Heparin

Chromogenix Monograph Series

CHROMOGENIX

Heparin

Contents

Monitoring Heparin Therapy – A Role For The Chromogenic Anti-Factor Xa Assay

Heparin preparations extracted from animals have been used clinically for over half a century as a potent anticoagulant therapeutic for the treatment and prevention of thrombotic disease. Bleeding and heparin-induced thrombocytopenia are the main adverse reactions associated with heparin therapy. These risks can be minimized by appropriate patient management and by laboratory monitoring using specific chromogenic anti-factor Xa assays. The clinical indications for these assays are reviewed together with a basic introduction of the clinical pharmacology of heparin.

Heparin is a naturally occurring, highly sulfated polysaccharide, characterized by a wide molecular weight range and powerful anticoagulant properties. Since its discovery by McLean in 1916, heparin has become a widely used anticoagulant for the treatment and prevention of thrombotic diseases and for maintaining blood fluidity in extracorporeal devices.[1-4] Material for clinical use is derived from porcine and bovine tissue and is prepared either as unfractionated (UF) heparin or as depolymerized low molecular weight (LMW) heparin.

The main complication with heparin therapy is that it occasionally causes life-threatening bleeding.[5] Laboratory monitoring with adjustments of dose-regimens is one of the options available which may improve the antithrombotic efficacy of heparin and reduce the risk of haemorrhage.[6] However, the ideal heparin test and its clinical relevance is still a controversial topic.[7-9]

UF heparin activity is usually estimated by a conventional clotting test such as the activated partial thromboplastin time (APTT), the thrombin clotting time (TCT), or the activated clotting time (ACT). These assays are non-specific and are termed 'global assays' as they reflect the ability of heparin to interfere with several steps in the coagulation cascade. APTT is today the most widely-used test for heparin, mainly because it is relatively simple and allows for automation. However, when a test such as the APTT is prolonged beyond the normal range or does not reflect the anticipated effect of heparin, a more specific assay is required.[10]

Observed Reaction	Incidence
Hemorrhage	1-33%
Thrombocytopenia	1-3%
Hypertransaminasemia	up to 93%
Osteoporosis (long term use)	n.d
Allergic reactions	n.d
Cutaneous reactions	n.d
Drug interactions	n.d

***Table 1**. Adverse effects associated with heparin therapy.[1-3]*
n.d = no data

The specific assays measure the effect of heparin-accelerated antithrombin on a single coagulation enzyme, either factor Xa or thrombin (factor IIa). The enzyme is determined by its clotting activity (chronometrically) or by its activity against chromogenic peptide substrates (amidolytically). A major advantage of the specific assays is that they isolate the biological activity of heparin to one reaction, thus minimizing the interference from other variables. The anti-factor Xa assay is the only practical way of measuring LMW heparins.[9,11-14]

This monograph presents four chromogenic anti-factor Xa kits for the determination of UF heparin/LMW heparin in human plasma, suitable for the routine clinical laboratory. For more detailed information, please contact your local Chromogenix distributor.

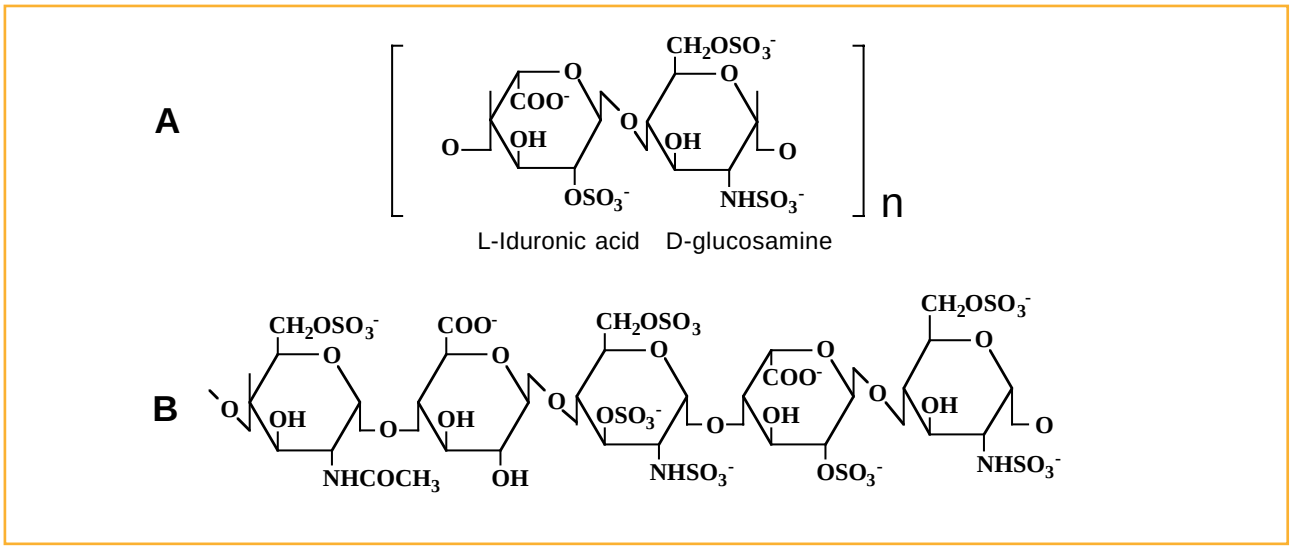


Figure 1. Heparin structure.
Heparin is a heterogenous mixture of polysaccharides, which chains are made up of alternating 1 to 4 linked, sulfated monosaccharide residues of L-iduronic acid and D-glucosamine. ***[A]*** *is the most frequent type of disaccharide unit, representing up to 90% of the structure of beef-lung heparin, and up to 70% of pig-mucosa heparin.* ***[B]*** *is the unique pentasaccharide binding site for antithrombin which occurs in about one-third of the heparin chains.*

Biochemistry of heparin

Structure and biological role

Heparin is a sulfated glycosaminoglycan (GAG) mixture, which consists of unbranched polysaccharide chains, composed of 15 to 100 alternating monosaccharide units of L-iduronic acid and D-glucosamine (Figure 1A). It has been found in mast cells in a large number of mammalian and non-mammalian vertebrates and is located mainly in tissues/organs that are in direct contact with the environment (i.e. lung, skin and intestine).[15]

The extravascular location of heparin and the failure to detect it in blood have suggested that heparin does not normally have a role in regulating blood coagulation. However, heparan sulfate, a heparin-related GAG located on endothelial cells which line the blood vessel wall, have been shown to have anticoagulant activity. This fact could explain the ability of heparin to interfere with blood coagulation.[16,17]

Anticoagulant activity

The basis for heparin's (and heparan sulfate's) anticoagulant activity in plasma is that it binds to antithrombin, the major inhibitor of the coagulation cascade in plasma (Figure 2). Binding induces a conformational change in the antithrombin molecule, which greatly accelerates the antithrombin inhibition of several serine proteases, including factors IXa, Xa, XIa, XIIa, kallikrein and thrombin.[16,17] The result is a stable 1:1 protease-inhibitor complex, which is rapidly removed from the circulation and catabolized.

The heparin-accelerated inhibition of thrombin and factor Xa constitutes the major portion of heparin's anticoagulant effect *in vitro*, and possibly also the antithrombotic effect *in vivo*.

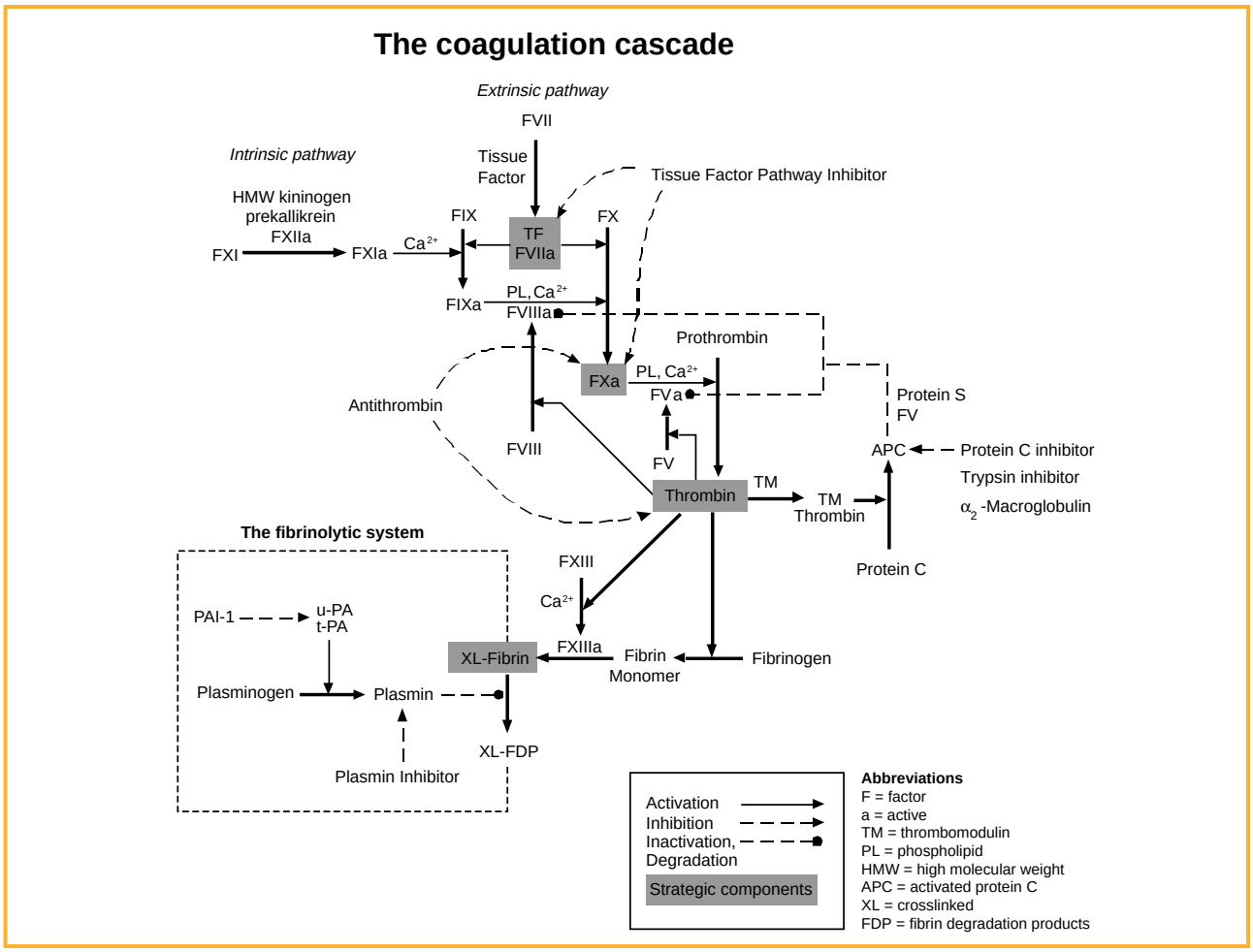


Figure 2. The coagulation cascade.
Blood coagulation is an enzymatic event initiated in response to tissue damage. Binding of circulating factor VII to exposed tissue-factor starts a cascade of reactions that ultimately leads to the formation of thrombin, which clots blood. The anticoagulant effect of heparin is mediated primarily by its binding to antithrombin, thereby accelerating the latter's inhibitory function of factor Xa and thrombin in plasma. Notes: The positive feedback reactions of factor Xa and the possible thrombin activation of factor XI are not shown. It has been suggested that factor V function in synergy with protein S as a second APC cofactor. Coagulation factors are represented by Roman numerals (a = activated). Abbreviations: HMWK= high molecular weight kininogen, PK= prekallikrein, K= kallikrein, TF= tissue factor, TFPI= tissue factor pathway inhibitor, PF3= platelet phospholipid, TM= thrombomodulin, PC= protein C, APC= activated protein C, PCI= protein C inhibitor, TI= trypsin inhibitor, α_2-M= α_2-macroglobulin.

Factor	Name	Size [kDa]	Concentration [µg/ml]	Factor	Name	Size [kDa]	Concentration [µg/ml]
I	Fibrinogen	330	3000	X	Stuart-Prower factor	59	8
II	Prothrombin	72	150	XI	Thromboplastin antecedent	160	5
III	Tissue factor	47	-	XII	Hageman factor	80	30
IV	Calcium	-	-	XIII	Fibrin-stabilizing factor	320	10
V	Proaccelerin	330	20	-	Protein C	62	4
VI	-	-	-	-	Protein S	70	10 (free)
VII	Proconvertin	50	0.5	-	Antithrombin	58	145
VIII	Antihemophilic factor	330	0.1				
IX	Christmas factor	56	5				

Table 2. Plasma coagulation factors and regulatory proteins.

Catalytic mechanism

Heparin accelerates the inactivation of thrombin, and presumably also factors IXa and XIa, by serving as a template to which both antithrombin and the protease bind to form a ternary complex (Figure 3).[17,18]

The accelerating function of heparin depends on the presence of a unique antithrombin-binding pentasaccharide sequence in a heparin GAG chain (Figure 1B). Binding to this pentasaccharide induces a conformational change in the antithrombin molecule, which facilitates the reaction with the target protease. For more information about the biochemical mechanism see reference.[19,20]

Thrombin initially binds to the antithrombin-heparin complex in a non-specific fashion to any site along the GAG chain, then it slides along the surface until it encounters the inhibitor. It has been found that this sliding mechanism for thrombin requires a GAG chain of at least 18 monosaccharide units (Mw > 5,400 Da). Surprisingly, the sliding mechanism is not required for the inhibition of factors Xa, XIIa or kallikrein. Instead, the inhibition works predominantly through the conformational change of antithrombin bound to heparin.

The accelerated inactivation of these enzymes can therefore be achieved by GAG chains as small as the unique pentasaccharide sequence (Mw = 1,756 Da).

Another important feature of heparin is that its affinity for the antithrombin-protease complex is much lower than that of unreacted antithrombin. Heparin will therefore dissociate from the complex once its job is done, being free to catalyze further antithrombin reactions.

Molecular weight aspects

Two forms of heparin are used clinically: unfractionated (UF) heparin with an average molecular weight of 15,000 (range 15 to 100 monosaccaharides) and low molecular weight (LMW) heparin with molecular weights between 4,000 to 6,500 (range 4 to 40 monosaccaharides). The reduction in molecular weight causes a marked change in the heparin activity. LMW heparin acts primarily on FXa, whereas UF heparin is an efficient catalyst for inhibition of both thrombin and factor Xa.

Unfractionated heparin

Two distinct fractions can be obtained from UF heparin by using affinity chromatography with immobilized antithrombin. The fraction that accounts for roughly 30% of the starting material, and nearly all the anticoagulant activity, is known as high-affinity heparin. The other fraction, which represents the majority of heparin chains, is the low-affinity heparin, with virtually no anticoagulant activity. The different anticoagulant activities of these two fractions are the result of the unique antithrombin-binding pentasaccharide sequence, which is absent in low-affinity heparin chains.

The anticoagulant activity or potency of UF heparin is expressed relative to the 4th international standard.[21] UF heparin preparations have specific activities of 150-190 IU/mg.

LMW heparin

The term low molecular weight (LMW) heparin refers to a heparin preparation obtained by fractionation of natural low-molecular weight material in UF heparin or by depolymerization of UF heparin.

Reduction in chain length of heparin reduces its affinity to plasma proteins, vascular matrix proteins, endothelial cells, marcrophages and platelets. As a result, LMW heparins have greater bioavailability, a longer plasma half-life, a more predictable therapeutic response to fixed doses and reduced platelet-associated side-effects.[22]

A characteristic feature of LMW heparins is that they have less ability to enhance thrombin inibition than to potentiate factor Xa inhibition compared to UF heparin. The difference may be described in terms of an activity ratio such as the anti-factor Xa/anti-factor IIa ratio. For UF heparins the ratio is 1:1 whilst for LMW Heparin the ratio is 1:2-1:4.

The anticoagulant activity or potency of LMW heparin is expressed relative to the 1st international standard for LMW heparin.[23] The specific activities among LMW Heparins varies between 80-120 anti-Xa U/mg and between 35-45 anti-IIa U/mg.[24]

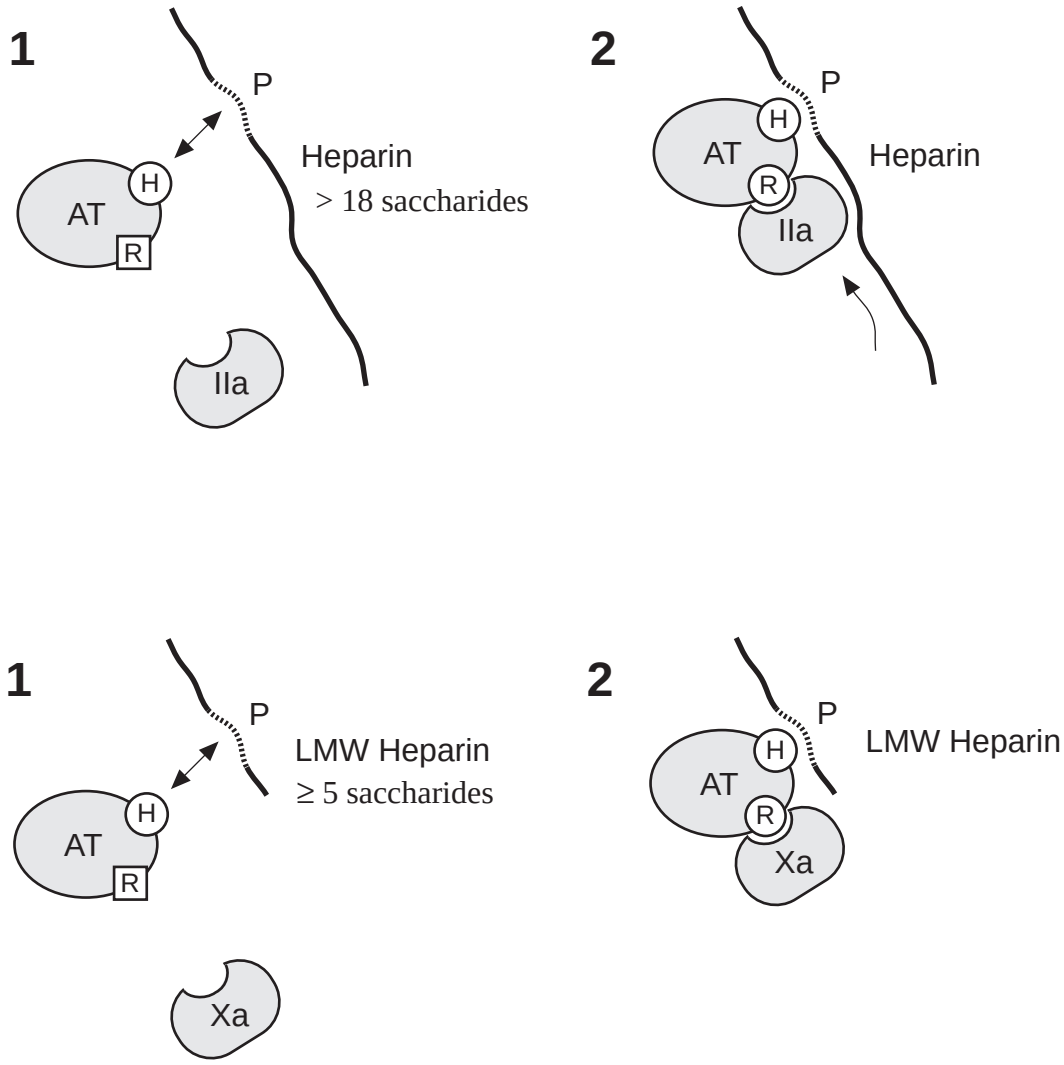


Figure 3. Model describing how heparin catalyzes the antithrombin-protease reaction
[H] *symbolizes the heparin binding site and* ***[R]*** *is the reactive site in antithrombin, normally in an unfavourable conformation for protease inhibition.* ***[P]*** *is the unique antithrombin binding sequence of heparin. Binding to this sequence induces a conformational change in antithrombin ,which facilitates its reaction with its target proteases.* ***Top:*** *The effect of heparin on the reaction between antithrombin and thrombin (IIa) involves binding both the enzyme and the inhibitor to the heparin chain, which thus needs to be of a certain length (≥18 monosaccharides). Thrombin binds to heparin in a non-specific manner (through positive surface charges) and 'slides' along the chain until it encounters the bound antithrombin.* ***Bottom:*** *Inactivation of factor Xa does not require a ternary complex formation (i.e. the sliding mechanism is not required) and is achieved solely through heparin binding to antithrombin. Heparin's affinity to the antithrombin-protease complex is much lower than that of free antithrombin and therefore heparin dissociates and binds to unreacted antithrombin, thus being able to catalyze further antithrombin reactions.*

Clinical use of heparin

Established uses for heparin include the treatment and prevention of various thrombotic disorders. Heparins are also used as an anticoagulant in extra-corporeal circulations or in dialysis devices (Table 3).

Therapeutic objective

The basic aim of using heparin preparations clinically is to reduce, delay or prevent the presence of thrombin. When heparin is used for prophylaxis (low-dose regimens), thrombin generation is mainly prevented. In the case of acute thrombosis, heparin is used for neutralizing thrombin that has already been formed, and for preventing further thrombin generation (high-dose regimens).

Venous thromboembolism

- Prophylaxis of DVT and pulmonary embolism.
  - 5,000 U/12 hour SC, or adjusted low-dose regimens
  - For some LMW Heparins the regimen is recomended on the weight basis instead of activity.
- Treatment of DVT (D.a)
  - IV bolus 5,000 U, followed by 24,000 U/24 hour IV
  - or 35,000 U/24 hour SC

Coronary heart disease

- Unstable angina (D.a)
  - IV bolus 5,000 U followed by 24,000 U/24 hour IV
- Acute myocardial infarction
  - - Prevention of mural thrombosis
  - 12,500 U SC/12 hour
  - - Prevention of reinfarction
  - IV bolus 2,000 U followed by 12,500 U/12 hour SC
- After thrombolytic therapy with t-PA (D.a)
  - IV bolus 5,000 U followed by 24,000 U/24 hour

Artificial thrombogenic surfaces

- Haemodialysis, surgical bypass, heart-lung machines (D.a)
  - Special dose regimens

Table 3. *Clinical use of heparin and corresponding dose regimens according to the European Consensus Statement on the Prevention of Venous Thromboembolism and ref 2. IV= intravenous route, SC= subcutaneous route, (D.a)= dose adjustment (monitoring).*

Mode of administration

Administration of heparin is performed by intravenous or subcutaneous routes, as intermittent injections or continuous infusion. The effect is immediate when given intravenously, whereas the action of subcutaneous heparin occurs within 20 to 60 minutes. The systemic absorption of heparin by oral or nasal administration is neglible.

Pharmacokinetics

The elimination rate of heparin from the blood is dose dependent. With low doses the clearance of UF heparin appears to rely on a saturable mechanism caused mainly by endothelial cell-uptake. At high doses a non-saturable mechanism predominates due to renal filtration.[25]

LMW heparins are cleared mainly by renal filtration, probably due to its lower affinity to endothelial cells.[26] As a result, LMW heparins have a two to four times longer half-life as compared to UF heparin at therapeutic doses, and ~90% bioavailability following subcutaneous injection as compared to only ~30% for UF heparins. This enables LMW heparins to be administered as a single daily injection.

Treatment of venous thromboembolism

Heparin has a long clinical history as the principle therapeutics in acute therapy for both deep vein thrombosis (DVT) and pulmonary embolism. Several studies have confirmed heparin's role in the treatment of thrombosis.[27-29]

After a 5 or 10-day course of heparin, treatment with the anticoagulant warfarin is usually started and then continued for several months.

Heparin resistance

Patients with thromboembolism that require more than 35,000 U/24 h to achieve the therapeutic range are classified as 'heparin resistant'. There are a number of possible causes, such as increased heparin clearance, increased levels of procoagulants, reduced antithrombin levels, and increased levels of heparin binding proteins (e.g. platelet factor 4 and histidine rich glycoprotein).[30]

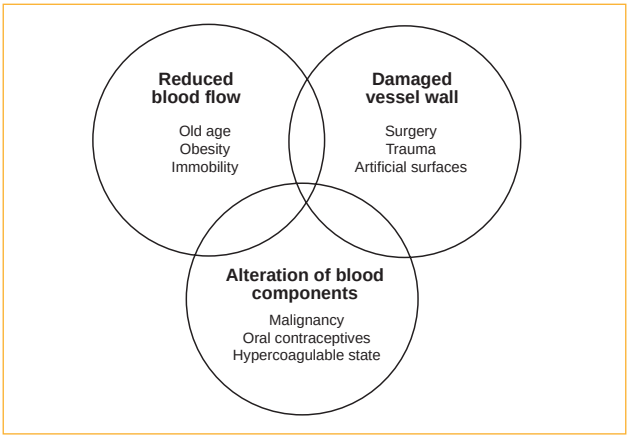


Figure 4. *The "Virchow Triad".*

Prophylaxis of DVT

A reduced blood flow, the alteration of blood components and abnormalities of blood vessels are predisposing risk factors believed to result in thrombosis (Figure 4).[3]

High-risk groups for the development of DVT include patients with acute myocardial infarction and patients who have undergone major surgery such as abdominal and orthopedic operations. In general surgical procedures the rate of DVT formation may be as high as 28% and even up to 50% after open prostatectomy or hip fracture.[3]

Numerous clinical trials have demonstrated the efficacy of heparin therapy in reducing the incidence of pulmonary embolism and DVT as well as the long term complications after major surgery.[31,32] The effectiveness of several commercial LMW heparins have also been investigated, most of which suggest a relative superiority (i.e. increased convenience) when compared to UF heparin. Although they are currently more expensive, they have proven to be cost effective for prophylaxis of DVT.[33-36]

Extracorporeal circulation

Exposure of blood to large artificial surfaces (e.g. haemodialysis, cardiopulmonary bypass) activates coagulation. This may lead to thrombus formation and impaired function or occlusion of medical devices. The normal procedure is to control coagulation by administering UF heparin, although recent developments, which may promise fewer bleeding problems, include the use of LMW heparins or the concept of using heparin-coated membranes.[37,38]

Complications

Haemorrhage

Haemorrhage is the main complication associated with heparin therapy, particularly when full dose heparin is injected intravenously.[5] Major bleeding has been reported to occur in 1 to 33% of patients receiving various forms of heparin therapy.[39] The risk is greater in the elderly, in patients with hypertension after trauma or surgery, and in patients with additional haemostatic abnormalities.

In summary, there are four variables reported to influence the risk of bleeding: the dose, the patient's anticoagulant response, the mode of administration and specific patient-related characteristics.

Thrombocytopenia

Heparin-induced thrombocytopenia is another adverse effect with a reported incidence of 1-3%.[1] The effect is usually moderate and is reversible once heparin administration is discontinued.[40]

Occasionally a more severe heparin-induced thrombocytopenia (platelet count less than 50,000/µl) may occur, causing acute arterial thrombosis ('white clot syndrome').[41]

Other toxicities and drug interactions

Following long-term heparin therapy, the development of osteoporosis (bone loss) can occur, with vertebral fractures as the predominant clinical sign. Most cases reported are in connection with pregnancy.[42] Hypertransaminasemia has been observed in as many as 93% of subjects receiving heparin.[43] Cases of skin necrosis have been observed with both UF heparin and LMW heparin.[44] The precise cause of these heparin-induced reactions is at present unclear.

Heparin has been shown to cause a prehaemorrhagic tendency in patients undergoing aspirin (salicylates) therapy.[45] Of particular note is the fact that heparin may be inhibited by concurrent intravenous nitroglycerin infusion during the treatment of patients with unstable angina or in the acute post-myocardial infarction period.[46]

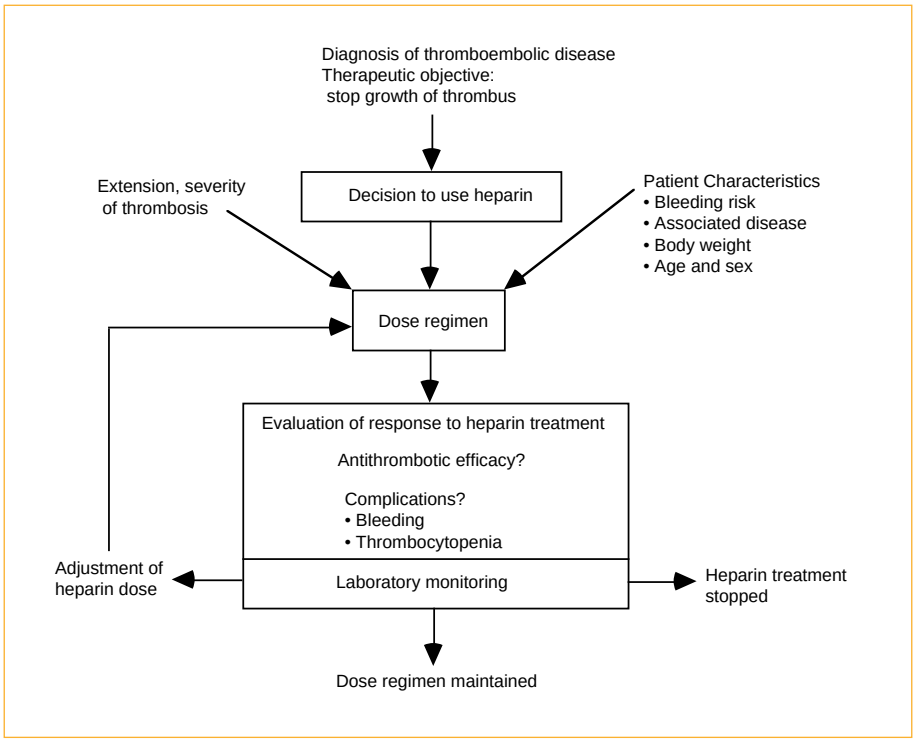


Figure 5. *The role of laboratory monitoring in heparin treatment according to Abildgaard.*[25] *Individual variation in response to heparin is the main reason for monitoring heparin therapy.*

Laboratory monitoring

The clinical relevance

The purpose of monitoring heparin therapy is primarily to minimize the risk of haemorrhage from overdosage and to optimize the antithrombotic effect by appropriate dose adjustments. However, relatively few studies have been performed that clearly evaluate the usefulness of laboratory monitoring.[6,39,47] The main reason is the complex pharmacokinetics of heparin and the relatively weak correlation between the antithrombotic effect (in vivo) and anticoagulant activity (in vitro). Nevertheless, it is generally accepted that high-dose intravenous therapy with UF heparin should be monitored because of the danger of haemorrhage.[4] For LMW heparins there is currently no definitive recommendation in favour of monitoring although, considering the comparable haemorrhagic risk of LMW heparins to UF heparins, as well as the risk of undertreatment, it may be useful to test the anti-Xa activity at least once at the beginning of treatment.[9,48]

Low doses of UF heparin or LMW heparin, given subcutaneously for prophylaxis of DVT seldom require monitoring, although it may be useful to document the anti-Xa level in the case of unexpected haemorrhagic episodes.[4,9]

Individual response to heparin

The risk of bleeding depends not only on the dose but also on the individual response. A large number of variables influence the antithrombotic and anticoagulant effect of heparin, including sex, age, weight, drug interactions, associated disease, extent of fibrin, vascular surfaces and the levels of various heparin-binding proteins. Elevated levels of heparin-binding proteins may contribute to heparin resistance in patients with inflammatory and malignant disorders.[2]

In the case of LMW heparin, which is cleared mainly through the kidneys, it has been reported that renal insufficiency lowers the clearance rate and may result in a dangerous accumulation.[49] Thus, it may be advisable to check the anti-Xa activity at the beginning of therapy in such patients irrespective of the severity of the impaired renal function.

The large variation in response to heparin calls for an individualization of the heparin dose regimen, according to the characteristics of the thrombosis and the patient (Figure 5).

Heparin assays

The determination of the anticoagulant activity of UFH and LMW heparin is performed using a wide variety of assay methods (Table 4). The most frequently used tests are the activated partial thromboplastin time (APTT) and the specific anti-factor Xa assays, using either a clotting or a chromogenic substrate method. When LMW heparin is being investigated, anti-factor Xa is recommended since APTT values are only minimally prolonged.

APTT

Activated partial thromboplastin time (APTT) is a conventional screening test that measures the prolonged clotting time of recalcified citrate-anticoagulated plasma in the presence of heparin, by using a phospholipid reagent and a surface activator, such as kaolin.[50] APTT is the most popular clinical test for heparin, mainly because it is considered to be a simple method that allows for automation. However, the therapeutic range measured as an APTT ratio differs between varius different commercial thromboplastin reagents. Therefore it is recommended to calibrate the therapeutic ratio for each APTT reagent, to be equivalent to a heparin level of 0.2-0.4 U/ml by protamine titration or to 0.3-0.7 U/ml by anti-FXa measurement.[1,51]

Since the APTT is a global test, it measures the overall coagulability of a blood sample and not the specific presence of heparin alone. Therefore this test has important limitations that must be taken into consideration (Table 5).[10]

- Combined warfarin/heparin therapy
- Combined thrombolytic/heparin therapy
- Lupus anticoagulant
- Elevated fibrinogen
- Increased factor VIII
- Antithrombin deficiency
- Elevated platelet factor 4
- Accidental heparin administration
- Low molecular weight heparin

Table 5. *Selected situations in which APTT monitoring may cause erroneous heparin dosage, indicating the need for more specific assays.*[10]

Whole blood clotting time (WBCT)
The first heparin test. Based on the time for whole blood to clot in a glass tube (Howell 1924). Today WBCT is used primarily for monitoring the heparinisation degree of blood in extracorporeal circulations.

Activated clotting time (ACT)
An attempt to adapt the WBCT to a more mechanized system.

Activated partial thromboplastin time (APTT)
Measures the clotting time of citrated plasma incubated with phospholipid and kaolin after recalcification. APTT is a global test, i.e it is based on the time for clot formation and thus claims to reflect the overall function of the coagulation system.

Pharmacopoeia methods
Standardized methodologies have been adapted by European (EP), British (BP) and United States Pharmacopoeias (USP) based on APTT on citrated sheep plasma as well as chromogenic Factor Xa and Thrombin based assays.

Anti-factorXa
Measures the ability of heparin to inhibit a single factor in the coagulation cascade. Two versions of the assay are used, one with the residual enzyme activity measured by a clotting assay and the other in which enzyme activity is measured by a factor Xa chromogenic substrate.

Thrombin inhibition, amidolytic
Same as the chromogenic anti-factor Xa assay except that residual thrombin activity is measured.

Thrombin clotting time (TCT)
One of the first heparin assays and still in use in many clinical laboratories. The TCT is performed by measuring the clotting time following the addition of excess thrombin to undiluted plasma.

Polybrene or protamine titration
These compounds neutralize heparin stoichiometrically. Heparin can be accurately measured by determining thrombin times using various concentrations of the neutralizer.

Table 4. *Laboratory tests relevant to heparin therapy*

Combined heparin and warfarin therapy

A frequent clinical situation is the cross-over to oral warfarin therapy from intravenous heparin therapy. It takes about three days for warfarin to reach therapeutic effect and the drug is usually administered concurrently with heparin during this period. Since warfarin prolongs APTT it may prompt the clinician to decrease heparin administration.[52]

Combined heparin and thrombolytic therapy

Heparin is commonly included in the thrombolytic treatment of myocardial infarction. Because the APTT is prolonged during thrombolytic thearpy with t-PA, it is not a clear indicator of heparin anticoagulation.[53]

Altered coagulation proteins

If the APTT is not prolonged as expected in patients receiving intravenous heparin, this may be a result of altered levels of coagulation proteins. There are four main situations in which this may occur: increased fibrinogen, increased factor VIII, increased platelet factor 4 and decreased antithrombin.[10,54]

Lupus anticoagulant

The lupus anticoagulants have been shown to react with anionic phospholipids and may therefore cause prolonged APTT.[55] As a result, the usual therapeutic range for heparin is no longer valid.

Anti-factor Xa assays

Unlike the APTT, the anti-factor Xa assays are more specific since they measure the ability of heparin-accelerated antithrombin to inhibit a single enzyme. Either plasma or purified antithrombin can be used, and residual enzyme can be measured by its clotting activity or amidolytically by a chromogenic peptide substrate.[21]

Clotting method

The clotting assay introduced by Yin et al in 1973 is based on the heparin-accelerated inhibition of factor Xa.[56] During the initial phase of the reaction, the amount of neutralized factor Xa is proportional to the heparin concentration if antithrombin is present in excess. Residual factor Xa is then measured using a clotting technique.

Several kits utilizing the above clotting methodology have been introduced, including Heptest® or Heparimat®. However, these assays appear to be highly unsuitable for determining plasma anti-factor Xa activity generated by LMW heparins due to their sensitivity to the residual anti-factor IIa activity of LMW heparins.[13]

Chromogenic method

In 1976 Teien and co-workers introduced a photometric version of the anti-factor Xa clotting assay.[57] This was later modified by adding purified antithrombin to the test sample, thereby reducing the influence of varying antithrombin concentrations.[58]

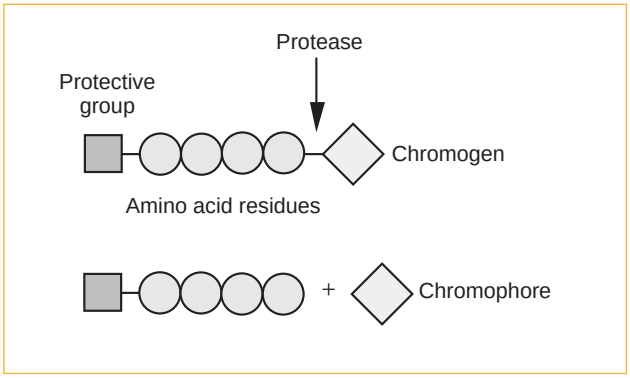


Figure 6. *Principle structure of synthetic peptide substrates.*

S-2222™
Bz-Ile-Glu-(γ-OR´)-Gly-Arg-pNA

S-2765™
Z-D-Arg-Gly-Arg-pNA

S-2772™
Ac-D-Arg-Gly-Arg-pNA

S-2732™
Suc-Ile-Glu-(γ-Piperidyl)-Gly-Arg-pNA

Figure 7. *Chromogenic substrates for factor Xa. Abbreviations: Bz; benzoyl, Z; benzyloxycarbonyl, R'= H (50%) and R'= CH_3 (50%), pNA; 4-nitroaniline.*

Their method was in principle the same as described above, except that residual factor Xa is measured by using a synthetic factor Xa chromogenic substrate. In addition to this two-stage assay also chromogenic one-stage assays have been introduced.
The chromogenic methods enables more precise determination of both UF heparins and LMW heparins, especially given that the methods have also been successfully automated.[58]

Peptide substrates used in the chromogenic anti-Xa assay are generally composed of 3-4 amino acids, with the chromogenic group para-nitroaniline (pNA) attached to the end (Figure 6–7). When the synthetic substrate is incubated with factor Xa it is cleaved and a chromophore (yellow colour) is liberated. This is measured at 405 nm, either during the reaction (kinetic method), or after stopping the reaction with acetic or citric acid (end-point method). The resulting photometric signal is inversely proportional to the heparin activity in the sample.

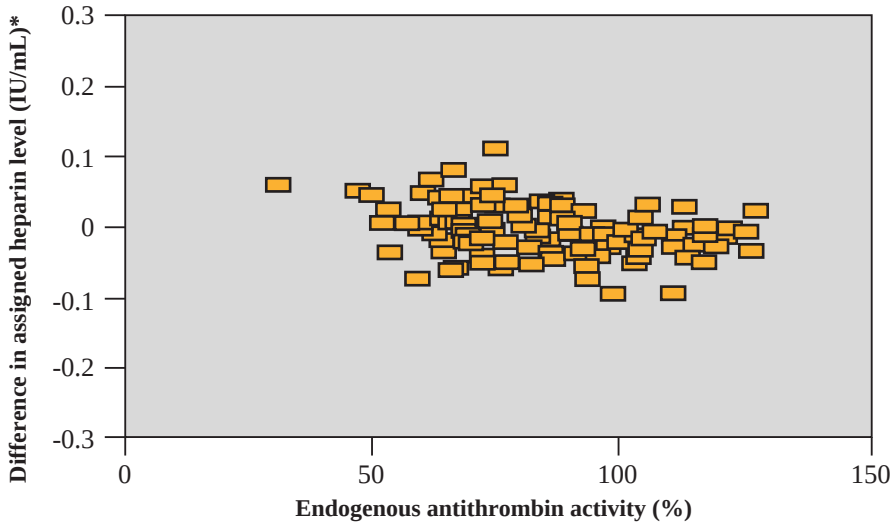


Figure 8. Lack of effect on the assigned heparin level from exogenously added antithrombin to plasma from heparinized patients. The heparin level was monitored twice using Coamatic Heparin with and without addition of exogenous antithrombin and the difference in the result between the two measurements was calculated.

Chromogenic anti-FXa assays are performed either as single-stage or as two-stage assays. In the latter, exogenous antithrombin is added to the test and the results are then considered to reflect the total heparin concentration. The single-stage assay utilizes only the endogenous antithrombin in the plasma sample, yielding results which are referred to as the effective heparin concentration.
The effect of adding antithrombin to the test is apparent in experiments utilizing heparin spiked samples. If the antithrombin activity in the plasma is below the normal range, a decreased heparin recovery is obtained, unless exogenuos antithrombin is added to the test.

However, when plasma samples from heparinized patients with antithrombin levels varying from 35% to 130% were tested with the single-stage method (COAMATIC® Heparin, page 15) addition of exogenous antithrombin had no effect on the resulting heparin level (Figure 8). Consequently, this kit method is insensitive to variation in the endogenous antithrombin activity in the plasma.

Heparin data

Trade names:	Heparin sodium, Liquemin-sodium, Lipo-Hepin, Panheprin Calciparin etc.
Biosynthesis:	Mast cells
Specific activity:	150-190 IU/mg
AT- high affinity chains:	30-35%
Molecular weight:	5,000 to 30,000 daltons
Structure:	Sulfated polysaccharide of 12 to over 100 saccharides
Half-life:	1 to 3 hours (dose-dependent)
Bioavailability:	~ 30%
Removal pathway:	Cellular (saturable mechanism), renal (insaturable mechanism)
Function:	Acceleratesprimarily the inhibition of thrombin and factor Xa

LMW heparin data

Trade names:	Fraxiparin, Enoxaparin, Fragmin, Sandoparin, Logiparin, Lovenox etc.
Method of preparation:	nitrous acid digestion, heparinase digestion, peroxidative cleavage, B-elimination of heparin ester, benzylation followed by alkaine hydrolysis, nitrous acid depolymerrisation
Specific activity:	80-120 IU/mg (anti-Xa assay)
AT- high affinity chains:	<30%
Mean molecular weight:	4,000 to 6,500 daltons
Structure:	Sulfated polysaccharide of 4 to 40 saccharides
Half-life:	4 hours (dose-dependent)
Bioavailability:	~ 90%
Removal pathway:	Renal
Function:	Accelerates primarily the inhibition of factor Xa

Diagnostic kits from Chromogenix

We invented the technology

The development of the first chromogenic peptide substrate S-2160 in the early Seventies, initiated the introduction of photometry in haematology.[60]

Today, Chromogenix has a wide range of chromogenic peptide substrates as well as complete assay kits with applications extending from routine analysis to front line research in both coagulation and fibrinolysis (Table 6).[61] The tests can be performed manually or on automated analytical systems with high specificity, sensitivity and accuracy. Important, non-chromogenic-based products include kits for APC resistance (an APTT test) and several ELISA kits (Table 7).

Heparin kits

COAMATIC® Heparin, COATEST® Heparin, COATEST® Heparin/LMW Heparin and COACUTE® Heparin are four kits for the specific determination of the anti-factor Xa activity of heparin and LMW Heparin in human plasma. The assays isolate the biological activity of heparin and thus minimize the interference from other variables.

HAEMOSTASIS
Factor VII
Factor VIII
Soluble fibrin
Antithrombin
Heparin/LMW heparin
Protein C
Plasminogen
t-PA
PAI-1
Plasmin inhibitor

Table 6. *Substances that can be determined with diagnostic kits based on synthetic peptide substrates (Examples from Chromogenix product range 1999).*

HAEMOSTASIS
APC resistance
Anti-Cardiolipin IgG, IgM
t-PA
PAI-1

Table 7. *Substances that can be determined with kits from Chromogenix, based on ELISA or clotting techniques .*

Clinical indications for the anti-Xa plasma heparin assays

- **Combined heparin/warfarin therapy**
- **Combined heparin/rt-PA therapy**
- **Altered plasma coagulation proteins**
- **Presence of circulating lupus anticoagulant**
- **Unrecognized heparin administration**
- **Renal failure**

COAMATIC® Heparin

Art. no. 82 33 93

COAMATIC® Heparin provides a chromogenic test for the specific determination of the anti-factor Xa activity of both unfractionated (UF) and low molecular weight (LMW) heparin. A high user convenience is obtained through its few components and easy performance.

The assay makes use of undiluted plasma and is performed in a single stage. This renders it especially appropriate for automated, state-of-the-art, coagulation instruments. The single stage assay format increases the instrument adaptation possibilities and the instrument flexibility since it allows for optimal use of a variable incubation time. The use of undiluted test plasma means a saving in time as well as in cuvette usage. Validated instrument applications are available for most coagulation instruments.

Calibrators and controls for this kit are available separately.

Measurement principle

Heparin or LMW heparin is analyzed as a complex with antithrombin present in the plasma sample. Factor Xa is added in excess to a mixture of the plasma sample and a chromogenic substrate. Two competing reactions then occur simultaneously:

-Inhibition of FXa by the antithrombin-heparin complex

-Hydrolysis of the substrate by FXa resulting in release of pNA. The resulting increase in absorbance is inversely proportional to the concentration of heparin in the sample.

The kit contains

S-2732, 15 mg	2 vials
Factor Xa, 35 nkat	2 vials

Storage and stability in solution

S-2732	2-8°C, 3 months
Factor Xa	2-8°C, 3 months

Measuring range

Linear standard curve on automated instruments in the range 0-1.0 IU/ml. Second order polynomial standard curve in the range 0-2.0 IU/ml.

Reproducibility

Heparin	CV% within series	CV% between series
0.7 IU/ml UFH	2.8%	1.2%
0.4 IU/ml UFH	3.4%	1.5%
0.7 IU/ml LMWH	3.6%	2.8%
0.4 IU/ml LMWH	2.4%	2.3%

Interfering substances

The results are not affected by haemoglobin up to 200 mg/dl, bilirubin up to 20 mg/dl or triglycerides up to 600 mg/dl. The presence of dextrane sulfate reduces the influence from heparin antagonists, eg platelet factor 4.

Determinations per kit

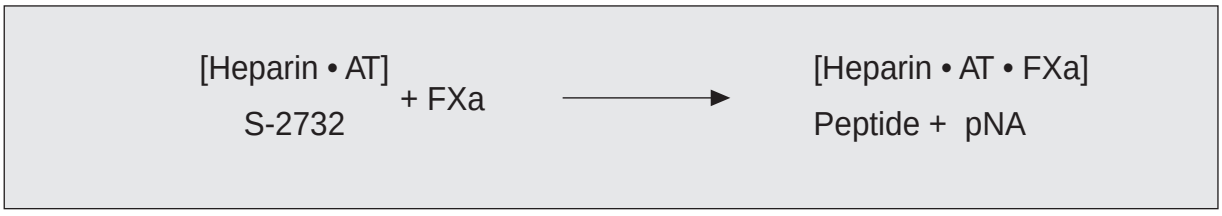
Microplate: 200
Instrument: 110-130

Calibration Plasma - LMW Heparin

Art. no. 82 35 00

Control Plasma - LMW Heparin

Art. no. 82 34 92



The measurement principle of COAMATIC® Heparin

COATEST® Heparin

Art. no. 25 55 39

COATEST® Heparin provides a two-stage chromogenic test for the specific determination of the anti-factor Xa activity of both unfractionated (UF) heparin and low molecular weight (LMW) heparin. The assay is less sensitive to heparin antagonists (platelet factor 4) than APTT and TCT.

Measurement principle

Heparin is analyzed as a complex with antithrombin (AT). In order to obtain a constant activity of AT, an excess of purified AT is added to the test plasma. Factor Xa (in excess) is neutralized in proportion to the amount of [heparin•AT] complex. The residual amount of factor Xa hydrolyzes the chromogenic substrate S-2222™, thus liberating the chromophore, pNA. The release of pNA (yellow colour) is inversely proportional to the amount of heparin in the sample and is read photometrically at 405 nm.

The kit contains

S-2222	1 vial
Factor Xa (bovine)	1 vial
Antithrombin (human)	1 vial
Buffer	1 vial
Normal plasma (human)	4 vials

Storage and stability in solution

S-2222	2–8 °C, 6 months
Factor Xa	2–8 °C, 1 month
	-20 °C, 6 months
Antithrombin	2–8 °C, 1 month
	-20 °C, 6 months
Buffer	2–8 °C, 2 months
Normal plasma	2–8 °C, 2 weeks
	-20 °C, 1 month

Measuring range

Above 0.7 IU/ml, dilute with normal plasma

Reproducibility

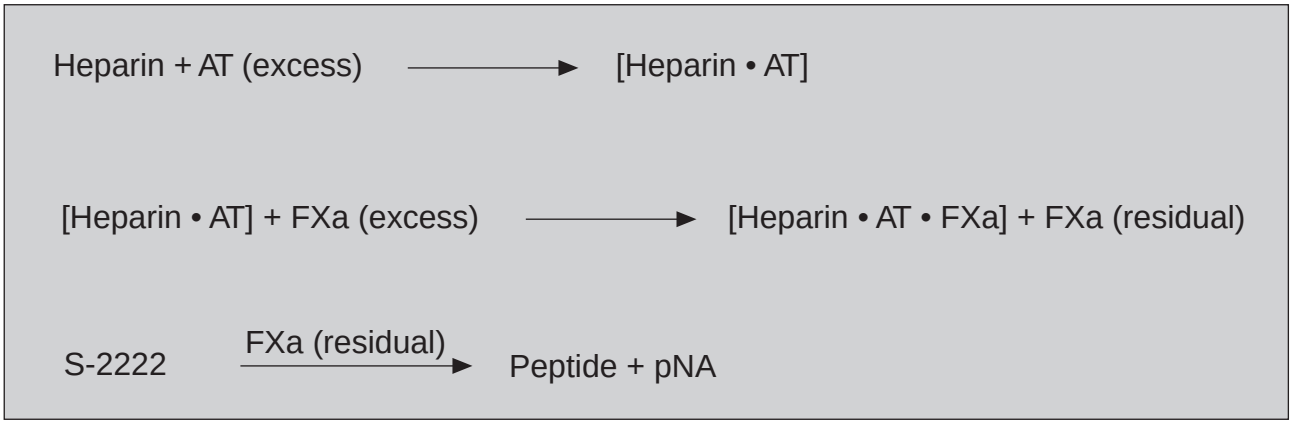
Heparin	CV% within series	CV% between series
0.7 IU/ml	2.3%	2.6%

Specificity and interfering factors

No drug interference reported.
FDP levels in pathological plasmas do not interfere with the test[40]

Determinations per kit

Manual 100, microplate 400, automated up to 285



The measurement principle of COATEST® Heparin

COATEST®
LMW Heparin/Heparin

Art. no. 82 13 63

COATEST® LMW Heparin/Heparin provides a chromogenic test for the specific determination of the anti-factor Xa activity of UF heparin and LMW heparin. The kit is mainly intended for the non-automated laboratory and allows rapid and reliable manual determination in a one-stage procedure.

Measurement principle

LMW heparin or heparin is analyzed as a complex with antithrombin (AT) present in the plasma sample. Factor Xa is added to a mixture of the plasma sample and the chromogenic substrate S-2732™ in a buffer. Two competing reactions then start. One is the inhibition of FXa by the [heparin•AT] complex, the other is the FXa catalyzed release of pNA (yellow colour) from the synthetic substrate. After a certain period of time most of the FXa is inhibited and the release of pNA has declined. Further release of pNA is stopped by the addition of acetic acid. The relationship between the photometric absorbance at 405 nm and LMW heparin/heparin activity is linear in the range 0.1-1.0 IU/ml when plotted on a logarithmic scale.

The kit contains

S-2732	1 vial
Factor Xa (bovine)	1 vial
Buffer	1 vial
LMW Heparin standard	1 vial

Storage and stability in solution

S-2732	2–8 °C, 6 months
Factor Xa	2–8 °C, 1 month
Buffer	2–8 °C, 2 months
LMW Heparin standard	2–8 °C, 6 months

Measuring range

Above 1.0 IU/ml, dilute with normal plasma

Reproducibility

Heparin	CV% within series	CV% between series
0.4 IU/ml	3.3%	4.2%
0.8 IU/ml	0.8%	6.9%
Fragmin	CV% within series	CV% between series
0.4 IU/ml	3.3%	5.9%
0.8 IU/ml	0.8%	5.7%

Specificity and interfering factors

The method is slightly dependent on the patient's antithrombin concentration. If results deviate from the expected activity, measurements of the patient's antithrombin level is recommended.

Determinations per kit

Manual method 50, microplate 125

The measurement principle of COATEST® LMW Heparin/Heparin

COACUTE® Heparin

Art. no. 82 16 60

COACUTE® Heparin provides a single-stage anti-FXa chromogenic test designed for the convenient, rapid and reliable determination of UF heparin and LMW heparin activity in human plasma. The kit is suitable for laboratories performing only a small number of tests. Although, it is also ideal as a stat or back-up assay for an automated system. Batch specific standard curves are provided in the kit.

Measurement principle

Plasma is diluted with a buffer. An aliquot of the plasma dilution is added to a cuvette containing a lyophilized mixture of factor Xa (FXa), the FXa specific chromogenic substrate S-2732™ and an excess of antithrombin. Following the immediate formation of an [AT•heparin] complex two competing reactions occur simultaneously: a) inhibition of FXa by the [AT•heparin] complex and b) reaction of FXa with the chromogenic substrate, resulting in cleavage of pNA. The pNA release measured at 405 nm is inversely proportional to the heparin level.

The kit contains

2 x 5 semi-micro cuvettes containing:
S-2732 + Factor Xa (bovine) + Antithrombin (human).
2 empty cuvettes to be used as blanks

Buffer	10 tubes
Acetic acid 5%	1 vial

Storage and stability after opening

Cuvettes	2–8 ˚C, 1 month
Buffer	2–8 ˚C, exp. date
Acetic acid 5%	2–8 ˚C, exp. date

Measuring range

0 – 1.0 IU/ml.
Above 1.0 IU/ml, dilute with normal plasma

Reproducibility

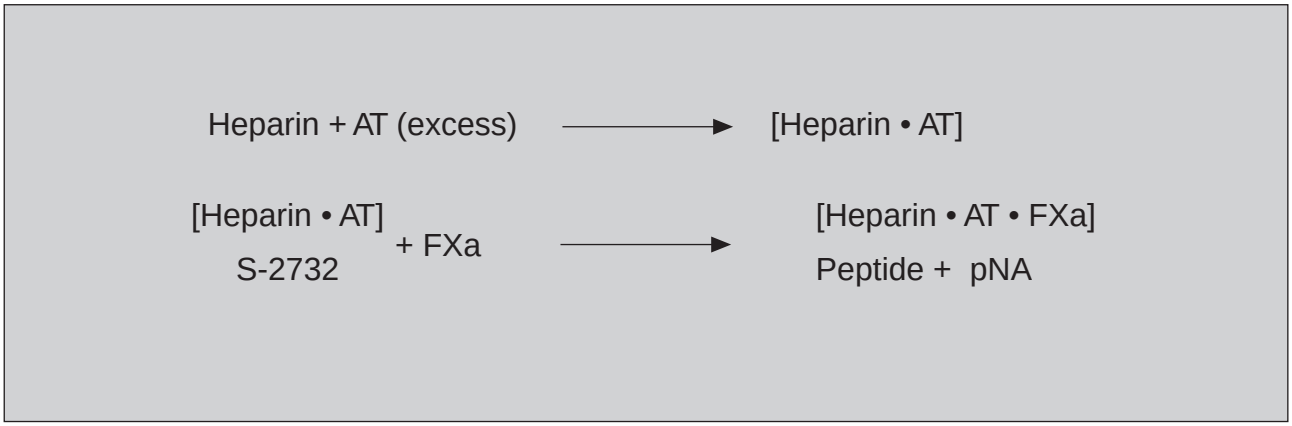
Heparin	CV% within day	CV% between days
0.8 IU/ml	1.3%	1.1%
0.2 IU/ml	2.0%	3.6%

Specificity and interfering factors

The method is less influenced by heparin antagonists (e.g. platelet factor 4) in plasma compared to other methods.

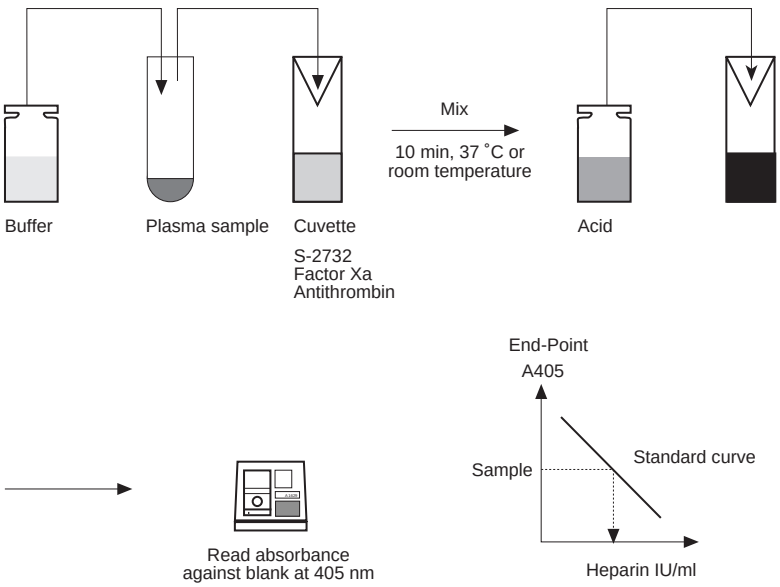
Applications & determinations per kit

Manual 10



The measurement principle of COACUTE® Heparin

COACUTE® Heparin



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Glossary

Antigen. A molecule that induces the formation of an antibody.

APC resistance. An hereditary defect caused by a point mutation in the gene coding for factor V and characterized by a poor anticoagulant response to activated protein C.

Embolism. Obstruction or occlusion of a vessel by a transported clot.

Endothelium. Cells lining blood vessels and lymphatics which control the passage of materials into and out of the bloodstream.

Enzymes. A protein with catalytic power.

Fibrin. An elastic filamentous protein derived from fibrinogen by the action of thrombin, which releases fibrinopeptides A and B from fibrinogen.

Fibrinogen. Factor I; a globulin of the blood plasma that is converted into the coagulated protein, fibrin, by the action of thrombin in the presence of calcium ions.

Fibrinolysis. The hydrolysis of fibrin by plasmin.

Glycoprotein. One of a group of protein-carbohydrate compunds

Haemostasis. Process that arrests the escape of blood from injured vessels.

Heparin cofactor II. A thrombin specific serpine with heparin co-factor abilities. Like antithrombin it forms a 1:1 complex with thrombin. The affinity between heparin and heparin cofactor II is much lower then the affinity between heparin and antithrombin.

Hepatocytes. Cells in the liver that are arranged in folded sheets. They produce many of the blood proteins.

Platelets. A small disk-shaped blood cell, containing granules in the central part and peripherally, clear protoplasma, but no nucleus. Numbering 200,000 to 300,000/µl.

Platelet factor 4. A heparin antagonist released from activated platelets. It interacts equally with high and low affinity heparin and like most heparin-binding proteins the interaction decreases with molecular weight.

Protamine. Protein derived from fish and used as a clinical antagonist for heparin. After injection it neutralizes all the anticoagulant avtivity of UF heparin and about 75% of the anti-Xa activity.

Proteases, proteinases. Enzymes hydrolyzing native protein, or polypeptides, making internal cleavages; they include pepsin, chymosin, trypsin, papain etc.

Proteoglycan. A macromolecular glycoconjugate composed of sulfated glycosaminoglycans covalently linked to a protein core.

Proteolysis. Enzymatic cleavage of protein.

Prothrombin. Factor II, zymogen of thrombin; a glycoprotein formed and stored in the parenchymal cells of the liver. Present in blood at approximately 100 µg/ml.

Receptor. A cell surface molecule which binds specifically to particular proteins or peptides in the fluid phase.

Sepsis. A clinical syndrome of serious bacterial infection.

Serine protease. Proteolytic enzyme with a serine residue at its enzymatically active site.

Serpin. Serine protease inhibitor.

Thrombocyte. Blood platelet

Thrombocytopenia. A conditon in which there is an abnormally small number of platelets in the circulating blood (usually less than 150,000/µl).

Thromboembolism. Refers to either thrombosis or embolism or a combination of both.

Thrombolytics. Biological and synthetic substances capable of activating the fibrinolytic system in plasma.

Thrombin. Active protease deriving from prothrombin (factor II). Induces conversion of fibrinogen into clot-forming fibrin monomers resulting in the coagulation of blood.

Thrombophilia. A disorder in which there is a tendency to develop thrombosis.

Thrombosis. The formation of a thrombus (blood clot).

Thrombotic. Relating to, caused by, or characterized by thrombosis.

Zymogens. The enzymatically inactive precursors of proteolytic enzymes.

Notes

Notes

While others are still exploring chromogenic products, we're creating the next generation.

COAMATIC®	The latest techniques applied specifically for use with automated instruments.
COATEST®	Innovative and well-documented products with a wide range of applications for automated instruments.
COACUTE®	Diagnostic kits for maximum convenience and rapid diagnosis. All reagents are freeze-dried in a single tests cuvette.
COASET®	A group of products designed for research applications.
COALIZA®	Complete ELISA-based kits for antigen determinations.

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