rightarrow$ D-glucosamine N,6-disulphate (IduA-2S $\rightarrow$ GlcNSO3-6S). The anticoagulant effect of heparin is, to a large extent, a direct consequence of its catalytic action of thrombin inhibition by antithrombin III. Most of the sensitive techniques for heparin assays are based either on their overall anticoagulant activity or more specific properties such as the inactivation of thrombin or Factor Xa. The competitive binding assay for heparin and other therapeutic sulphated polysaccharides in plasma, serum and urine was reported by Dawes and co-workers using radioactive heparin for binding the sample to a polybene-solid phase.

This study produced and characterized a monoclonal antibody against protamine sulphate, heparin and heparin like substances. Consequently, this research developed a method for the determination of heparin and heparin like substances by testing this antibody with human plasma from both normal and thalassemia subjects. The developed assay may be useful in evaluating the possible causes of abnormal bleeding.

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Materials and Methods

Immunization in Balb/c mice and hybridoma development

Female Balb/c mice aged 6-8 weeks were immunized with a protamine sulphate and polyolpolysulphate that was supplied from Luitpold-Werk, Munich, Germany. Antigen solution was prepared by adding polyolpolysulphate powder to photomine sulphate solution (Luitpold-werk, Munich, Germany) to a final ratio of 1:3 (W/W). Each mouse was given 500 μl of antigen complex solution mixed with 500 μl of complete Freund’s adjuvant subcutaneously. After 14 days, a mixture of the same complex with the incomplete Freund’s adjuvant was injected intraperitoneally into all mice (500 μl each). They were then injected intraperitoneally with the antigen complex in an incomplete Freund’s adjuvant, on day 35, and received 100 μl of antigen complex without adjuvant on day 56. On day 59 their spleenocytes were fused with the myeloma cell line (X63 Ag 8.653) using polyethylene glycol as a fusing agent. Tissue culture media from individual hybridoma cell lines were screened against POPS and protamine sulphate complex by using an ELISA technique. Positive wells were cloned as a monoclon by a limiting dilution technique, expanded in a bulk cell-culture, and grown in serum-free media for production of the monoclonal antibody (mAb).

Purification of a monoclonal antibody from serum-free medium

A monoclonal antibody was purified by thiophilic adsorption (T-gel) column chromatography as described elsewhere in this study.(7,8) Briefly, hybridomas were separated from tissue culture serum-free media by centrifugation. Potassium sulphate was added to the media to a final concentration of 0.5 mol/L and was equilibrated with washing buffer (50mmol/L phosphate and 0.5 mol/L potassium sulphate, pH 8.0). It was then applied to a T-gel column (a gift from Dr Jan Carlsson, Pharmacia Diagnostics AB, Uppsala Sweden). Following this, the unbound protein was eluted from the column with washing buffer. The monoclonal antibody was adsorbed into the column and was eluted with a phosphate buffer (50 mmol/L, pH 8.0). Fractions were pooled, dialyzed against double distilled water, and lyophilized to provide purified mAb for testing.

Biotinylation of a monoclonal antibody

Biotinylation of mAb was performed by a standard method.(9) Briefly, mAb, which had been prepared as described above, was dissolved in 0.1 Msodium hydrogen carbonate buffer pH 8.5 and mixed at a ratio of 3:1 (w/w) with N-hydroxysuccinimidobiotin (Sigma Chemical) (34.1 g/L in DMSO) at room temperature for 1-2 hours. The mixture was applied to a Sephadex G-25 column, which was eluted with PBS, pH 7.4. The excluded protein peak was collected, aliquoted and stored at -20°C as a stock solution of the biotinylated monoclonal antibody (B-mAb).

Enzyme-linked immunosorbent-inhibition assay (ELISIA) using the labeled avidin-biotin (LAB) technique

Characterization of the mAb obtained from the hybridoma was performed by using an ELISIA-LAB technique. Various concentrations of the preparations of DS, xylan, heparin, heparan sulphate, keratan sulphate, dermatan sulphate, hyaluronan, chondroitin sulphates (4- and 6-sulphate and 4, 6-disulphate), or calf thymus DNA in 6% BSA-PBS were incubated with appropriate dilutions of B-mAb in PBS-Tween 20 (5 mL/L) at room temperature for at least 1 hour. The reaction mixtures were applied to plates in which the protamine sulphate-POPS was bound and BSA blocked. The level of B-mAb, which bound to the wells, was determined by the addition of peroxidase conjugated
streptavidin (Amersham, UK; 1:1,000 in PBS) followed by peroxidase substrate (O-PD in citrate phosphate buffer, pH 5.0), and the absorbance at 492/690 nm was determined by a microplate reader. For this assay, triplicate results were averaged and the percentage inhibition was calculated from the mean. Inhibition curves were constructed from these data using log/linear co-ordinates.

**Human plasma**

The plasma samples were prepared from venous blood using 3.8% trisodium citrate in normal saline solution as an anticoagulant (at 10% v/v). The subjects were healthy and non-hospitalized individuals between the age of 18-47 years. They were thalassemia patients selected from OPD # 29, Maharaj Nakorn Chiang Mai hospital who exhibited repeated episodes of epistaxis and no abnormal bleeding. All the studies were in accordance with the current revision of the Helsinki Declaration of 1975. The blood sample were centrifuged at 1,000 g for 10 minutes, and plasma samples were separated. An equal volume of pronase solution (1 g/L) was added to each plasma samples and then incubated at 37°C overnight. The digested samples were boiled for 3 minutes and centrifuged as described above, and the resulting supernatant fluids were collected as pretreated samples for a further assay.

**The quantitation of heparin in human plasma samples by HEPTEST®**

The HEPTEST® kit was purchased from Sigma Chemical. The assay procedure consisted of incubating an undiluted test plasma sample with an equal volume of Factor Xa for 120 seconds at 37°C; thereafter this reaction mixture was recalcified by the addition of RECALMIX®. The time required for the plasma mixture to clot was converted to heparin (units/ml) using a standard calibration curve. This assay kit was used to quantitate the amount of heparin that had been added with therapeutic heparin at different levels (calibrated from the manufacturer). These levels, which ranged from between 0.125 to 1.0 U/mL, were calibrated in an individual plasma sample, and compared with the amount determined from the method described above.

**Results**

**The production of a monoclonal antibody**

Several hybridomas were obtained from the fusion of spleen cells and myeloma using polyethylene glycol as a fusing agent. Hybridomas from the highest titer of ELISA-positive wells (6 clones) were expanded and cloned by the limiting dilution technique. Some of them showed a strong reactivity with pure protamine sulphate, while others did so with the complex of protamine sulphate and POPS or heparin. One of the latter clones was selected to produce a monoclonal antibody for further analysis. The mAb in the supernatants was characterized by sub-typing immunoglobulins by specific antibodies and the immunoblotting technique. It was found that this clone produced IgM immunoglobulins and K-light chains.

**The characteristics of a monoclonal antibody**

A monoclonal antibody was purified by using T-gel column chromatography. It was characterized by an ELISA-LAB technique. Figure 1 shows typical inhibition curves against various inhibitors. It was found that this mAb had a strong binding to polyolpoly-sulphate and heparin, but interacted weakly with heparan sulphate. Heparinase was used to digest heparin to confirm the above observation (Figure 2) and the mAb did not cross-react with naturally occurring sulphated polysaccharides such as human keratan sulphate, bovine nasal cartilage proteoglycan, and DNA (Figure 3). On the contrary, the mAb showed a strong reaction against naturally occurring polysulphated oylsaccharides
Fig. 1. An inhibition curve for a biotinylated monoclonal antibody and heparin, heparan sulphate and polyolpolysulphate (POPS). The inhibitor was incubated with optimal dilution of the antibody and then added to the protamine complex coated plate. The enzyme conjugated streptavidin was used as a probe, which was demonstrated by an enzyme substrate, and absorbance was determined by a microtiter plate reader.

Fig. 2. An inhibition curve for demonstrating the effects of heparinase The samples were subjected to digestion with heparinase, boiled prior to being assayed in the competitive inhibition mixture with a biotinylated monoclonal antibody and then added to the coated plate, followed by the standard procedure as described in the method section.
such as chondroitin sulphate E, fucoidan and carrageenan type IV at a concentration at 50% inhibition (IC50) as shown in Table 1. Furthermore, it reacted with semi-synthetic polysulphated polysaccharides like pentosan polysulphate, dextran sulphate, and glycosaminoglycan polysulphate, but not with their precursors.

**Optimal conditions for the standard method for quantitation of heparin and heparin like substances in plasma samples**

The optimal concentration and dilution of B-mAb were determined by performing a chequerboard titration of antigen and B-mAb. The optimal concentration of antigen complex for the coating plate was 100 mg/L and the optimal dilution for B-mAb was 1:2,000.

With these conditions and normal human plasma samples, the intra- and inter-assay coefficient of variations (CVs) were 3.6% and 13%, respectively. The recovery of 20-10,000 ng/ml of porcine heparin (diluted in 1% BSA) was 96% and 94%.

**Table 1.** A table showing the various type of inhibitors and their concentration at 50% inhibition of antibody activity using competitive assay.

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>IC50 (ng/ml)</th>
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<tbody>
<tr>
<td>POPS</td>
<td>60</td>
</tr>
<tr>
<td>Glycosaminoglycan polysulfate</td>
<td>220</td>
</tr>
<tr>
<td>Heparin</td>
<td>300</td>
</tr>
<tr>
<td>Pentosan polysulfate</td>
<td>300</td>
</tr>
<tr>
<td>Dextran sulfate</td>
<td>300</td>
</tr>
<tr>
<td>Chondroitin sulfate E (4,6-disulfate)</td>
<td>600</td>
</tr>
<tr>
<td>Carrageenan</td>
<td>1000</td>
</tr>
<tr>
<td>Fucoidan</td>
<td>1050</td>
</tr>
<tr>
<td>Heparan sulfate</td>
<td>1050</td>
</tr>
<tr>
<td>Calf thymus DNA</td>
<td>6000</td>
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**Fig. 3.** An inhibition curve to demonstrate the reactivity of a biotinylated monoclonal antibody against proteoglycan fragments and calf thymus DNA compared with standard heparin. The various concentrations of an individual inhibitor were incubated with an optimal dilution of a biotinylated monoclonal antibody, and then added to the coated plate, followed by enzyme conjugated streptavidin and substrate according to the standard ELISA method.
that had been added to pooled human plasma was 139±17%.

**Quantitation of heparin in normal human plasma samples**

A typical calibration curve for the quantitation of heparin in plasma is shown in Figure 4. In plasma samples (N = 53) from normal healthy volunteers aged 18-47 years without a history of abnormal bleeding, the mean heparin concentration (± SD) was 6.50 μg/L. (±1.56)

**Comparison between the developed method and HEPTEST®**

Two methods had been used to quantitate heparin in a parallel manner using a single human plasma sample with heparin added at various quantities. As mentioned before, heparin was calibrated against a known concentration supplied by the manufacturer. For the ELISIA method, endogenous concentrations of heparin were subtracted from the samples without added heparin (Figure 5).

**Quantitation of heparin in Thalassemia patients**

Fifty-two plasma samples from thalassemia patients with and without a history of abnormal bleeding were assayed for heparin by the above method. The degree of abnormal bleeding was based on the frequency of repeated epistaxis, ranging from 0 (no epistaxis) to +4 (epistaxis occurs every 1-2 weeks). As shown in Figure 6, there was no significant difference and correlation in the plasma heparin levels between normal individuals (6.50 ± 1.56 μg/mL) and thalassemia patients either with or without epistaxis (6.18 ± 1.92 μg/mL; p > 0.05).

**Discussion**

The monoclonal antibody possesses reactivity against both natural occurring and semi-synthetic polysulphated polysaccharides like: fucoidan, carrageenan (both found in sea algea), chondroitin sulphate E (found in squid cartilage and the granule of mast cells), pentosan polysulphate (from sulphatation of xylan), dextran sulphate (from sulphatation of dextran), and glycosaminoglycan polysulphate (from oversulphatation of chondroitin sulphate). This antibody did not react to xylan, a precursor of pentosan polysulphate, dextran, a precursor of dextran sulphate, or various types of chondroitin sulphate, which are precursors of glycosaminoglycan polysulphate. The only polysulphate polysaccharides that occurred naturally in plasma were heparin and heparan sulphate of which the latter showed less sulphatation. However, heparan sulphate had a weak recognition of this antibody and its level did not affect this method. The enzyme that digested heparin was used to confirm its specific activity, as shown in Figure 2.

The newly developed method described here can be used for the quantitation of heparin in human plasma. In separate experiments it was found that the results obtained from EDTA or citrate plasma were not statistically different. Since EDTA is routinely used as an anticoagulant in a hematological laboratory, it would be convenient to have a heparin assay as additional to many hematological tests. The most appropriate amount of EDTA for sample preparation needs further investigation, due to the possible effects on enzyme digestion. The quantitation was set up with plasma samples because heparin binds to coagulants such as fibrinogen or fibrin and could incorporate into blood clots. Blood coagulation releases substances such as thrombospondin from platelets. This makes serum containing these substances 200 times greater than plasma. Since thrombospondin has binding sites for heparin, it could affect this assay, and should be the subject of future research.

The plasma samples in this method must be digested with broad spectrum proteolytic enzymes like pronase, since proteins in serum
**Fig. 4.** A calibration curve for the quantitation of heparin in human plasma using a biotinylated monoclonal antibody. The calibrator was therapeutic heparin dissolved in 6% BSA in PBS, pH 7.4.

**Fig. 5.** Comparison of ELISIA and HEPTEST® for the quantitation of heparin in plasma.
or plasma could bind to the protamine sulphate complex used for a coating plate. The enzyme does not only remove non-specific binding proteins, but also degrades the protein core, which covalently binds to heparin. This is why heparin substances are observed via this developed immunoassay method in normal samples, while no heparin substance is observed in the typical clotting or anti-factor Xa assay for normal plasma. The contamination of heparinase in this pronase was confirmed from recovery studies. If there was a contamination of heparinase in the pronase, it would have depleted the amount of heparin that had been added to the samples in those studies. A high percentage of recovery could be due to the contamination of heparin in bovine serum albumin. Most of the high recoveries were in the submicrogram range, which would not greatly affect the level of heparin in plasma samples, as they contained heparin in the microgram range. In the protein concentrations of samples after digestion, when boiling, and centrifugation were about 10 g/L, this value was used to dilute the heparin in the assay so that the concentrations of the standards were similar to those samples.

The heparin in digested samples was confirmed by performing repeated digestion with heparin specific heparinase, and it had no inhibition activity against this antibody, while samples that did not undergo digestion still contained this activity. The levels of normal human plasma heparin substances obtained from this assay were comparable to previous ones reported by an extraction and electrophoresis technique.\(^{(10)}\)

The ability of endothelial cells to synthesize heparan sulphate has been demonstrated\(^{(11-12)}\) and it was suggested that one should investigate these cells as the source of the plasma “heparinoid” constituent. An additional potential source of circulating

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**Fig. 6.** A scatter graph of the level of heparin and degree of repeated epistaxis in thalassemia patients using the developed method.
heparinoid molecules is the platelet itself. Adhesion-dependent synthesis of heparin by platelets was reported in 1982. Further investigations should address the difference in sex and age of the subjects. The comparison of this method with others, such as coagulation-based assays (activated partial thromboplastin time (APTT) test, HEPTEST®, synthetic enzyme substrate, competitive binding assay and inactivation of thrombin), is of particular interest, especially in terms of the structural, chemical and functional relationships to the immunological activity. The HEPTEST® is based on the inhibition of Factor Xa by antithrombin III and is accelerated by heparin. Under optimum conditions the amount of Xa activity neutralized during a predetermined time period is directly proportional to the concentration of heparin in the reaction mixture. It was found that both the developed method and HEPTEST® were comparable and had a correlation coefficient of 0.84. Furthermore, this method can be used for kinetic studies of polysulphated polysaccharide drugs, which has been used for HIV-related infection.

There was no significant difference in the heparin level between healthy and thalassemia subjects. Furthermore, there was no correlation between the level of heparin in thalassemia patients and the severity of repeated epistaxis (Figure 6). These additional studies may provide an insight into the cause of epistaxis in thalassemia patients. There is a possibility of vitamin deficiency or other related factors and these will be the subjects of future investigations.

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