Assay of Heparin in Plasma Using a Chromogenic Substrate and Its Clinical Applications

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SUMMARY

The concentration of heparin in plasma was measured using a chromogenic substrate. It appeared that the measurement of heparin concentration in plasma was important in therapeutic control and evaluation of heparin. There was a correlation between the activated partial thromboplastin time (APTT) and heparin concentration in plasma, but since the gradients of regression line differed in each of the cases, measurement of APTT alone would give a different estimate of heparin concentration in each case. Fibrinopeptide A (FPA) is considered as the best indicator for evaluation of therapeutic effects of heparin. Therapeutic heparin concentrations were defined as ranging from 0.2 to 1.2 IU/ml because the normalization of FPA was observed and there happened no hemorrhagic accidents in that range.

Additional Indexing Words:
Heparin   Chromogenic substrate   Activated partial thromboplastin time   Antithrombin III   Fibrinopeptide A   Disseminated intravascular coagulation

Thrombosis or disseminated intravascular coagulation (DIC) is induced by pathological activation of coagulation or coagulation-fibrinolysis in various diseases. Therefore, for the treatment of thrombosis or DIC, prevention of such pathological activation is very important as the treatment of the underlying diseases.

Heparin is frequently used in cases with thrombosis or DIC to prevent the consumptions of platelets and coagulation-fibrinolytic factors which are induced by abnormal fibrin formation. Hemorrhagic symptoms are often improved by the use of heparin in DIC cases, but there is also the risk that its use may induce new hemorrhage in turn. It is important, therefore, to determine and control the level of heparin during the treatment. Clinically, 2

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types of assay for heparin hitherto have been used. These include whole blood clotting time and activated partial thromboplastin time (APTT) (Godal, Teien and Abildgaard). Although both of these assays are relatively insensitive to heparin but reflect the overall influence of the anticoagulative activities on whole blood or plasma, the sensitivity of APTT differs by the order of magnitude from that of the whole blood clotting time, as pointed out by Godal. The point about APTT is that, unlike anti-Xa assay, it is considerably influenced by the levels of other clotting factors, especially in patients with thrombotic disease, and is therefore unreliable as a measure of heparin concentration. Direct determination of plasma heparin concentration was, therefore, done and effectiveness of various routine clinical tests as an indicator of heparin effect was evaluated in the present article.

Methods

1. Measurement of heparin in plasma

As shown in Fig. 1, standard curves were prepared using standard heparin dilution, antithrombin III (AT III), factor Xa (FXa), and a chromogenic substrate (S-2222; Bz-Ile-Glu(r-OR)-Gly-Arg-p Nitroaniline–HCl) (KABI Co). The heparin concentration was calculated from the absorbance for platelet poor plasma prepared from patients’ blood and the standard curves as described by Teien.

2. Measurement of fibrinopeptide A (FPA)

FPA was measured by the FPA radioimmunoassay kit (IMUCO Co) as de-
scribed by Nossel et al.2),3) (normal: <2 ng/ml)

3. Measurements of platelet factor 4 (PF₄) and β-thromboglobulin (β-TG)
PF₄ was measured by the PF₄ radioimmunoassay kit (ABBOTT Co) as described by Handin et al.4) (normal: 9.2±7.2 ng/ml, mean±S.D.) β-TG was assayed by the βTG Riapac (RADIOCHEMICAL CENTRE Co) as described by Ludlam et al.5) (normal: 26.9±16.8 ng/ml, mean±S.D.)

4. Coagulation-fibrinolytic tests
APTT was measured with celite and cephalin suspension (Warner-Lambert) (normal: 38–45 sec). Prothrombin time was determined with Lyoplatin reagent (Mochida) (normal: 10–13 sec). Fibrinogen concentration was determined by modified tyrosine method (normal: 150–350 mg/100 ml). Fibrin degradation product (FDP) was determined with FDPL test (Teikokuzoki) (normal: <5 μg/ml). AT III (normal: 28–32 mg/100 ml) and plasminogen (normal: 10–17 mg/100 ml) were determined with M-partigen (Behringwerke). Platelet count was determined by Brecher-Cronkite method (normal: 130,000–320,000/mm³).

RESULTS

1. Standard curves
Because the standard curves differed from one type of heparin to another,
the standard curves prepared with the same kind of heparin used actually for the treatment were used for the measurement of heparin concentration. When the p-nitroaniline (PNA) concentration is plotted on the ordinate and heparin concentration on the abscissa, the regression line of the standard curve for Japanese Pharmacopoeia heparin was \( Y = -0.540X + 0.890 \). The coefficient of correlation of the measured values was \( r = -0.9908 \), which was significant at \( p < 0.001 \) (Fig. 2). While the therapeutic range of heparin was defined as 0.1–0.7 IU/ml by Teien and Abildgaard with the polybrane titration method, the range of 0.2–1.2 IU/ml was considered, using a chromogenic substrate, to be effective and safe in our cases.

2. Investigation of clinical cases during heparin therapy

a) 37-year-old, male, acute respiratory distress syndrome (ARDS) with DIC (Case 1 in DIC group)

Fig. 3. Changes of concentration of heparin in a case of ARDS with DIC (Case 1 in DIC group).
The patient had marked dyspnea. Blood gas analysis showed $\text{PO}_2$ 36.8 mmHg, pH 7.448. Platelet count was 56,000/mm³, FDP 20 µg/ml, fibrinogen 113 mg/ml, AT III 15 mg/100 ml, FPA 3.6 ng/ml, and blood sedimentation rate 10 mm/hr. DIC was suspected as a complication of ARDS. The concentration of heparin in plasma was 0.194 IU/ml when 10,000 units of heparin were administered intravenously over 3 hrs. FPA (pretreatment: 3.6 ng/ml, 3 hrs: 1.7 ng/ml, 12 hrs: 0 ng/ml) and FDP (pretreatment: 20 µg/ml, 3 hrs: 20 µg/ml, 12 hrs: 5 µg/ml) improved to normal range 3 hrs or 12 hrs after the intravenous injection respectively. Concentrations of heparin in plasma at 3, 6 and 12 hrs after subsequent subcutaneous injection of 12,500 units were 0.222, 0.148 and 0.111 IU/ml respectively. Plasma heparin level increased at 3 hrs after each injection. AT III or fibrinogen also improved to normal range about 1 w after the heparin treatment (Fig. 3).

![Fig. 4. Changes of heparin concentration in a case of DIC after the surgical operation of cerebral teratoma (Case 5 in DIC group).](image-url)
b) 11-year-old, male, DIC following surgical operation of cerebral teratoma (Case 5 in DIC group)

From the second day after the surgery, there were remarkable oozing from the digestive tract and oliguria. The patient went into shock and blood transfusions were given. APTT was 110 sec, FDP 40 μg/ml, AT III 16.4 mg/100 mg, and blood sedimentation rate 7 mm/hr. Then 6,250 IU of heparin were administered subcutaneously and repeated gastric lavage with trans aminomethycyclohexane carboxylic acid (t-AMCHA) was carried out to no avail. There was no increase in the concentration of plasma heparin and the patient died (Fig. 4).

c) 70-year-old, male, lung cancer with renal failure (Case 5 in non-DIC group)

During radiotherapy, thrombosis in the right femoral artery and sub-
endocardial infarction were found as its complications. From 3 to 12 hrs after subcutaneous injection of 12,500 units of heparin, the concentration of heparin in plasma was maintained effectively (Fig. 5).

d) 38-year-old, male, acute myelocytic leukaemia (AML) with DIC (Case 3 in DIC group)

The patient had high fever, general fatigue, and lumbago. The white blood cell count in peripheral blood was 2,300 and as many as 6% of it was myeloblastic leukaemic cells, while 70% of nucleated cells in bone marrow. Although DCMP (Daunorubicin, Cytosine arabinoside, 6MP, presonisolone) treatment had been undergone, the patient did not get into remission but gradually developed many subcutaneous bleedings. APTT was 50 sec, FDP 80 μg/ml, FPA 11.4 ng/ml, platelet count 20,000/mm³, and blood sedimentation rate 13 mm/hr. DIC was suspected and heparin administration was

![Fig. 6. Changes of heparin concentration in a case of AML with DIC (Case 3 in DIC group).](image-url)
begun intravenously and subcutaneously. FPA improved immediately after the use of heparin and a decrease of FDP followed thereafter. Then, after a little while, fibrinogen and the other coagulation-fibrinolytic factors became normal and DIC was improved (Fig. 6).

e) 59-year-old, woman, right deep vein thrombosis (Case 4 in non-DIC group)

The patient was operated on for cholelithiasis on November 28, 1978 and was attacked by sudden right chest pain and cough on December 16, which were followed by spontaneous remission in a few days. Then she was attacked by the pain and swelling of the right lower extremity on January 17, 1979. On admission, it was demonstrated by chest X-ray and scintillation scans of the lung that S9 of the right lung had been suffered from infarction.

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Fig. 7. Changes of heparin concentration in a case of rt. deep vein thrombosis (Case 4 in non-DIC group).
Deep vein thrombosis of the right extremity was also demonstrated by veno-
angiography. Subcutaneous administration of 250 IU/Kg of heparin Na, and
intravenous administration of $24 \times 10^4$ Units of urokinase (UK) and 3,000
mg of dextran sulfate (DS) were started soon after the diagnosis. Although
heparin treatment had been continued and the concentrations of plasma hepa-

Fig. 8. Correlation between heparin concentration and APTT in cases
without DIC.
Case 1: male, lung cancer with femoral artery thrombosis
Case 2: male, lung cancer with anterior spinal artery thrombosis
Case 3: female, lung cancer with femoral artery thrombosis
Case 4: female, deep vein thrombosis
Case 5: male, lung cancer with renal failure
Case 6: male, rt. femoral artery thrombosis
rin were near the maximum level of the therapeutic range defined by Teien and Abildgaard, recurrent thrombotic episodes were seen and FPA was elevated at that time. After the third fibrinolytic therapy, FPA was stabilized in normal range and the patient got well, while the other coagulation-fibrinolytic factors remained abnormal for several days or weeks thereafter. During the treatment, slight subcutaneous bleedings were seen when the concentration of plasma heparin rose above 1.8 IU/ml (Fig. 7).

f) Correlation between heparin concentration and APTT in patients without DIC

When APTT and heparin concentration in these cases were plotted on

![Correlation between heparin concentration in plasma and APTT in cases with DIC.](image)

Case 1: male, ARDS with DIC
Case 2: male, gastric cancer with DIC
Case 3: male, AML with DIC
Case 4: male, APL with DIC
Case 5: male, DIC after surgical operation of cerebral teratoma
Case 6: male, APL with DIC
the ordinate and abscissa respectively, the coefficients of correlation were 0.915 \((p<0.01)\), 0.747 \((p<0.01)\), 0.838 \((p<0.01)\), 0.97 \((p<0.01)\), and 0.918 \((p<0.01)\) respectively in 5 patients, all of which but one (Case 5) were significant. However, the gradients differed among the patients (Fig. 8).

g) Correlation between heparin concentration and APTT in patients with DIC

The coefficients of correlation in 6 cases, in which the administration of heparin was effective except in 1 case (Case 5), were 0.947 \((p<0.01)\), 0.902 \((p<0.01)\), 0.958 \((p<0.01)\), 0.693 \((p<0.1)\), 0.809 \((p<0.05)\), and 0.861 \((p<0.01)\) respectively, all of which but 1 were significant (Fig. 9).

h) Changes of FPA after the administration of heparin

The levels of plasma FPA of 6 cases in which heparin was considered to be effective were 17.5±5.3 ng/ml at the beginning of heparin treatment, 2.9±0.9 ng/ml at 3 hrs, 1.4±0.5 ng/ml at 6 hrs and 3.9±2.5 ng/ml at 12 hrs after the injection of heparin respectively. On the contrary, the levels of plasma FPA of 6 cases in which heparin was considered to be ineffective were 15.4±4.5 ng/ml at the beginning of heparin treatment, 11.9±4.5 ng/ml at 3 hrs, 15.5±4.1 ng/ml at 6 hrs and 14.3±5.2 ng/ml at 12 hrs after the treatment (Fig. 10).

i) Changes of the other coagulation-fibrinolytic factors after the administration of heparin

![Fig. 10. Changes of FPA after the administration of heparin.](image-url)

the solid line: changes of FPA in effective cases of heparin
the dotted line: changes of FPA in ineffective cases of heparin
the shaded area: the normal range of FPA
When the changes in FPA and in FDP following heparin injection were compared there was a definite difference in the sensitivity as the indicator for the appearance of thrombotic episodes or the evaluation of therapeutic effects of heparin. When FDP was to be normalized, it took longer time than normalization of FPA, left more than a few hours or even days behind the latter (e.g. Cases 1 and 3 in DIC group). Normalization of fibrinogen, AT III, platelet count and other factors tended to delay further.

**DISCUSSION**

The concentration of heparin in plasma of patients during heparin therapy was measured with a chromogenic substrate-Bz-Ile-Glu-Arg-pNA (S-2222) as described by Teien et al. This method is highly specific, sensitive, and can be used to obtain reliable heparin level measurements in plasma. The standard curves tended to differ slightly according to the type of heparin. Therefore, the concentration of heparin in patient's plasma should be calculated with the standard curve prepared with the same kind of heparin used. The therapeutic range of heparin had been defined as 0.1–0.7 IU/ml by Teien and Abildgaard with the polybrane titration method from the point of view that APTT should be set between 40–120 sec in heparin therapy. But it was demonstrated in our cases that the range of 0.2–1.2 IU/ml of plasma heparin should be maintained in heparin therapy because normalization of FPA, which meant the complete inhibition of fibrin formation and the final purpose of heparin administration, was seen in the range of 0.73±0.51 IU/ml (mean±S.D.) of heparin concentration in 13 times in 7 cases. In this heparin concentration, FPA improved from 9.4±4.6 ng/ml (mean±S.D.) to 1.4±0.5 ng/ml (mean±S.D.) and APTT prolonged to 146±102 sec (mean±S.D.) and there happend no side effect such as bleeding. So, we are convinced that 0.2–1.2 IU/ml of plasma heparin concentration is the effective and safe therapeutic range except in cases wounded or during bleeding.

From the results in our patients, it is evident that it is possible to maintain the concentration of heparin constantly in a therapeutic range by subcutaneous injection of 250 IU/Kg of heparin Na every 12 hrs. However, when these injections were continued, a gradual increase in the concentration of plasma heparin was observed and the degree of increase following each injection also rose gradually in all patients. These results suggest that there must be a risk of heparin excess unless the concentration in plasma is measured frequently during treatment.

In Case 5 of DIC group, there was no increase in heparin concentration in plasma after subcutaneous injection. This happened to occur probably
because the patient was in shock, and there were disturbance of peripheral circulation and continuous oozing. Therefore, it is suggested that an intravenous drip of heparin is desirable in patients with shock.

APTT has been widely used as a guide for control of heparin level. There was a correlation between concentration of heparin in plasma and APTT, and such correlation was recognized also in DIC cases, in which heparin was effective. However, when the regression lines were compared among patients, the gradients were not always similar but differed among them. Therefore, even when APTT was at the same level, the heparin concentration was not always the same. Then, regression line of APTT-heparin correlation should be prepared by measuring APTT in the mixture solution of heparin and preheparin plasma from each patient, when APTT is used as the control index. The concentration of heparin in plasma should be estimated from the regression line and APTT of patient after heparin administration. There is a major possibility that the heparin control method only using APTT may lead to critical hemorrhages and sufficient therapeutic results may not be obtained.

In connection with AT III, a heparin co-factor, it has been pointed out that AT III level may decrease markedly during thrombotic episodes. Either heparin or AT III may be rate limiting in the inhibition of thrombin, and more heparin is required for the same antithrombotic effect when AT III level in plasma is depressed. Among our patients, including those with DIC, the lowest value of AT III was 15 mg/100 ml. This patient was an ARDS case, in which heparin was effective. Further investigations are planned for cases with low AT III levels.

The results in 12 cases suggest that FPA is more sensitive indicator of thrombin action than FDP, Fibrinogen, AT III, platelet counts, and the other factors and provides direct information concerning the effectiveness of heparin treatment in preventing fibrin formation.

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REFERENCES