Flow Injection Determination of Heparin by Inhibition of Ribonuclease (Rnase)

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Summary: Heparin is a mucopolysaccharide from mammalian tissues. It is an anticoagulant drug used universally. Of naturally occurring mucopolysaccharides only heparin competitively inhibit Ribonuclease (RNase) in its physiological range. Because of high bleeding risks due to the continuous consumption of heparin its determination in blood is critical. Many assays have been devised to measure this substance in blood. This paper describes manual and flow injection methods for the determination of heparin by the inhibition of ribonuclease. Manual spectrophotometric method was found very simple and sensitive. By this method heparin can be determined in its physiological range. Calibration graph was linear in the range 1.0-16 μg ml^{-1}. The limit of detection was 1.0 μg ml^{-1}. However, sensitivity decreased in flow injection method while using immobilised Rnase.

Introduction

In health, blood is kept in a fluid state by various mechanisms which may be upset in the sense of thrombus formation in certain circumstances. A thrombus may be defined as an abnormal mass formed inside a blood vessel. It causes many problems:

(i) Abnormalities of blood vessel walls.

(ii) Abnormalities of blood flow.

(iii) Hyperviscosity.

In order to overcome these problems certain types of anticoagulants have been tried clinically. Among them, heparin is an anticoagulant drug used universally.

Heparin is a mucopolysaccharide from mammalian tissues. It is a linear polymer composed of disaccharide repeating units obtained from sulphated glucuronic acid and disulphated galactosamine linked through α(1→4) linkages. Disaccharide units are linked through α(1→4) linkages [1].

Heparin was discovered by Mclean [2] and recognised as an anticoagulant by Howell and Holt [3]. Because of the high potential risks of bleeding due to continuous dosage of heparin, heparin determination in blood is critical [4]. Many assays have been devised to measure this substance in blood and tissue extracts.

Methods Available for Heparin Determination

Heparin can be monitored by using its chemical and biological properties. There are several methods for determination of heparin.

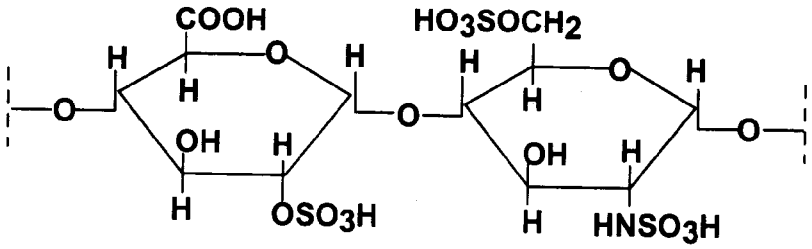
Among them the most commonly used methods are following:

(i) Metachromatic interactions of heparin with various dyes.

(ii) Turbidimetric interactions with polycations.

(iii) Degradation of heparin.

(iv) Methods based on enzyme inhibition.



STRUCTURE OF HEPARIN

(i) Metachromatic interactions with dyes

The mechanism of metachromatic interaction of dyes with heparin is based on the fact that dye molecules show resonance due to which partial charge is delocalized on the molecule. This permits loose interaction between dye molecule and heparin, and thus the amino group on the dye molecule is blocked, reducing electron delocalization. With less electron mobility the dye molecule absorbs light energy at a shorter wavelength. Klein et al. [5] Yin *et al.* [6] and Band and Lukton [7] have reported such a type of method for heparin determination, employing different dyes like Auramine and Azure A. The decrease in absorbance is noted at various concentrations of heparin. The concentrations of heparin measured by these methods [5,6 and 7] are 0-10, 0.1-5.0 and 0.169-16.9 units ml^{-1}, respectively.

(ii) Turbidimetric interactions

Heparin is a negatively charged colloid. It interacts with polycations to form a turbidity. That turbidity is measured by absorbance or by titration. Katayama *et.al* [8] has reported a method in which Cat Floc (polydiallyldimethylammonium chloride) is used as a polycation. The turbidity formed is titrated with the polyanion. This assay detects 10-40 units ml^{-1} heparin. Bohn [9] has reported a method based on mixing heparin with N-cetylpyridinum chloride; the resultant turbidity is measured at 580 nm. But these methods have a drawback that they are not capable of distinguishing heparin from other naturally occurring glucosamino glycans like heparin sulfate and keratin sulfate. Purification steps are quite lengthy for routine clinical studies, so they are not appreciated for clinical purposes.

(iii) Heparin degradation

Heparin can be degraded by acid hydrolysis or with an enzyme, heparinase The resultant oligosaccharides are detected by chemiluminescent reaction with lucigenin [10]. In the case of heparin degradation by acid hydrolysis, heparin cannot be hydrolysed completely with acid. However, the selectivity of enzyme hydrolysis can improve the method, but the enzyme is quite expensive and does not seem economical for heparin determination.

For this reason determination of heparin has typically been based on the unique physiological activity of heparin. The most commonly used methods for this purpose are based on the principle that heparin accelerates the inactivation of α-thrombin and Factor Xa by antithrombin [11,12].

Residual protease (Thrombin and Factor Xa) activity determined with a chromogenic substance is correlated with heparin concentration.

Factor Xa+AT 111→Factor Xa-AT 111+Factor Xa (residual)

where AT 111 is antithrombin 111

Residual Factor Xa then hydrolyses a chromogenic substrate methoxycarbonyl-D-cyclo-hexylglycyl-glycyl-arginyl-p-nitroaniline to release p-nitroaniline. These assays are extensively used clinically and are sensitive to very low levels of heparin (0.01-1.0 unit ml^{-1}). Almost all of these methods are done manually so they seem to be expensive for routine examination.

(iv) Methods based on enzyme inhibition

Of naturally occurring mucopolysaccharides only heparin competitively inhibits ribonuclease in its physiological range [13], and ribonuclease inhibition is also exploited for the determination of heparin [14, 15, 16].

These methods consist of simply mixing the ribonucleic acid with ribonuclease and the mixed solution is immediately transferred into a cuvette. Changes in absorbance of RNA due to hydrolysis is measured in the presence and absence of heparin for about 5-10 mins.

The use of ribonuclease inhibition has the advantage of simple experimental conditions. There is no need for a long incubation period or high temperature, so this reaction is carried out under mild conditions. The most important point is that other naturally occurring glucosamino glycans present in plasma do not interfere [17] so there is no need for a long purification process. By this method 0.5-5.0 unit ml^{-1} heparin can be determined. Therefore the system is simple and the analysis is completed in a few mins rather than in hours.

In an attempt to improve the economy of the system the present work describes the conversion method of analysis into a flow injection method with

an immobilised enzyme, so that the system could become economical and fast for daily use.

Flow injection determination of drugs by the inhibition of enzymes has been successfully studied by Ghous [18, 19, 20, 21]. The method has also been exploited for the determination of heparin by the inhibition of RNase.

The heparin determination method given by Zollner [15] is used for the soluble enzyme and extended further by immobilising the enzyme and using that enzyme in a flow injection system.

Results and Discussion

Effects of Reaction Conditions

Experiments were conducted to find the effect of different reaction conditions on ribonuclease inhibition by heparin in the batch method. Fig.1 is a simple representation of the change in absorbance in the presence and absence of heparin.

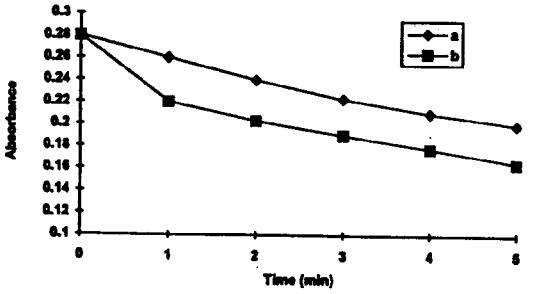


Fig.1 Change in absorbance of RNA in the presence of heparin with time. (a) assay mixture contained 1.0 ml heparin (5 μg ml^{-1}), 0.5 ml RNA (1.0 mg ml^{-1}) and 0.5 ml RNase (50 μg ml^{-1}). (b) absorbance of RNA in the absence of heparin under the same conditions.

Effect of pH

It was found that at constant substrate, inhibitor and enzyme concentrations the inhibitory effect increases with increase in pH. Fig. 2 indicates that heparin has maximum inhibitory effect between pH 6.0 and 6.5 but by increasing the pH the absorbance of RNA decreases. As a compromise a pH of 6.0 was used during this work.

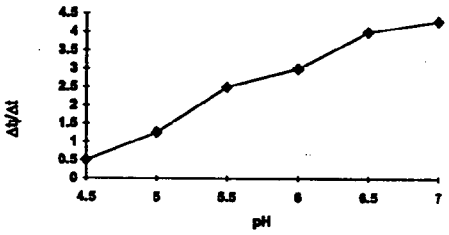


Fig. 2 Effect of pH on RNase inhibition by heparin (5 μg ml^{-1}). Substrate conc. 2.0 mg ml^{-1}

Effect of Heparin Concentration

Ribonuclease inhibition at various concentrations of heparin was determined. Fig.3A shows the change in absorbance of RNA in the presence of different concentrations of heparin and Fig. 3B shows a plot of the ratio $\Delta t_i/\Delta t$ in the presence of different concentration of heparin.

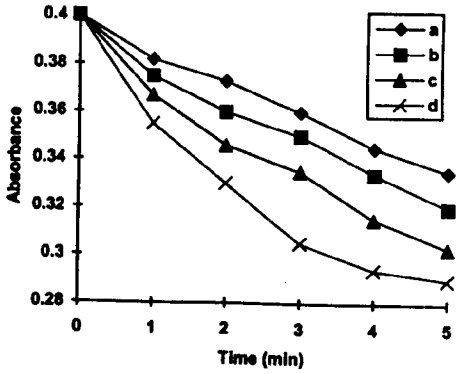


Fig. 3A. Effect of heparin concentration on ribonuclase inhibition. (heparin concentration was (a) 10, (b), 5.0 and (c) 2.5 μg ml^{-1}). (d) shows absorbance in the absence of heparin. RNA conc. 2.0 mg ml^{-1} and RNase conc. was 50 μg ml^{-1}.

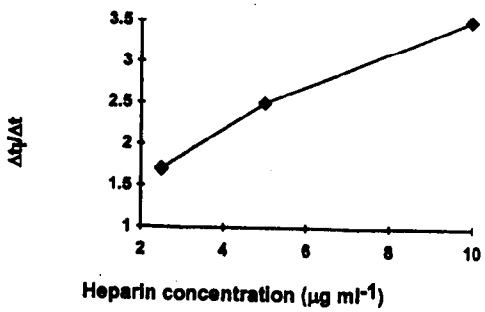


Fig. 3B. Effect of heparin concentration on the ratio of uninhibited and inhibited reaction.

Effect of Incubation Time

The effect of incubation time of RNase with heparin was examined. Inhibition increases with increase in incubation time, the maximum change appearing in two minutes. The results are shown in Fig. 4A and 4B.

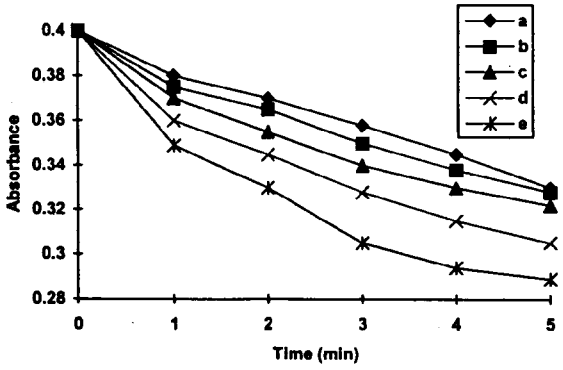


Fig. 4A. Effect of incubation time on ribonuclase inhibition by heparin. (Substrate 2.0 mg ml^{-1}, heparin 5 μg ml^{-1}, incubation time = (a) 2 min, (b) 4 min , (c) 5 min , (d) 0 min. (e) is the substrate absorbance with out heparin. RNase conc. 50 μg ml^{-1} and pH was 6.0.

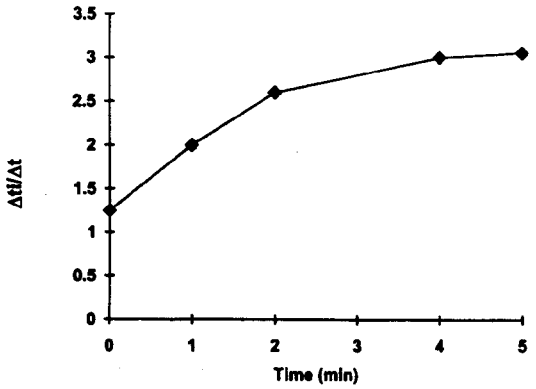


Fig. 4B. Effect of incubation time on ratio of inhibited and uninhibited reaction.

Effect of Substrate Concentration

The effect of substrate concentration was studied over the range 0.5-2.5 mg ml^{-1}. The result shows that by increasing substrate concentration inhibition decreases Fig. 5.

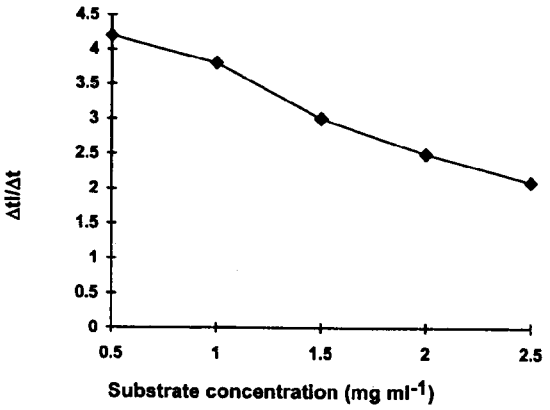


Fig. 5 Inhibition of RNase by heparin (5 μg ml^{-1}) at various concentrations of substrate.

Analytical Performance

Under the optimised conditions (pH 6.0, incubation time 2 min and substrate conc. 1.0 mg ml^{-1}) a calibration graph was constructed for heparin which was linear in the range 1.0 to 10 μg ml^{-1}. The limit of detection was 1.0 μg ml^{-1}. Fig. 6.

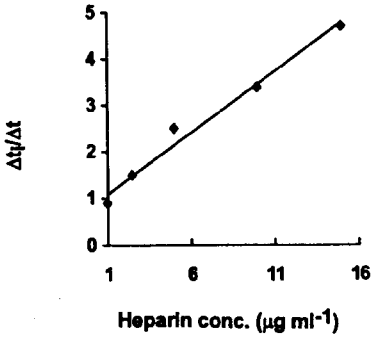


Fig.6 Calibration graph for heparin.

Effect of Reaction Conditions

The effects of different concentrations of substrate (RNA), pH, change in loop size of heparin solution and concentration of heparin were examined.

Effect of pH

The effect of pH from 4.5 to 7.0 on immobilised RNase inhibition was investigated by using 0.2 M NaAc buffer of various pH values. The peak height difference (measure of inhibition) increases with increase in pH .The biggest peak

height difference was obtained at pH 5.5. The results are shown in Fig. 7.

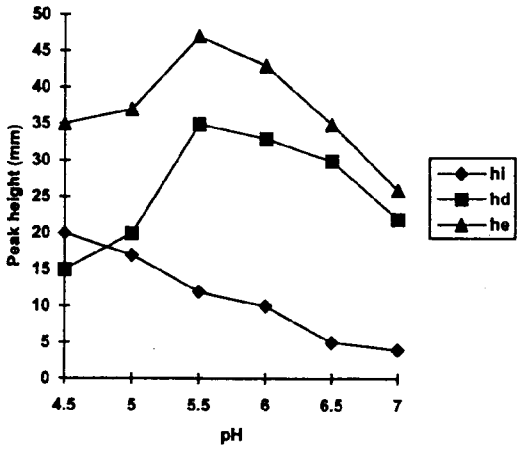


Fig.7 Effect of pH on immobilised ribonuclease inhibition (heparin 0.5 mg ml^{-1}). RNA conc. was 1.0 mg ml^{-1}. Where hi and he indicate peak height in the presence and in the absence of heparin and hd shows difference in peak height (hi-he).

Effect of Substrate Concentration

The effect of substrate concentration was studied over the range 0.5-2.0 mg ml^{-1}. The difference in peak height increases with decrease in substrate concentration from 1.0-2.0 mg ml^{-1};a 1.0 mg ml^{-1} solution of RNA is used in further work Fig. 8

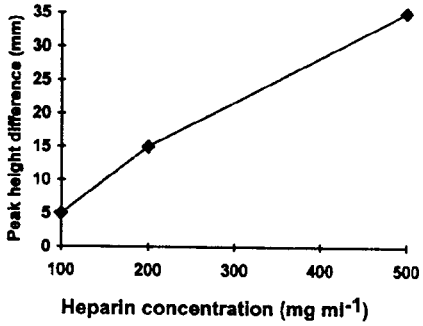


Fig.8 Effect of substrate concentration on RNase inhibition with 0.5 mg ml^{-1} heparin (pH 5.5).

Analytical Performance

Using the optimised conditions (pH 5.5, sample loop volume 130 µl, substrate conc. 1.0 mg ml^{-1} and volume 50 µl) a calibration graph for 0.1-0.5 mg ml^{-1} heparin is shown in Fig.9. This is not as sensitive as the conventional batch method, because after immobilising the enzyme, it is not inhibited by lower concentrations of heparin. It is interesting that in the batch method only 50 µg ml^{-1} enzyme is used and ≥ 10 µg ml^{-1} heparin is determined. As heparin is not a strong inhibitor, more heparin is needed to inhibit a significant proportion of immobilised enzyme, thus accounting for the poor sensitivity. Because of the lack of sensitivity, further work was not carried out. The system could no doubt be improved by immobilising a much smaller amount of enzyme and by packing less amount in the column, however this will make the detection of any enzymatic reaction much more difficult.

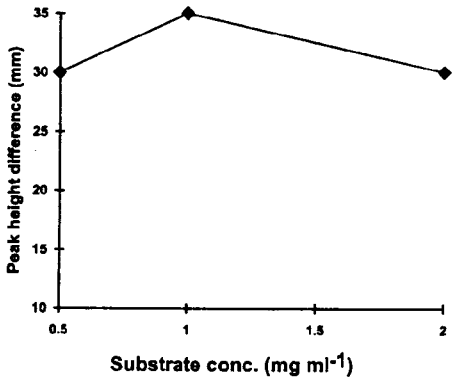


Fig.9 Effect of heparin concentration on RNase inhibition.

Experimental

(soluble enzyme)

In order to get a clear picture for the enzyme activity and inhibition reaction, Zollner batch method is repeated here with further optimisations.

Reagents

Ribonucleic acid. Type 111 from baker's yeast, ribonuclease A (E.C.3.1.27.5) from bovine pancreas (168 U mg^{-1}), and heparin ammonium salt (158.7 USP units mg^{-1}) from porcine intestinal mucous (all obtained from Sigma Chemical Co).

Solution Preparation

Ribonucleic acid (RNA), 0.2 percent solution, was prepared by mixing 0.2 g of RNA in sodium

acetate buffer (0.2 M, pH 6.0). Ribonuclease, 50 μg ml^{-1}, was prepared by dissolving 5.0 mg of RNase in 100 ml of distilled deionized water. A heparin standard solution was prepared by dissolving 0.5 mg of heparin in 50 ml of distilled deionized water. All solutions were kept in a refrigerator at 4°C.

Apparatus and Procedure

RNA absorbance was measured at 300 nm with a LKB Ultraspec 11 spectrophotometer. Two cuvettes (light path 10 nm) were used.

This method is based on the absorbance of RNA at 300 nm. When it is mixed with ribonuclease, it is hydrolysed into oligonucleotides, and the absorbance at 300 nm decreases. When heparin is mixed with RNase it competitively inhibits RNase so that enzyme activity is decreased toward its substrate and substrate absorbance is decreased less. The ratio of uninhibited and inhibited reaction is determined by measuring the time necessary for a particular decrease in absorbance in the presence and absence of inhibitor.

Basic Procedure

0.5 ml of RNase was mixed with 1.0 ml of NaAc buffer (0.2 M, pH 6.0) and 0.5 ml of RNA so that volume was 2.0 ml. The assay mixture was quickly transferred into a cuvette and the change in absorbance at 300 nm was noted with the passage of time, usually from 0-5 min (Δt). In a second step 0.5 ml of RNase, 1.0 ml of heparin and 0.5 ml of RNA were mixed together and again the change in absorbance with time (Δt_i) was noted. The inhibitory effect was measured by plotting the ratio $\Delta t_i/\Delta t$ against concentration of heparin.

(immobilised enzyme)

Once it was observed that the enzyme inhibition method for determination of heparin with soluble enzyme was sensitive it was decided to immobilise the enzyme and to use it for heparin determination.

Reagents

Ribonuclease, ribonucleic acid and heparin (ammonium salt), obtained from Sigma were as described for the soluble enzyme.

Controlled pore glass (CPG 240, 80-120 mesh, mean pore diameter 22.6 nm) was also obtained from Sigma.

Enzyme Immobilisation

Enzyme (40 mg) was immobilised on controlled pore glass (0.5 g) by the method described by Ghous [22], and packed in a glass column (5.0 mm long and 2.5 mm i.d.)

Apparatus and Procedure

The absorbance was measured at 300 nm with an LKB Ultraspec 11 spectrophotometer equipped with a flow cell (vol 30 μl, light path 10 mm) and connected to a Chessel chart recorder. The peristaltic pump was a Gilson Minipuls 2. Two Rheodyne RH-5020 rotary injection valves were used. The manifold tubing was 0.5 mm i.d. PTFE. The manifold used is shown in Fig.1 [20]. Two injection valves were connected in series. RNA (1.0 mg ml^{-1} solution) was loaded in a 50 μl sample loop and injected by the first injection valve into the carrier stream of sodium acetate buffer (0.2 M, pH 5.5). It was allowed to pass through the column and the absorbance at 300 nm was recorded. In the second step 1.0 mg ml^{-1} RNA solution was loaded in the 50 μl of sample loop of the first injection valve and injected into the carrier stream of NaAc buffer (0.2 M, pH 5.5) exactly 5 sec after heparin injection (0.5 mg ml^{-1}, 130 μl) from the second valve. This is passed through the column together at the same flow rate as before (1.0 ml min^{-1}). Again the absorbance was recorded. There was an increase in absorbance in the presence of heparin which indicated that enzyme is inhibited by heparin. The effect of inhibition was measured from the difference in peak height of the uninhibited and inhibited reactions.

Conclusions

The determination of heparin by using the soluble enzyme in batch method was found to be successful. But when using immobilised enzyme in a flow injection system RNase was not inhibited by low concentrations (0.5 μg ml^{-1}) heparin. High concentrations (0.1 mg ml^{-1}) of heparin were required to inhibit the immobilised RNase, which showed that immobilised enzyme incorporated in a flow injection system may not be used for heparin determination in routine studies.

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