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Examination of Laboratory for Monitoring Heparin Anticoagulant Therapy

Yetti Hernaningsih and Ersya Bayung Maulidan

Abstract

Heparin-derivative anticoagulants include unfractionated heparin (UFH), low molecular weight heparin (LMWH), pentasaccharide (fondaparinux), and ultralow molecular weight heparin (ULMWH). Heparin contains an active pentasaccharide sequence that binds to antithrombin (AT). This bond produces conformational changes that accelerate its binding with AT and inactivation of coagulation factors XIIa, XIa, Xa, and IXa and thrombin (IIa). Thrombin and factor Xa are the most sensitive to inhibition by the heparin-AT complex, and the strength of inhibiting thrombin is ten times more sensitive than factor Xa. The UFH anticoagulant response is monitored using activated partial thromboplastin time (APTT), a measurement that is sensitive to inhibition of thrombin and factor Xa. Protamine titration examination is the standard for measuring UFH concentrations in plasma. Recommendations from the American College of Chest Physicians (ACCP) suggest that the APTT target range for the UFH therapy is equivalent to 0.2–0.4 IU/mL with protamine titration or 0.35–0.7 IU/mL with an anti-Xa examination. A new examination is thrombodynamics (TD), measuring the level of development of clots. This method is considered most able to mimic the coagulation process that occurs in vivo compared to other examinations.

Keywords: heparin, anticoagulant, PPT, APTT, anti-Xa, protamine, thrombodynamics

1. Introduction

At present the use of anticoagulants is very wide; about 0.7% of the population in the west receives anticoagulant treatment. Basic anticoagulant therapy is a vitamin K antagonist; a derivative of warfarin, which is most commonly used, is coumadin (warfarin). This drug has been used for more than 50 years and is consistently able to eliminate recurrent venous thrombosis at adequate doses. However, warfarin has disadvantages, namely, the interaction with other drugs and with food, slow onset and excessive effects, and a narrow therapeutic range. Drug responses and pharmacodynamics are varied and unpredictable, so routine monitoring is needed. For most patients who take drugs in the long term, this is quite troublesome [1].

The current world medical need is to find anticoagulants that are more effective and safer than warfarin for both doctors and patients in long-term use. Responding

to this need, a new drug is needed that can change molecules that are difficult to absorb to be easily absorbed through the digestive tract; this is used as the basis for making oral preparations of unfractionated heparin (UFH). In theory the use of the oral form of heparin or low molecular weight heparin (LMWH) is given at fixed doses, two or three times a day, and does not require overly frequent coagulation monitoring checks or dosage adjustments which are too tight, and the potential for interactions between drugs and medications is also low, making this drug an anticoagulant needed for long-term use [1].

Coagulation monitoring for patients receiving heparin therapy is very important. This is intended to obtain a range of heparin therapy that is effective in reducing the incidence of thrombus and bleeding. The effective use of heparin anticoagulant therapy must increase the activated partial thromboplastin time (APTT) value from 1.5 to 2.5 times. This value is equivalent to levels of heparin 0.2–0.4 U/mL based on protamine titration and is equivalent to anti-Xa levels 0.3–0.7 U/mL [2]. This chapter will discuss laboratory tests that are used to monitor patients receiving heparin therapy.

2. Development of heparin

Heparin is the oldest anticoagulant used in medicine. Heparin was discovered by McLean in 1916 while trying to isolate thromboplastic agents. Heparin is a polysaccharide from the class of glycosaminoglycans (GAG) which naturally appears on all mast cells. Further research in 1935 resulted in clinical use of heparin. Since then, heparin has been widely studied for various applications and modifications [3].

Unfractionated heparin (UFH) is a product of GAG purified from animal tissue, most often from pig intestines. Heparin provides indirect anticoagulant properties by binding to antithrombin III (ATIII) and facilitating the inhibitory effects possessed by AT on thrombin and activated X factor (factor Xa). It is known that only UFH contains at least 18 saccharide sequences that can affect AT activity and thrombin, whereas UFH with a series of certain pentasaccharides can inhibit the activity of factor Xa [3] (**Figure 1**).

The heterogeneity of the structure of the UFH causes extensive bioactivity and physiological activity. Some heparin chains bind to other plasma proteins and have an effect on bone metabolism resulting in osteoporosis or heparin-induced thrombocytopenia (HIT) and other unpredictable effects that require continuous monitoring. Further research and discoveries resulted in low molecular weight heparins (LMWH) in the late 1970s to early 1980s; this was to find anticoagulants which were more predictable in their activities [4]. LMWH, such as enoxaparin, dalteparin, and tinzaparin, is made by chemical control or enzymatic cutting of UFH in a depolymerization reaction.

This controlled process produces fragments with lower molecular weight and more predictable action than UFH. As a result, side effects are lighter than UFH, monitoring needs are decreased, and bioavailability increases, making LMWH potentially used for outpatients. This makes LMWH the standard of care replacing UFH except in certain cases such as kidney failure and acute coronary syndrome where UFH is still preferred because the liver clearance is lighter and better reversible with protamine sulfate [5].

Ultralow molecular weight heparin (ULMWH) was discovered in early 2000 through a process of chemical synthesis. The reason is to get agents with lighter side effects but have the same or better anticoagulant effect which causes a higher anti-factor Xa ratio to antithrombin activity [6].

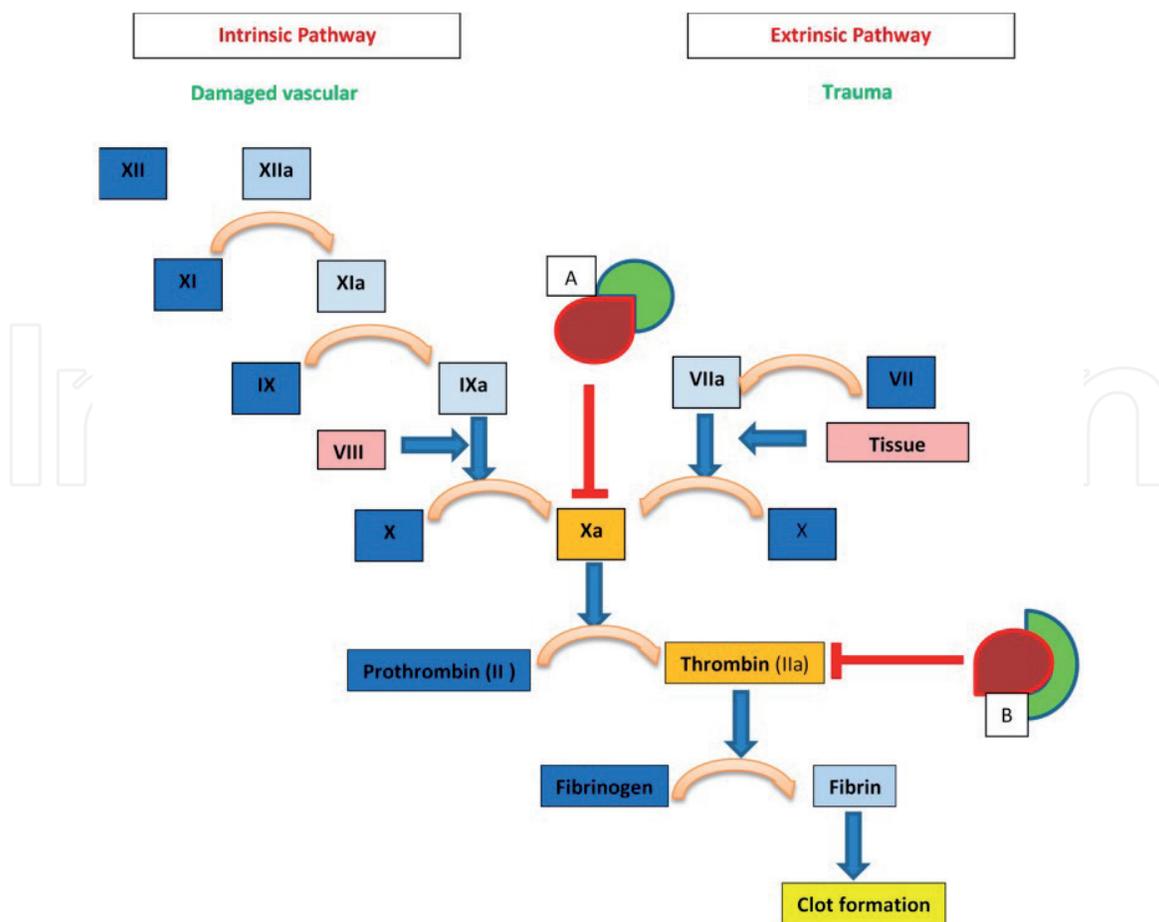


Figure 1. Mechanism of heparin in the coagulation cascade. Box A: AT (red) binds to a heparin fragment (green) with any series length provided that certain pentasaccharide bonds can inhibit factor Xa. Box B: AT (red) binds to heparin (green) with a chain length > 17 U of disaccharide that can inhibit thrombin (factor IIa) [3].

3. Structure and biosynthesis of heparin

Heparin is a polydisperse and highly sulfated GAG with a molecular weight between 5 and 40 kDa. The structure of the complex contains repetitive disaccharide units that contain uronic acid residues (L-iduronic (IdoA) or D-glucuronic acid (GlcA)) and N-acetyl-D-glucosamine. The biosynthesis process of heparin starts in the endoplasmic reticulum and the Golgi apparatus of mast cells. The tetrasaccharide link attaches to the residue of serine in the core protein, serglycin, and then adds a unit of D-glucuronic acid (1→4) N-acetyl-D-glucosamine disaccharide. Disaccharide sulfonation and epimerization of glucuronate to iduronate are carried out by various enzymes in the biosynthetic pathway. In total there are 12 enzymes involved in this pathway, which act together to form the desired molecule. These involved enzymes have many isoforms, which cause heterogeneity of heparin and allow these enzymes to directly biosynthesize associated GAG, heparin sulfate. The degree of sulfation and sulfate residue allocation depends on the spectrum of activity of the product. In mast cell degradation, peptidoglycan heparin changes to GAG heparin through protease and β-endo glucuronidase activity [3].

The first glycosaminoglycan-protein bonding region is formed due to glycotransferase activity. Repeated disaccharide units undergo elongation by GlcA and GLcNAc transferase. Modified chains include N-deacetylation and N-sulfonation, O-sulfonation, and epimerization, which then occur due to specific enzyme activity. The monosaccharide symbol in this figure follows the symbol nomenclature for glycan (SFNG) system [3] (Figure 2).

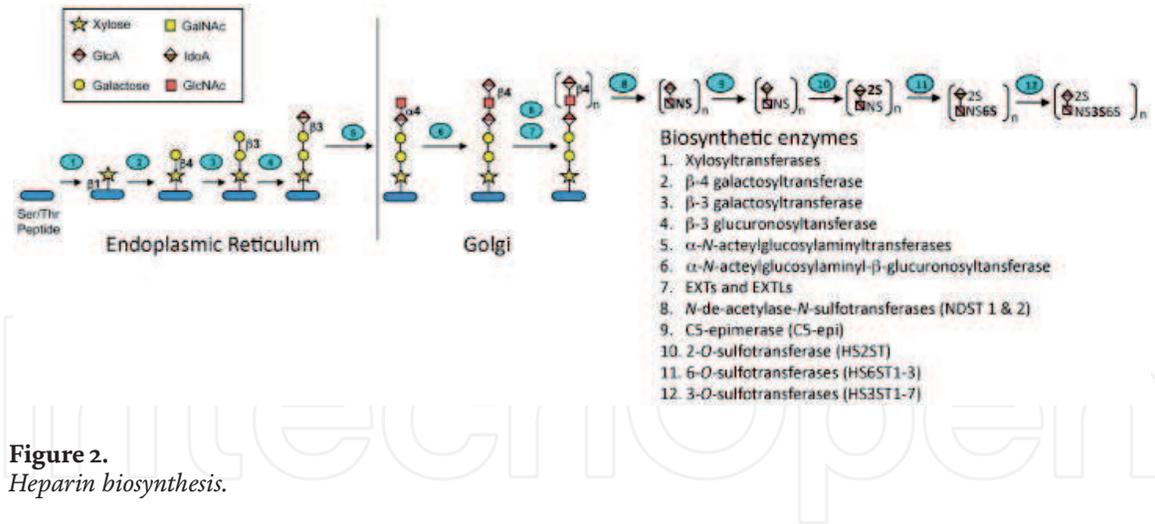


Figure 2.
Heparin biosynthesis.

4. Mechanism of heparin as an anticoagulant

Heparin has anticoagulant effects through interactions with coagulation factors and inhibitors. Coagulation is a complex process involving proteins, platelets, and cellular components such as endothelium and monocytes. The balance of hemostasis is maintained if the activity of procoagulant can be balanced with an inhibitor. The main inhibitor of plasma coagulation factor is AT, which acts on active coagulation factors such as FXIIa, FXIa, FXa, FIXa, FVIIa, and FIIa. These coagulation factors are serine proteases, so AT is a serine protease inhibitor (serpin).

Heparin, acting as a catalyst, provides anticoagulation activity by potentiating other AT and serpin inhibitor activities (**Figure 3**). The interaction of heparin with AT requires a certain sequence of pentasaccharides, but not so with other serpins.

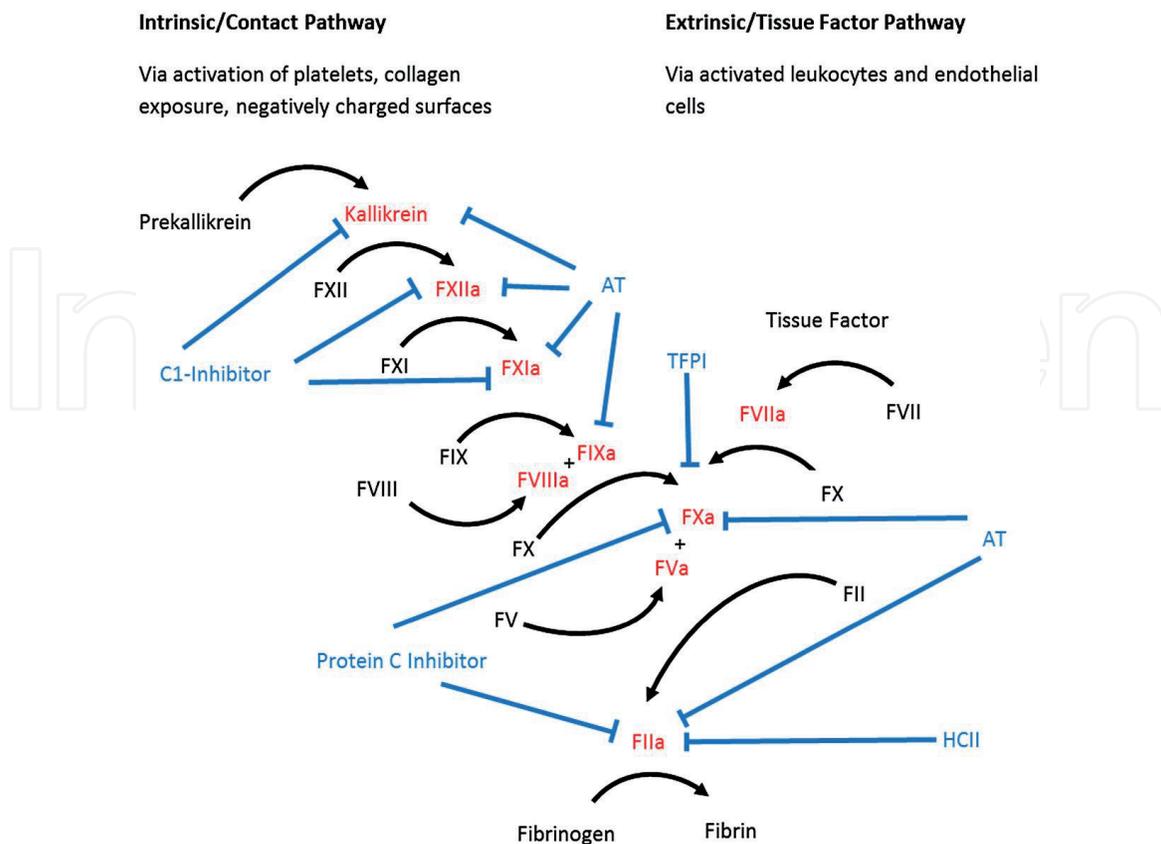


Figure 3.
Interaction of the coagulation factor with serpin. The blue line shows the place of inhibition of each serpin [11]. TFPI, tissue factor pathway inhibitor; AT, antithrombin; HCII, heparin cofactor II.

Another important heparin-binding inhibitor on the extrinsic pathway is tissue factor pathway inhibitors (TFPI) [7–9].

Antithrombin (AT) under conditions when it does not bind to heparin is a slow-acting serpin, because the reactive center loop is partially folded to the center of the β -sheet structure. When binding to heparin, AT inhibition activity will increase significantly, which is a characteristic of serpin. The high-affinity pentasaccharides in heparin bind to antithrombin through two stages: first, the initial binding process involves three monosaccharide units and initiates conformational changes to AT, and then after the interaction is complete, AT conformation after activation by heparin is stabilized. This conformational change is transmitted to the AT structure, causing the opening of the reactive center loop and an increase in exosite exposure from AT which binds directly to FXa [10].

4.1 Inhibition of antithrombin in factors IIa and Xa

Active AT conformation caused by pentasaccharides is sufficient to increase inhibitory activity in FXa, a protease that converts prothrombin to thrombin (FIIa). Factor Xa interacts directly with AT in specific exosite exposed when binding to heparin. In addition, in the presence of calcium, a single heparin chain can bind directly to AT and FXa, increasing AT-FXa interactions, but this is not absolutely necessary. This calcium-dependent effect can account for reports of calcium-induced-specific molecules during the FXa inhibition process by AT [12]. Heparin-mediated FXa binds to AT, indicating the longer heparin chain will increase its affinity with the presence of Ca^{2+} and will strengthen inhibition [13].

Unlike FXa, the potentiation of thrombin inhibition by AT requires an additional 13 saccharide chains attached to the nonreductive end of the pentasaccharide sequence, so thrombin and AT are bound to the same heparin molecule. Thrombin interacts with heparin in exosite II, which is basically a different method with AT; no specific heparin is needed for this interaction [14].

4.2 Inhibition of antithrombin in other coagulation factors

Antithrombin also inhibits several other protease coagulant factors. The way antithrombin blocks FIXa is similar to FXa, which binds to the same exosite on AT. The high-resolution crystal structure of the pentasaccharide-FIXa-AT complex shows that one pentasaccharide binds to AT and the second binds to exosite from FXa, which allows a relationship between the two proteins with one molecule of heparin [13].

The ability of heparin to increase AT inhibition in FXI and kallikrein in the intrinsic coagulation pathway is relatively limited compared to FXa and thrombin. It should be noted that FXI activity in AT mutants that bind to the heparin site is still slightly potentiated by heparin, suggesting that there is a direct interaction between heparin and FXI involved in the binding sites of potential heparin found in the FXI catalytic domain [11].

Antithrombin inhibits the FVIIa complex in the extrinsic pathway, and this effect is reinforced by fondaparinux, LMWH, and UFH. Direct and calcium-dependent interactions are found between FVIIa and heparin [11].

4.3 Other serpins activated by heparin

Heparin cofactor II (HCII), another serpin potentiated by heparin, is a coagulation inhibitor that only inhibits thrombin. HCII is also reported to inhibit chymotrypsin and neutrophil cathepsin G. Heparin cofactor II is found in plasma

at the same level as AT, but HCII cannot replace AT if there is a deficiency. HCII deficiency has no effect on the coagulation system but results in increased formation of an occlusive arterial thrombus after endothelial damage. In vivo HCII is potentiated by dermatan sulfate, which is found in the walls of blood vessels. HCII activated by dermatan sulfate may play a role in preventing excessive thrombosis of injured blood vessels [15].

Heparin and dermatan sulfate both potentiate inhibition of thrombin through HCII in several stages. Unlike AT, HCII does not require a certain series of heparin to interact. Other polyanions can also bind to HCII. The HCII bond with heparin causes conformational changes similar to AT while also releasing thrombin-binding N-terminal tail. The combination of reactive center loop expulsion initiated by GAG by exposure to exosite protease-binding is controlled by AT and HCII in the opposite way [16, 17].

Protein C inhibitors (PCI) regulate the activity of activated protein C (APC), which is the active form of zymogen protein C. Protein C is converted to APC by thrombin and in its active form acts as an anticoagulant by inactivating FVa and FVIIIa, in the presence of protein cofactors S. PCI regulates coagulation inhibitors, in this case acting more as a supporter of coagulation than inhibiting coagulation. The bond of heparin to PCI strengthens the inhibition of APC and FXa in the presence of calcium. A long chain of heparin is needed to strengthen APC inhibition, which suggests both PCI and protease require simultaneous bonding with heparin. The basic structure of the PCI complex with heparin and thrombin if separated, binding sites for heparin will appear involving the H helix, which is located close to the reactive loop [18].

The importance of protease nexin (PN)-1 in the last biology and hemostasis is known. In vitro serpin is known to have an effect of approximately 100 times faster than AT, and heparin increases about 3 times. In vivo PN-1 does not contribute to the activity of heparin as an anticoagulant because its concentration in plasma is very low; on the contrary, PN-1 is found to bind to the cell surface in several organs and tissues, including blood vessel walls. Protease nexin (PN)-1 is detected in platelet granules on the platelet surface and secreted during platelet activation. In this context, the contribution of PN-1 to antithrombotic activity from heparin in vivo is ignored [19].

The crystal structure of PN-1 is obtained from the breakdown of complexes with heparin and thrombin. This protein has a typical serpin fold with the heparin-binding site in helix D. Contrary to AT, heparin-binding site PN-1 and thrombin are not parallel when the reactive center loop of PN-1 productively interacts with the active part of thrombin. The initial formation of the ternary complex between PN-1 and thrombin and heparin can be said to be the initial phase of two-phase interaction, with loss of heparin-thrombin interactions when covalent complex PN-1 thrombin is formed. Heparin is not released when forming the PN-1-thrombin complex; this shows that the PN-1-thrombin complex is still bound to HS on the cell surface [11, 20].

Protein Z-dependent proteinase inhibitors are known to inhibit both FXa and FXIa. Heparin speeds up this reaction 20–100×. The heparin-binding site on protein Z-dependent protease inhibitors involves basic residues in helix D (such as AT) and helix C (unlike AT), and the presence of unstructured N-terminal ends can indicate similarities with HCII [21].

Other serpins, c1inh, inhibit both the complement cascade and the intrinsic pathway, where the coagulation system and innate immune system interact. Clinical deficiency leads to congenital angioedema through excessive contact system activity. The effects of c1inh are not limited to complement and contact systems. Heparin potentiates the activity of the c1inh, and the crystal structure of the c1inh indicates

a different model of activity against different protease and heparin-binding sites against AT [22].

4.4 Non-serpin inhibitors: tissue factor pathway inhibitors

Tissue factor pathway inhibitors (TFPI) are structurally serpin, but not serine protease inhibitors. Tissue factor pathway inhibitors (TFPI) are major inhibitors in the extrinsic pathway. The heparin-binding TFPI α isoform contains an acidic N-terminal region, three Kunitz domain pairs, and a basic C-terminal end. Kunitz domain is involved in anticoagulant activity, with the first domain inhibiting the FVIIa-tissue factor complex and the second domain inhibiting FXa. The C-terminal circuit in TFPI has a high affinity for heparin. Heparin injection releases TFPI bound to the endothelium to the circulation. Heparin bound to TFPI potentiates inhibitory activity in both free FXa and FXa in the FVIIa-TF-FXa complex [11].

5. The drug derivate heparin

5.1 Unfractionated heparin

Unfractionated heparin (UFH) is one of the most commonly used parenteral anticoagulants to treat or prevent thromboembolism and has been used for almost a century. This drug is used in various methods, such as systemic use, through a catheter, extracorporeal, or on the surface of a medical device to prevent thrombotic complications. Heparin depends on the presence of antithrombin (AT) to inhibit clotting factors, so heparin is called an anticoagulant which acts indirectly. Heparin does not have fibrinolytic activity and will not lyse the thrombus [23].

Heparin contains an active pentasaccharide sequence that binds AT. The active pentasaccharide sequence responsible for catalyzing AT is found in one third and one tenth of the UFH and LMWH chains. After heparin binds and activates AT, heparin can release AT and bind other ATs, thus providing a continuous anticoagulant effect. This bond produces conformational changes that accelerate binding of AT and inactivation of coagulation factors XIIa, XIa, Xa, and IXa and thrombin (IIa). Thrombin and factor Xa are the most sensitive to inhibition by the heparin/AT, and tenfold thrombin complex is more sensitive to inhibition than factor Xa [23, 24].

The inhibition of UFH on thrombin requires binding of coagulation enzymes and AT through high-affinity pentasaccharides, whereas inhibition of factor Xa requires only heparin binding to AT. By deactivating thrombin, heparin not only prevents fibrin formation but also inhibits platelet activation induced by thrombin and coagulation factors V and VIII. In addition to its anticoagulation effects, heparin increases the permeability of blood vessel walls, suppresses smooth muscle proliferation, suppresses osteoblast formation, and activates osteoclasts [23, 24].

5.1.1 Pharmacokinetics and pharmacodynamics

Intravenous (IV) or subcutaneous (SC) injection is the route available for UFH administration, and IV is the most frequently used route. When given by SC injection for therapeutic anticoagulation, the dose must be large enough (30,000 U/day) to compensate for the low bioavailability of UFH, as can be seen in **Table 1**. UFH is already bound to plasma proteins, which results in variations in anticoagulant responses [25].

| Characteristics | Heparin | LMWH |
|--|--|------------------|
| Origin | Pig intestine | Pig intestine |
| Molecular weight (Da) | 15,000 | 5000 |
| Target | Xa:IIa | Xa > IIa |
| Bioavailability (%) | 30 | 90 |
| Half-life (h) ^a | IV depends on doses 1–3 SC depends on doses 2–5 | 3–7 |
| Reversal by protamine | Complete | Partial (60–80%) |
| Renal excretion | Depends on dose | Yes |
| Occurrence of heparin-induced thrombocytopenia (%) | <5.0 | <1.0 |

^a In normal renal function.

Table 1.
Pharmacological properties of UFH and LMWH [26].

UFH clearance depends on the dose and occurs through two independent mechanisms. The initial phase is a fast and saturated bond in endothelial cells, macrophages, and local proteins where UFH is depolymerized. The second phase is slower and unsaturated clearance through the kidneys. At therapeutic doses, UFH is cleared mainly through depolymerization, where higher molecular weight chains are cleared faster than those with lower weight. When clearance tends to the kidneys, an increase or extension of the dose of UFH provides a disproportionate increase in both the intensity and duration of the anticoagulant effect. Anticoagulant responses to UFH administration are usually monitored using activated partial thromboplastin time (APTT). APTT must be measured every 6 hours with IV administration and the dose adjusted until the patient has reached a stable level of therapy. After a stable condition is reached, the frequency of monitoring can be extended [24, 26].

5.1.2 Monitoring

The UFH anticoagulant response is monitored using APTT, a measurement that is sensitive to inhibition of thrombin and FXa. APTT examinations have a large variety of reagents (even the same reagents have different lots) so that they have varying sensitivity to the anticoagulant effect of UFH. Each laboratory must ensure that their therapeutic range of heparin and APTT is based on levels of heparin measured by anti-Xa (target range 0.3–0.7 U/mL) or protamine titration (0.2–0.4 U/mL). APTT must be measured every 6 h based on UFH half-life and the dose adjusted until the patient reaches the therapeutic level based on the APTT target range. When APTT values are obtained in the treatment range twice in a row, monitoring can be extended to one or two times a day depending on the clinical scenario. Weight-based dose nomograms, consisting of bolus doses and infusion droplet speeds with regular monitoring using APTT, are recommended for the treatment of thromboembolic disease [25].

The UFH dose nomogram differs in each hospital due to differences in thromboplastin reagents, calibration, and interlaboratory standards in APTT measurements. This causes the need for alternative monitoring methods. Functional heparin, also known as anti-Xa, has been promoted as a more reliable measure of UFH because it is not sensitive to factors other than UFH, such as concomitant use of warfarin, sodium citrate in the sample tube, impaired lupus anticoagulant (LA), increased factor activity VIII, and liver disease [25].

Acquired inhibitors, such as LA, cause an extension of APTT, which results in not being able to accurately measure the level of anticoagulation due to UFH.

In this case, if APTT is maintained within the usual therapeutic range, it may result in underdose of UFH and cause development or recurrence of thrombosis. Simultaneous testing of APTT and anti-Xa levels is needed to estimate the APTT value of therapy in patients receiving heparin [27].

5.2 Low molecular weight heparin (LMWH)

LMWH is a polysaccharide derived from the pig's intestine containing an active pentasaccharide sequence which is needed for anticoagulant activity as in UFH. LMWH is produced from UFH through chemical or enzymatic degradation. Each LMWH product is prepared by a different method. Clinical development of LMWH driven by certain observations includes the reduction of thrombin activity in relation to anti-factor Xa activity, the ratio of benefits or risks that are more favorable in animal studies, and good pharmacokinetic properties. The molecular weight of the LMWH is approximately one third of the molecular weight of UFH (4000–5000 Da). Because of their smaller size, LMWH has a lower affinity for thrombin because they cannot bind AT and thrombin together. However, LMWH has the same affinity as UFH for FXa [23].

Factor Xa does not require heparin to stabilize its interaction with AT, so smaller molecules such as LMWH deactivate factor Xa equivalent to larger molecules such as UFH. The length of the polysaccharide chain of at least 18 saccharides, including the order of active pentasaccharides, is needed to bridge between AT and thrombin. About 25–50% of LMWH molecules are above the length of this chain. All LMWH chains contain active pentasaccharide sequences, so 100% can inactivate factor Xa [23].

5.2.1 Pharmacodynamics and pharmacokinetics

There are several biological consequences of the small size of LMWH compared to UFH, a decrease in binding of LMWH to other plasma proteins, macrophages, and endothelial cells. This resulted in a more predictable dose-response relationship and a longer plasma half-life for LMWH. In contrast to UFH, routine plasma monitoring is not needed which makes it easier for outpatient management. The lower incidence of HIT has also been investigated because of the reduced bond to PF4 and platelets. LMWH has also reduced bonds in osteoblasts resulting in decreased incidence of osteoclast activation and lower bone destruction [23].

All LMWH products have half-lives ranging from 3 to 7 h and bioavailability 87–90%. Anti-Xa peak activity occurs 3–5 h after SC injection with predictable dose-based responses. All agents are metabolized through desulfation or depolymerization, and all agents metabolized are excreted through the kidneys [25].

5.3 Pentasaccharides (fondaparinux)

Fondaparinux is a chemically synthesized anticoagulant that is specifically developed as a selective indirect inhibitor for FXa. Factor Xa is an important target for anticoagulant therapy given its position at the meeting of the intrinsic and extrinsic coagulation pathways. Its inhibition significantly decreases thrombin formation [28] (**Figure 4**).

Factor Xa has one function in the coagulation cascade, as a gatekeeper to the path along the coagulation cascade. Conversely, thrombin (FIIa) has many roles in the coagulation process, including activation and mediation of endogenous anti-coagulation by binding to thrombomodulin and activation of protein C. Factor Xa may be a purer target than thrombin [29].

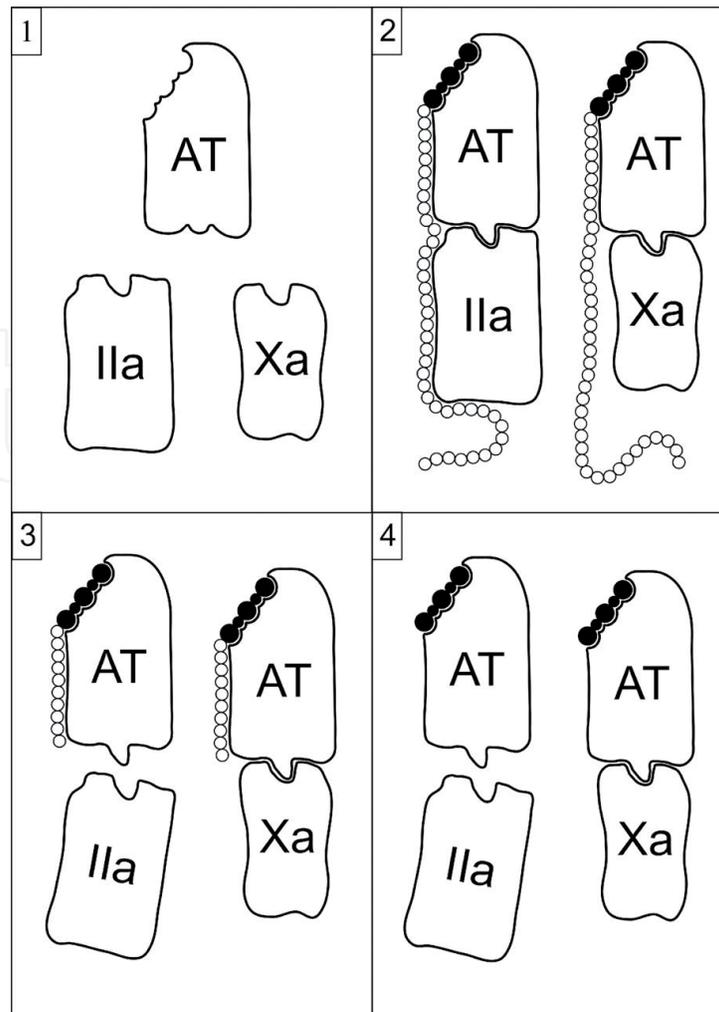


Figure 4. Formation of antithrombin complexes with FIIa and FXa. (1) Antithrombin (AT), active thrombin (FIIa), active X factor (FXa). (2) UFH (chain shape) forms a complex with AT both in FIIa and FXa. (3) Shorter polysaccharides of 18 U form AT complex with FXa but not with FIIa. (4) The sequence of pentasaccharides forms a bond with FXa only [30].

5.3.1 Pharmacodynamics and pharmacokinetics

Fondaparinux binds non-covalently and reversibly to AT, increasing AT anticoagulant activity by up to 300 times. The AT-fondaparinux complex then binds and neutralizes FXa, which reduces prothrombin (FII) conversion to thrombin (FIIa), thereby inhibiting clot formation. Fondaparinux is then released and can catalyze other AT molecules. When AT plasma becomes saturated, excess unbound circulating fondaparinux (which has no anticoagulant activity) is excreted through the kidneys.

Because it does not affect pre-existing thrombin circulation, fondaparinux may in theory have some residual hemostatics, if needed, at the site of injury. Fondaparinux has no effect on the examination of fibrinogen platelet function, thrombin time, or antithrombin tests. Fondaparinux can affect PT and APTT and can interfere with factor VIII testing. Although not routinely recommended, if measurements of fondaparinux are needed (e.g., changes in kidney function, weight, or extreme age), the most accurate plasma concentration is measured using the anti-factor Xa test. The results of this examination are in IU/mL, which is directly proportional to the plasma concentration of fondaparinux. The results were extrapolated to the mcg/mL plasma concentration using a standard curve calibrated by fondaparinux. The test must be calibrated specifically for fondaparinux, because the use of calibration for UFH or LMWH will produce inaccurate results.

The use of fondaparinux and drugs that affect concomitant coagulation (e.g., antiplatelet, NSAIDs) results in pharmacodynamic drug interactions that can increase the risk of bleeding and should be avoided as much as possible. After stopping fondaparinux, the anticoagulant effect will last up to 4 days and even longer in patients with low clearance [31].

Fondaparinux is not absorbed through the gastrointestinal mucosa, so it must be given parenterally. Subcutaneous administration showed rapid absorption and complete absorption with 100% bioavailability. The peak plasma concentration is reached about 2–3 h after subcutaneous administration. A stable state is achieved after 3–4 doses of administering fondaparinux once a day [25].

Fondaparinux is highly protein bound and cannot be distributed to tissues without binding to proteins. The volume of distribution is 7–11 L, which is close to blood volume. Fondaparinux does not undergo metabolism in the liver and is not susceptible to the pharmacokinetics of drug interactions with the cytochrome P450 isoenzyme system substrate [25].

Reduced bonding with macrophages and endothelial cells increases the half-life of plasma fondaparinux compared to UFH and LMWH. Elimination of fondaparinux is influenced by several patient parameters, including kidney function, age, and low body weight. These factors must be evaluated regularly, because they can block the use of fondaparinux or require increased monitoring for signs and symptoms of drug accumulation [25].

5.4 Ultralow molecular heparin

Therapy using LMWH provides clear pharmacokinetic advantages over UFH, so LMWH has become the standard of care for prevention and treatment of venous thromboembolism (VTE) in patients with and without cancer. The development of the ULMWH drug is based on the theory that, because of the much higher ratio of anti-Xa and anti-IIa activity, ULMWH will be associated with similar or better antithrombotic efficacy from the efficacy achieved by LMWH products but with lower bleeding and HIT risks. ULMWH has a molecular weight of <4000 Da and an increase in anti-factor Xa activity compared to LMWH. There is only one ULMWH marketed outside the United States (bemiparin), and another, RO-14, is currently in clinical development [32].

5.4.1 Pharmacokinetics and pharmacodynamics

Bemiparin was approved for once-daily use of subcutaneous VTE primary prophylaxis in medical patients and patients undergoing general or orthopedic surgery and for prophylaxis in patients with deep vein thrombosis (DVT). Bemiparin originates from alkaline depolymerization and UFH fractionation from pig intestinal mucosa. Pharmacokinetic studies in healthy volunteers given bemiparin showed an increase in anti-factor Xa activity depending on the dose given [32].

6. Heparin therapy monitoring

Treatment using UFH requires routine monitoring to see its functional activity due to the high variation in plasma concentrations and functional activities after fixed doses in each patient. This is due to several reasons, including AT levels in plasma that are different in each patient, UFH elimination with two mechanisms that also differ in each individual, and neutralization and bonding of heparin with various activated plasma proteins and platelets. It is estimated that 10% of people

are not sensitive to heparin. The nonlinear pharmacodynamics of UFH make it difficult to predict. Unfractionated heparin (UFH) is usually measured by APTT or activated coagulation time (for high levels of heparin), but this examination has a low sensitivity and is not standardized; besides, this examination is also not sensitive to the use of low-dose UFH as prophylaxis. The results also depend on the reagent and the instrument used. The reference value for this examination must be determined by each laboratory, so it is difficult to compare the results obtained from each laboratory [33].

Monitoring the therapy of coagulation UFH activity should be measured 6 h after bolus and 6 h after dose change. It takes 6 h for the heparin to reach a stable phase, so monitoring heparin therapy less than 6 h after administration gives the wrong results for determining the dose. Protamine titration is the gold standard for heparin monitoring, but this examination is not widely available and expensive, so it is only used for research [34].

Unlike UFH, LMWH has a more stable pharmacokinetics, and its bioavailability reaches 100% at any dose with subcutaneous administration. The maximum LMWH concentration in plasma is directly proportional to the LMWH dose, and many experts believe that routine coagulation monitoring during therapy is not necessary because the clinical dose of LMWH can be corrected based solely on the patient's body weight. In a healthy population after a fixed dose of LMWH, it turns out that heparin concentration varies and only partially correlates with the patient's body weight. More precisely correction needs to be done in some cases (e.g., low or high BMI; critical condition; kidney failure (creatinine clearance <30 ml/min); when changing anticoagulants; age (children or elderly >75 years); and pregnancy) [35].

Anti-Xa activity measurement is the most widely used examination for LMWH therapy management. This examination has a high sensitivity (lower limit of determination using chromogenic substrate is <0.03 anti-Xa IU/mL). This method is not a method of global coagulation examination because it only measures the concentration of one factor (Xa) but does not react with AT deficiency or changes in concentration from other factors that influence patient hemostasis. This method is difficult to predict thrombosis or bleeding. Until now there is still no universal and reliable method for monitoring heparin properly [30].

6.1 Activated partial thromboplastin time (APTT)

The APTT examination is currently used by most laboratories for monitoring UFH therapy. This examination uses plasma citrate from the patient and is measured based on clot formation. Phospholipids and activators are added to platelet-poor plasma (PPP) patients and then incubated. Calcium is added and then the clotting time is measured. This examination has several advantages, fast, inexpensive, and widely available, but this does not directly measure the level of heparin. Many APTT reagents are available, and each reagent has a varied response to heparin therapy, and besides that several physiological factors can influence the results of this examination [36].

The clinical condition of patients can also affect the results of APTT, but it does not correlate with the presence of bleeding or thrombosis. The most frequent cases are patients receiving vitamin K antagonist therapy. Patients with international normalized ratio (INR) of more than 1.3 because warfarin therapy can also affect APTT results in heparin monitoring. Other cases that also affected were patients with antiphospholipid antibodies, which could affect clotting tests. Coagulation factor deficiencies, such as in patients with liver disease, or consumptive coagulopathy in disseminated intravascular coagulopathy (DIC) will affect APTT results. These conditions cause heparin anticoagulation activity not to be measured properly [36].

The conditions mentioned above can prolong the APTT results and result in underdose anticoagulant doses. Factor VIII and fibrinogen are the most frequent causes and can significantly extend or shorten the APTT baseline. Patients with acute disease are also known to have a deficiency in antithrombin. This condition can lead to excessive anticoagulation when using APTT for monitoring [36].

Preanalytic variables also play a role in the APTT response in monitoring heparin. The wrong APTT results can be due to improper sampling, and less samples also make too much citrate concentration in the tube. Underdose therapy causes a high risk of thrombosis [37].

Basu et al. [38] conducted a study on patients with venous thromboembolism who received heparin therapy and found the risk of recurrent thromboembolism associated with the APTT ratio which did not reach 1.5–2.5 times the normal value. This ratio is used as a standard for therapeutic ranges. Based on this study, the authors mention that the ratio is equivalent to levels of heparin 0.2–0.4 U/mL based on protamine titration and is equivalent to anti-Xa levels of 0.3–0.7 U/mL. The range of anti-Xa levels is higher because of heparin clearance. Smaller heparin molecules are cleared more slowly, so LMWH has a stronger inhibitory effect on factor Xa than thrombin. Examinations that measure anti-factor Xa activity, such as anti-Xa examination, will detect higher levels than examinations that measure antithrombin activity, such as protamine titration, which utilizes thrombin time [30].

Other studies evaluated the upper limit of anti-Xa examinations related to the incidence of bleeding. This study produced anti-Xa levels of more than 0.74–0.88 U/mL related to the incidence of bleeding complications [30]. The use of the APTT standard ratio for the range of heparin therapy is difficult to apply because the response of each APTT reagent to heparin is different. Researchers from the joint study found that APTT corresponds to a concentration of heparin 0.2–0.4 U/mL with protamine titration, based on the APTT reagent used. APTT reagents from other laboratories showed different sensitivity to heparin, and a ratio of 1.5–2.5 times the normal value did not correlate with the concentration of heparin in the therapeutic range. Various types of laboratories and reagents can produce an APTT ratio of 1.6–3.7 times the normal value, which is equivalent to a level of heparin 0.3 U/mL to a ratio of 2.4–6.2 times the normal value equivalent to the level of heparin 0.7 U/mL with anti-Xa [39].

6.2 Anti-Xa activity assay

Variations in results were also obtained for each anti-Xa examination if compared with protamine titration as a reference but far smaller compared to APTT. The therapeutic range for this examination will remain to be 0.3–0.7 U/mL, although with different machines and reagents. In contrast to APTT, anti-Xa results are not affected by poor sampling, also are not affected by factor VIII or fibrinogen, and are not affected by deficiency factors in patients with liver disease and consumptive coagulopathy [30].

Anti-Xa assay is not a test of factor X activity or factor X level antigen. This examination is also called an anti-factor Xa test or a functional test of heparin. The principle of this examination is to monitor indirect factor Xa inhibitors, such as LMWH and fondaparinux, or direct factor Xa inhibitor drugs such as rivaroxaban. These anticoagulants require monitoring in certain patient populations and in certain clinical settings. Each anticoagulant requires its own anti-X curve, and this must be done by each laboratory [37].

Anti-Xa activity assay uses the chromogenic method. The known factor Xa is added to platelet-poor plasma, wherein there is heparin. Heparin strengthens the inhibition of antithrombin to factor Xa, and the uninhibited factor Xa chromogenic substrate is added. This process produces color detected by a spectrophotometer and directly proportional to the level of factor Xa. The amount of color correlates with the level of heparin in the plasma with the correct heparin curve [36].

Like other tests, the anti-Xa assay is imperfect, with the chromogenic method; this examination will be affected by the conditions of the hemolysis, jaundice, and lipemic samples, which will affect the ability of the machine to measure and distinguish chromogenic reactions. Anti-Xa reagents which are not added with antithrombin will make false low heparin concentrations in the condition of patients with severe antithrombin deficiency, but there can be a misdiagnosis of antithrombin deficiency if antithrombin is added. Viewed from the laboratory side, anti-Xa assay is considered expensive and needs special attention in the process [36].

6.3 Protamine titration

Protamine is a small protein, rich in arginine, and positively charged, has a similarity to histones, and is involved in folding and stabilizing DNA in sperm heads. Protamine can neutralize the effects of heparin through electrostatic bonds between cation arginine groups from protamine and heparin anions with a ratio of 1:1. This results in a neutral protamine-heparin aggregate which can be seen in the form of a white suspense formed in a few seconds. Binding of heparin by protamine will release the AT-heparin complex, resulting in AT activity returning to its original state [40].

Protamine titration is the gold standard for measuring UFH concentrations in plasma. The results of this examination are quite promising, but it is still not considered a good examination in terms of clinical management of UFH because it is still not automatic. UFH clinical trials determined that heparin concentrations of 0.2–0.4 U/mL with protamine titration were equivalent to APTT lengthening 1.5–2.5 times the normal values, providing safe UFH levels and good patient outcomes [41].

The principle of this examination is to measure thrombin clotting time (TCT), which is the time required for clot formation after the addition of thrombin to plasma. The presence of UFH in plasma will result in TCT prolongation depending on the dose used. Protamine competed in binding to UFH, and the results of protamine titration measurements showed the amount of protamine needed to restore TCT to baseline [42].

This method is neither expensive nor difficult, but because it is done manually, the workload is quite heavy. The results given by this examination are very dependent on two important factors, the operator that works and the origin of thrombin. Protamine titration measurements depend on the operator when identifying clot formation in the test tube, so efforts to standardize this are difficult. The reproducibility of results can be achieved by limiting operators who work on checks and equating perceptions between operators about the process of forming clots and when the test is certain to be completed.

First time this method was found, thrombin used came from rabbits or young cattle. Its concentration is determined by identifying the amount of thrombin which results in TCT 18–20 s. At present the thrombin used is from humans, so it can increase the specificity of this test. Thrombin originating from humans has different interactions with plasma patients than thrombin from rabbits and young cattle. Thrombin originating from humans also needs to be determined by

calculating the amount of thrombin needed to achieve TCT 18–20 s. Each laboratory must determine the concentration of thrombin that is needed based on thrombin available in each lab [41].

UFH therapy is optimal if the levels are 0.2–0.4 IU/mL based on protamine titration. Recommendations from the American College of Chest Physicians (ACCP) suggest that the APTT target for the UFH therapy range is equivalent to 0.2–0.4 IU/mL with protamine titration of 0.35–0.7 IU/mL with anti-Xa assay [23].

This method is carried out at room temperature, plasma samples and protamine solutions that have been titrated at each concentration, and thrombin stored in ice until it is transferred to the test tube. First take a normal plasma, and leave it at room temperature for 30 s. Then the titration solution is added, and thrombin is then calculated when the clot is formed. The time obtained will be used as TCT baseline. Assay was repeated using a sample of patients with different protamine concentrations starting from the highest concentration first (0.9 IU/mL). After finding the smallest concentration that still provides TCT baseline, this result illustrates the level of heparin in the blood [41].

6.4 Thrombodynamics

A new examination for the coagulation function, thrombodynamics (TD), is examined under different circumstances. This TD measures the level of clot formation. Coagulation is triggered on the surface of an activator which is bound to a space and extends to the plasma layer. The scattered beam increases in the area formed by the clot, so clot formation and time needed can be measured. This method is considered most able to mimic the coagulation process that occurs *in vivo* compared to other examinations. This examination is very sensitive to the conditions of either hypocoagulation or hypercoagulation. **Figure 5** shows the

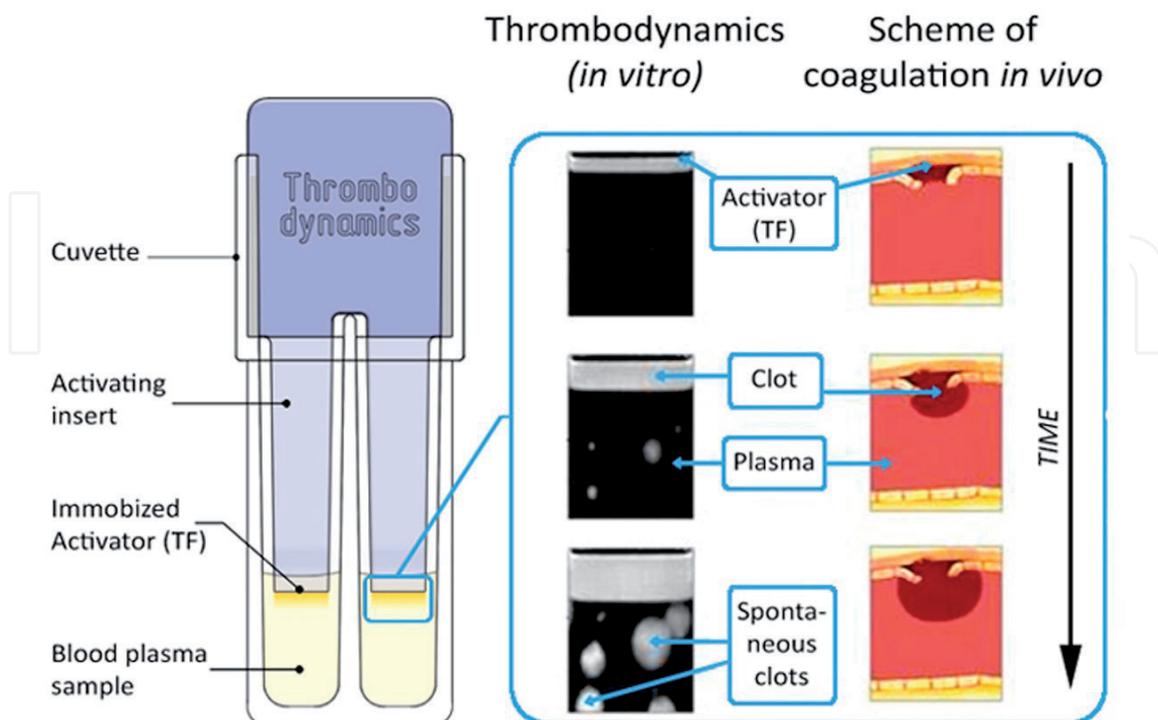


Figure 5. Examination of thrombodynamics simulates the *in vivo* coagulation process. (a) Plastic cuvette schemes and activators that have been added for TD measurements. (b) Photos of the sequence of clot growth from spontaneous activator *in vitro* and clot in a portion of the sample. (c) Schematic image of clot growth from damage to blood vessel walls *in vivo* [44].

test tube scheme used in TD examination and the formation of clots and compared with the process of clot in vivo [43].

Clot formation curve depends on several parameters obtained: Tlag (time of formation of clot); Vi (initial speed of formation of clots) and V (speed of formation of clots) in clot formation (slope of clot formation curve versus time at 2–6 min and 15–25 min from the initial formation of clots for Vi and V); and CS (clot size 30 min after the coagulation process is activated). Other important parameters that are also measured are the maximum optical density of the formed clot (D) which shows the quality of the clot and the time required for the appearance of spontaneous clot (Tsp). The last two parameters have important clinical values because spontaneous clot (which grows not from activators on the surface) is only found in serious hypercoagulability states [44].

Heparin anticoagulants form complexes with ATIII and inhibit factors II, X, and IX. Research by Sinauridze et al. measure Tlag, Vi, and V parameters in post hip surgery patients who get heparin. The result is only Vi and V have significant different results, whereas Tlag has results that are not much different before and after heparin is given [44].

7. Conclusion

Heparin monitoring is needed to achieve an adequate dose. Laboratory tests for heparin monitoring include APTT examinations especially for UFH, anti-FXa activity assays especially for LMWH, protamine titration especially for UFH, and thrombodynamic tests that better reflect in vivo conditions.

Abbreviations

| | |
|--------|--|
| ACCP | American College of Chest Physicians |
| APC | activated protein C |
| APTT | activated partial thromboplastin time |
| AT | antithrombin |
| C1inh | C1 inhibitor |
| DIC | disseminated intravascular coagulopathy |
| DNA | deoxyribonucleic acid |
| GAG | glycosaminoglycans |
| HC II | heparin cofactor II |
| HIT | heparin-induced thrombocytopenia |
| INR | international normalized ratio |
| IV | intravenous |
| LMWH | low molecular weight heparin |
| NSAID | nonsteroid anti-inflammatory drug |
| PCI | percutaneous coronary intervention |
| PCI | protein C inhibitor |
| PF4 | platelet factor 4 |
| PN-1 | protein nexin-1 |
| PPP | platelet-poor plasma |
| SC | subcutaneous |
| Serpin | serine protease inhibitor |
| SFNG | symbol nomenclature for glycans |
| STEMI | ST-segment elevation myocardial infarction |
| TCT | thrombin clotting time |
| TD | thrombodynamics |

| | |
|-------|-----------------------------------|
| TFPI | tissue factor pathway inhibitor |
| UFH | unfractionated heparin |
| ULMWH | ultralow molecular weight heparin |
| VTE | venous thromboembolism |

Nomenclature

Symbols

| | |
|------|--|
| CS | clot size at 30th minute of the measurement (μm) |
| D | clot density, it is an optical parameter, which is equal to intensity of light scattering from a fibrin clot (au) |
| Tlag | time between contact of plasma sample with activator and start of clot growth. Tlag characterizes the initiation phase of blood coagulation (min) |
| Tsp | time of spontaneous clot formation in plasma sample volume, which had no initial contact with activating insert. Spontaneous clotting is induced by circulating activators, active coagulation factors, and microparticles (min) |
| V | average rate of clot growth. The parameter characterizing the propagation phase of blood coagulation ($\mu\text{m}/\text{min}$) |
| Vi | initial rate, it characterizes the initiation phase of clot growth ($\mu\text{m}/\text{min}$) |

Superscript

| | |
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| a | in normal renal function |
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