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Ultrasonic Measurements of Blood and Plasma coagulation

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국문초록

지혈 시스템은 인체 내에서 두 가지 상반된 기능을 한다. 필요할 때마다 출혈을 멈추게 하기도 하고, 순환시스템 내에서 혈병의 방해 없이 혈액의 흐름을 유지시키는 기능을 한다. 이 시스템은 연속적인 효소 반응의 복잡한 응고 과정으로서 성능을 미세하게 조절하는 상호 기능을 하는 과정이다. 혈액응고인자 한 두개의 이상 농도는 과응고, 혈전증, 지혈장애로 인한 부적절한 섬유소 형성을 초래한다. 따라서 이 논문의 주된 목적은 초음파를 이용하여 혈액의 전 응고과정에 대해서 측정하는 것이다.

이번 실험의 목적은 전혈과 혈장 응고의 전과정과 헤파린(Heparin) 같은 항응고제 역할을 분석하는 초음파 기술을 개발하고, 지혈 시스템의 생리학적 과정을 음향학적 특성으로 연구하는 것이다. 이 목적을 달성하기 위해서 수온 37°C의 수조에서 중심주파수 5MHz 인 트랜스듀서(Transducer)로 초음파의 펄스-반향 방법을 이용해 혈액과 혈장 샘플의 음속과 감쇠계수를 측정하였다. 실험은 10 명의 사람 피를 사용 하였으며, 적혈구용적율은 평균 41%이다. aPTT(Activated Partial Thromboplastin Time)와 항응고제를 포함한 혈장의 응고 또한 Lab View 프로그램을 이용해서 실시간으로 측정하였다.

실험결과는 전혈 응고 시 음속이 1584m/s 에서 1595m/s 까지 증가했고, 감쇠계수는 2.2dB/cm 에서 4.3dB/cm 까지 증가했다. 혈장 응고 시에는 음속이 1535 m/s 에서 1541m/s 로 증가했으며, 감쇠계수는 1.25dB/cm 에서 3.2dB/cm 로 증가했다. 전혈과 혈장의 음속과 감쇠계수의 시간에 대한 기울기는 혈액 응고의 3 단계인, 초기 효소 단계, 확장 단계, 그리고 종결 단계인 생리학적 단계와 유사하다. 헤파린과 같은

항응고제를 투여한 혈장의 응고 시에는 음향 특성의 변화 양상이 달라지는 것이 밝혀졌다. 결론적으로, 전혈과 혈장의 응고에 대한 초음파 측정과 항응고제를 투여한 혈장의 응고과정을 함께 연구하면 혈액의 응고과정을 이해하는데 도움을 줄 것이다. 이러한 과정들은 항응고제를 개발하고 효과를 평가하는 다양한 기술에 적용될 수도 있다.

ABSTRACT

The haemostatic system has a dual paradoxical function in the body. It should stop bleeding whenever needed, but also keep the blood flowing in the circulatory system without any obstructing blood clots. The system is complex with series of enzymatic reactions, known as the coagulation cascade and intertwined processes that interact to produce a fine-tuned regulation of the performance. Abnormal concentration of one or more factors of blood coagulation results in an inadequate formation of fibrin by excessive coagulation ability, thrombophilia, and haemostatic disorders. Therefore, the main focus is to measure the whole process of coagulation using ultrasound.

The aims of this work are to develop an ultrasonic method for analyzing the whole process of blood and plasma coagulation and anti-coagulant activity of heparin, and to investigate the physiological phases of haemostatic system in terms of acoustic properties. To achieve this goal an ultrasonic pulse-echo method has been used for measurement of sound speed and attenuation coefficient in blood and plasma samples with a center frequency of 5 MHz at temperature of 37°C. The experiments were carried out using ten samples of human blood as an average hematocrit of 41 %. Plasma coagulation with aPTT (Activated Partial Thromboplastin Time) and with an anticoagulant has also been evaluated for haemostatic studies using Lab view program in real time.

Experimental results demonstrate that the average of sound speed and attenuation coefficient of whole blood were increased from 1584 to 1595 m/s, 2.2 to 4.3 dB/cm respectively, while the ones in plasma were increased from 1535 to 1541 m/s, 1.25 to 3.2 dB/cm during coagulation. The slope of

sound speed and attenuation of blood and plasma was changed at different physiological phases of enzymatic, propagation, and termination phases. Plasma coagulation with an anti-coagulant such as heparin was found to change the acoustic properties less with different patterns. In conclusion the combination of ultrasonic measurements of blood and plasma coagulation with/without an anticoagulant might help for a better understanding of coagulation processes, which may be applied in various techniques for the evaluation of the effects and development of anticoagulants and the drugs.

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Chapter I

Introduction

1.1 General

Thrombotic conditions affect more then 600,000 people in the world (Brown 1984). This means their blood has a tendency to clot without any injury (Brown 1984). Due to their conditions, patents will have a need for monitoring either during cardiac surgical procedure or on a regular basis during anticoagulant therapy.

Blood coagulation is a complex physiological process that helps to maintain hemostasis within the vascular system. It should stop bleeding whenever needed, but also keep the blood flowing in the circulatory system without any obstructing blood clots. It consists of series of enzymic reactions following activation of blood clot mechanism. There is a delicate balance among blood cells, coagulation factors and external tissue factors. The activation of these series of enzymatic reactions is known as coagulation cascade (Guyton 2000) as shown in Figure 1.1. These reactions are typically catalyzed by 20 different substances, most of which are plasma glycoproteins, with each step initiating another reaction until fibrin formation (Brown 1984). Abnormal concentration of one or more factors of blood coagulation results in an inadequate formation of fibrin.

According to physiological processes, blood coagulation is characterized by three phases, enzymic phase, propagation phase, and termination phase (Mann 1992) as shown in Fig 1.2. Extrinsic factor Xase is a principle player

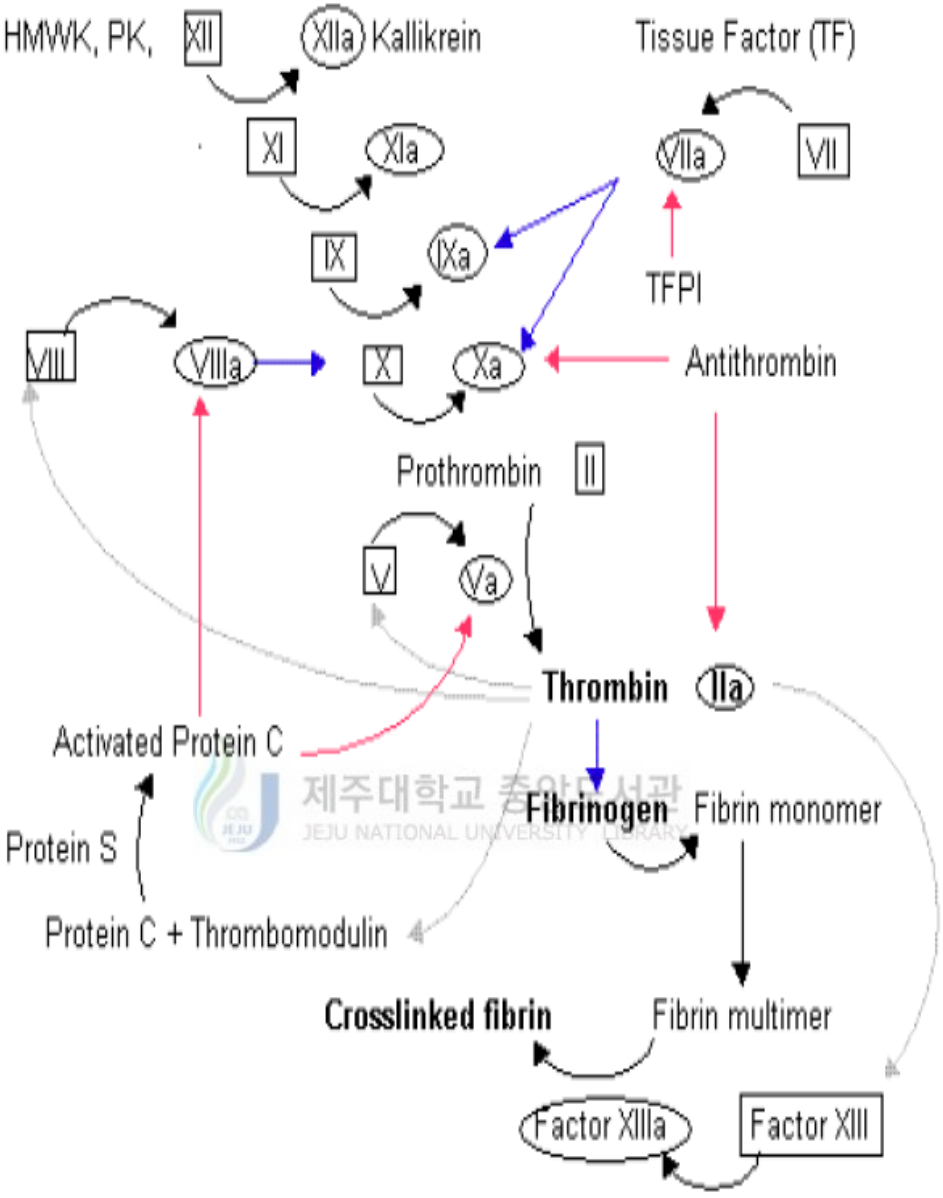
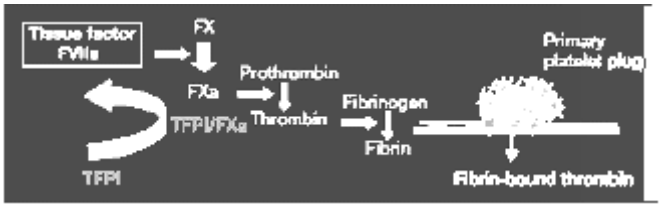
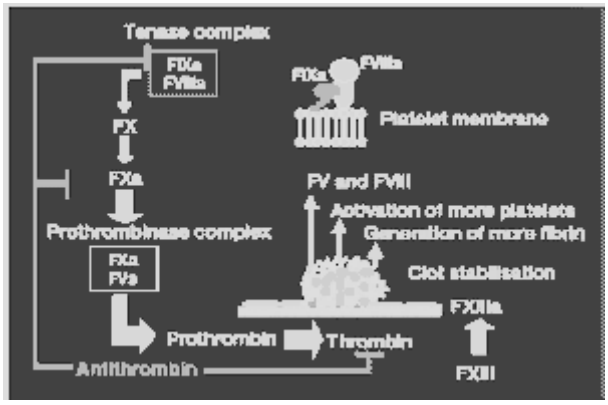


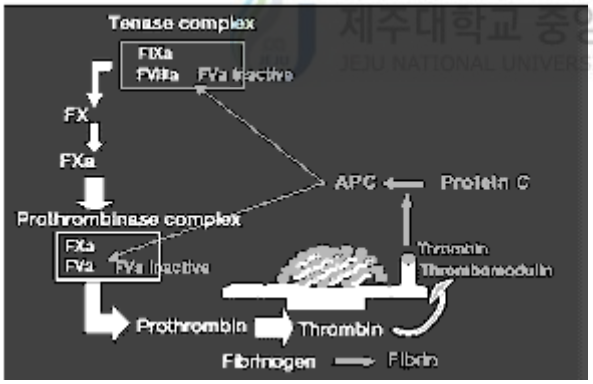
Figure 1.1 Coagulation cascade pathway (Guyton 2000)



Enzymatic phase



Propagation phase



Termination phase

Figure 1.2 Physiological phases of blood coagulation process (Mann 1992)

of enzymic phase, in which a tiny amount of thrombin is produced and prothrombin activator catalyzes the conversation of prothrombin to thrombin. Vigorous thrombin generation occurs during propagation phase in intrinsic Xase and continues reaction with prothrombinase (Mann 2002), starting to cleave fibrinogen and activate the factor XIII. Termination phase occurs through the stoichiometric inhibitors antithrombin III (AT-III) and tissue factor pathway inhibitor (TFPI) and through the protein C system. The clotting time is considered to mark the end point of enzymatic phase and the onset of propagation and termination phases.

Coagulation process is the primary stage that comprises the development of anti-coagulant drugs in medical industries. More over, the coagulation process is often used as a reference for determining the activity of anticoagulants. Therefore, an accurate determination of coagulation process is very important for an optimal evolution of anticoagulation drugs. On the other hand, abnormalities in the platelets, coagulation factors and blood vessel result in excessive bleeding. Therefore measurement of blood coagulation process may benefit to understand both types of conditions to improve diagnostic and therapeutic process.

1.2 Previous Studies

In last decades, monitoring of blood and plasma coagulation has been carried out in order to determine the coagulation process or/and clotting time. The investigations are based on electrical (Miale 1965), optical (Davey and Malley 1972), and laser (Pieiere et al 2004) methods. These systems were unable to measure the whole process of coagulation. Therefore, an ultrasonic method was introduced for monitoring the change of whole process of blood coagulation (Shung et al. 1984; Volesis 2002).

Measurement of acoustic properties of whole blood and plasma may allow us to detect the characteristics of the blood clotting deficiencies related to various diseases, to analyze anticoagulant activity of anticoagulant, to improve diagnosis of some diseases and to suggest the appropriate treatment.

1.2.1 Whole blood during coagulation

The first digital ultrasonic interferometer was introduced for testing small amount of whole blood sample during coagulation (Grybauskas et al. 1978). They investigated velocity and absorption of whole blood during coagulation. Further, ultrasonic quantitative parameters, including sound speed and attenuation were measured, and they have been demonstrated to be useful in the sensitive detection of the process of coagulation (Shung et al. 1984; Voleisis et al. 2002; Wang et al. 2002). In addition, high frequency ultrasound to measure the quantitative back scattering parameters and acoustic velocity as a function of hematocrit percentage of whole blood during coagulation were measured (Ossant et al. 2004). These previous researchers measured the whole blood coagulation at various temperatures using citrated blood with an addition of calcium chloride for coagulation. They did not investigate whole blood coagulation in real body temperature, as natural processes of coagulation. Previous measurements of sound speed and attenuation on whole blood coagulation have shown that coagulation process may be affected at different temperatures conditions, with different hematocrit percentage, using citrated whole blood coagulation with thrombin or calcium chloride (shung et al.1984; Ossant et al. 2004; Huang et al. 2005).To consider all the previous researches, it is crucial to develop another feasible approach to measure the whole blood coagulation at

different hematocrit percentages with a natural process of coagulation at real body temperature.

1.2.2 Plasma during coagulation with aPTT

The determination of plasma coagulation time is an essential part of monitoring therapeutic anticoagulants. Standard methodologies for the measurement of plasma clotting are available to evaluate elapsed time from the beginning of an induced coagulation process up to the formation of a stable fibrin polymer. Currently, accepted methodologies are based on optical and mechanical. Optical detection can be preformed either with naked eyes or as a reduction in transmittance when light passes through the sample (Davey and Malley 1972). Mechanical transduction is typically carried out with a vibrating or rotating metal ball. Cessation of ball moving was considered as the endpoint of coagulation. An ultrasonic method has been studied from last few years to measure the plasma clotting time. Alves and Machado used acoustic streaming method using backscatter signals from spherical glass particles (in 1991) and using ultrasonic shear wave method (in 1994) to detect the plasma clotting time.

Deviation from normal time range of plasma clotting of previous researches indicating that some factors is not adequately performing its function in the process, and further tests will be required for its identification. Each test initiates the coagulation process at a different stage and the measured time provides clue about the malfunction factor. An ultrasonic method to measure whole coagulation process has been employed for the monitoring of plasma coagulation. During plasma coagulation, the coagulum changes due to the formation of a fibrin clot. In turn, this changed coagulum shifts the characteristic resonance amplitude of the ultrasonic

transducer enabling real-time continuous monitoring of this biological event. By monitoring the signal output as a function of time, a distinct blood clotting profile can be seen.

1.2.3 Plasma during coagulation with aPTT and heparin

The activated partial thromboplastin time (aPTT) and the activated clotting time (ACT) are the most extensively applied measurements of the heparin effect. Coagulation analyzer (such as the Hemochron system) is the principle player of measurement of aPTT and ACT. Various new and improved coagulation analyzers (devises) were available on market in recent years (Cheng et al. 1998; Hepel et al. 2003). Hand held prototype of small capillary coagulation analyzer have been recently developed. They are based on the drawing of blood or plasma into small capillary, and coagulation is detected using either a laser (biotrack, ciba-corning) or pressure sensing system (Nyco Med). However these systems are quite complex and require a laser or pressure sensing equipment. The other method was a piezoelectric quartz crystal (PQC) sensor for determination of blood and plasma coagulation with heparin (Chang et al. 2000; Cheng et al. 1998). None of the previous studies investigated the properties of heparin during coagulation with plasma using ultrasound. In order to investigate the whole process of plasma coagulation with heparin as a function of time, ultrasonic pulse echo method was used in this study. This study investigated the whole process of plasma coagulation with heparin and effects of specific factors and/or process during coagulation in terms of sound speed and attenuation.

1.3 Specific Aims of this Research

This research primarily is to measure ultrasonic velocity and attenuation coefficient measurement of whole blood and plasma during coagulation. Specifically, the studies can be divided into three aims. The first aim is to measure the acoustic properties of whole blood during coagulation at real body temperature with natural process when exposed to the air. The acoustic properties of whole blood during coagulation at body temperature of 37°C are needed due to the increasing interest to identify the three different phases of coagulation as indicated in physiology. Human blood coagulation at real body temperature, at a certain hematocrit and natural process without any chemicals may be higher sensitivity than at the room temperature with addition of calcium chloride.

The second aim is to thoroughly investigate the whole process of plasma coagulation with aPTT. Until now no investigations are carried out to measure the whole process of plasma coagulation. A feasible method for measurement of acoustic properties of plasma during coagulation with respect to time was developed. Structural variation of whole process of coagulation was investigated since the standard clinical method may not be applicable to know each stage of coagulation.

The third aim is to extend the study to the plasma coagulation with commercial available heparin, to study the effects of specific factors and/or process of heparin as an anticoagulant using ultrasound. The ultrasonic properties of plasma during coagulation with heparin may identify coagulation/clotting time from the ultrasound signals.

1.4 Overview

There are six chapters in addition to the chapter of introduction.

Chapter 2 provides the theoretical background for measurement of ultrasonic velocity and attenuation coefficient from both blood and plasma during coagulation. Attenuation coefficient measurement model includes three layer transmission techniques to interpret the attenuation in time domain.

Material and Methods for measurement of acoustic properties of whole blood and plasma during coagulation was presented in Chapter 3. Properties of both blood and plasma were introduced briefly. Measurement of acoustic properties of blood and plasma coagulation using a pulse echo method was discussed in detail.

Chapter 4 describes the measurement of acoustic properties of whole blood during coagulation and analysis of three distinguishable phase of blood coagulation in terms of sound speed.

Chapter 5 shows the results on measurement of sound speed and attenuation coefficient of plasma during coagulation with aPTT. Analysis of whole process of coagulation was discussed.

Chapter 6 covers the measurement of sound speed and attenuation coefficient of plasma coagulation with heparin. Specific factors and process of plasma coagulation with heparin in terms of sound speed and attenuation were investigated.

Chapter 7 summarized the findings in this research and also provides some recommendations for future work.

At last, appendix includes the preliminary experiments with other anticoagulants (brown and red algae) from seaweed were presented.

Chapter II

Theory for measurement of sound speed and attenuation coefficient

2.1 Introduction

The theories about sound speed and attenuation coefficient were described in this chapter for a better understanding the experiments on blood and plasma coagulation with/without heparin. It includes the theory about the change of sound speed and attenuation coefficient within the blood/plasma sample during coagulation. Sound speed and attenuation coefficient are important ultrasonic parameters to derive blood and plasma coagulation constants and characterization of whole process of coagulation.

2.2 Ultrasonic velocity

The technique used to measure ultrasonic velocity of blood /plasma during coagulation was classical differential pulse-echo method. A change in sound pulse transit time when a sample is incepted with in the path was measured by using known speed of sound to infer the sound speed of the sample. Consider a source of an ultrasonic pulse S, and a received ultrasonic pulse R as shown in Fig.2.1. Suppose a right rectangular sample container of homogeneous blood/plasma sample is inserted in the path of beam, such that its flat faces are perpendicular to the direction of propagation of the pulse. The dotted box in Fig. 2.1 is defined as thickness of the container (L_m). Triggering is done via the pulse generated at the source so that the received pulse displayed on an oscilloscope looks like that as shown in Fig.2.1.

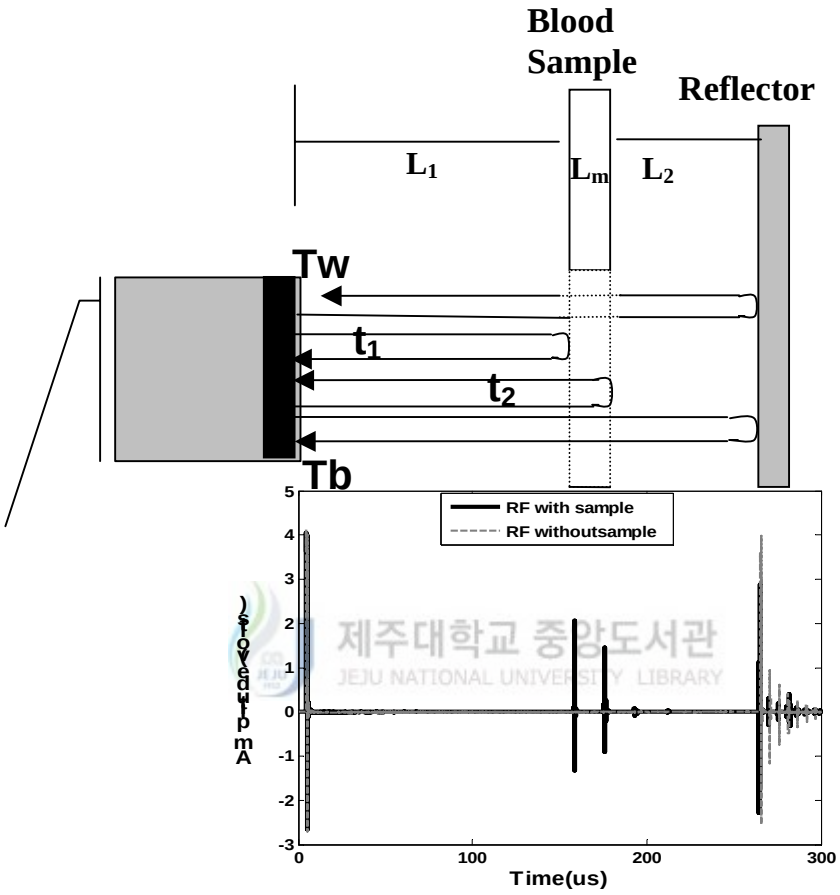


Figure 2.1 Time shift mechanism between wrap layers and reflected signal with and without sample

The time shift (t_2-t_1) in the position of the RF pulse from sample the front and rare face and the time shift (Tw-Tm) from the steel reflector with and without the sample in the path (Kuo 1990) as shown in Fig .2.1.

$$L_m = L - L_1 - L_2 = L - (t_1/2)\, C_w - [(T_m - t_2)/2]\, C_w \quad (2.1)$$

$$T_w/2 = L/C_w = 1/C_w\,(L_1 + L_m + L_2) \quad (2.2)$$

$$T_m/2 = 1/C_w\,(L_1 + L_2) + L_m/C_m \quad (2.3)$$

From Equation (2.2) and (2.3)

$$T_m - T_w/2 = L_m\,(1/C_m - 1/C_w) \quad (2.4)$$

Substituting Equation (2.1) into (2.4), we have

$$T_m - T_w/2 = (T_w/2 * C_w - t_1/2 * C_w - (T_m - t_2)/2 * C_w) * (1/C_m - 1/C_w) \quad (2.5)$$

Therefore

$$C = Cw\,[((Tw - Tm)/(t_2 - t_1)) + 1] \quad (2.6)$$

, where C_w is the sound speed of water, T_w and T_m are the time of flight from the transducer to the reflector without the sample and with sample, respectively. t_1 and t_2 are the time from the front and the rear face of the sample, respectively, and C is the sound speed of blood or plasma sample.

2.3 Ultrasonic attenuation

The amplitude of a plane ultrasonic wave decreases as the propagating distance increases. The amplitude of the ultrasonic wave which has propagated the distance of *x* is represented as

$$A(X) = A_0 e^{-\alpha x} \tag{2.7}$$

, Where α is the attenuation coefficient of the propagation media. X is distance traversed. A_0 is the attenuation of the wave and A is the amplitude after a distance x has been traversed. Like the sound speed, the attenuation coefficient is dependent upon the physical properties of the medium. However, unlike the sound speed, the attenuation coefficient varies greatly with frequency. It can be write as $\alpha = \alpha_a + \alpha_s$. Where α_a is the absorption coefficient and corresponds to the conversion of wave energy into internal energy (heat) of the medium. α_s corresponds to the scattering of the primary beam energy into all the different directions. The dimension of α is length $^{-1}$, and it can be expressed in nepers/cm. In medical acoustics it is more common to express the attenuation coefficient in dB/cm. To measure the attenuation coefficient as a function of total transmition coefficient of blood and plasma, the amplitude of the reflected echoes with and without sample were compared (Madsen et al.1999), as shown in Fig. 2.2.

$$\alpha = (20 / d) \log_{10} [A_0 / (A / T_{total})] \tag{2.8}$$

, where A_o and A are the peak to peak signal amplitude without and with the sample, respectively.

2.4 Reflection and Transmission of Plane Waves Normally Incident on a Layer between two Media

When a plane wave is normally incident on the plane interface, the wave incident on the front and rear surface of a sample container is reflected, and the reflected wave returned into the transducer, and some of it propagate and reflect from a perfect reflector The wave from the reflector meets the boundaries and reflects and transmits repeatedly.

$$R = \frac{r_2 - r_1}{r_2 + r_1} \tag{2.10}$$

1+R=T, we have

$$T = \frac{2r_2}{r_2 + r_1} = \frac{2r_2 / r_1}{r_2 / r_1 + 1} \tag{2.11}$$

The intensity transmission and reflection coefficients, T_I and R_I, respectively, are in the followings (Kinsler et al. 1980, pp. 124-131, p. 462),

$$R_I = I_r / I_i = |R|^2 \tag{2.12}$$

$$T_I = I_t / I_i = (r_1 / r_2)|T|^2 \tag{2.13}$$

$$R_I = \left(\frac{r_2 - r_1}{r_2 + r_1}\right)^2 = \left(\frac{r_2 / r_1 - 1}{r_2 / r_1 + 1}\right)^2 \tag{2.14}$$

$$T_I = \frac{4r_2 r_1}{(r_2 + r_1)^2} = \frac{4r_2 / r_1}{(r_2 / r_1 + 1)^2} \tag{2.15}$$

Where r_1 is the characteristic acoustic impedance of the medium in which the incident and reflected wave are traveling, and r_2 is that of the medium in which the transmitted wave is traveling. In ultrasonic testing, Eqs. (2.12) and (2.13) are generally used to obtain intensity reflection and transmission coefficients between a transducer and a test material. Since the case of the material being tested in this laboratory, a thin (100 μm) layer of OHP (Overheard Projector) film was used in this experiment for an acoustic window and this thin layer should be considered to compute the intensity reflection and transmission coefficients as shown in Figure 2.3.

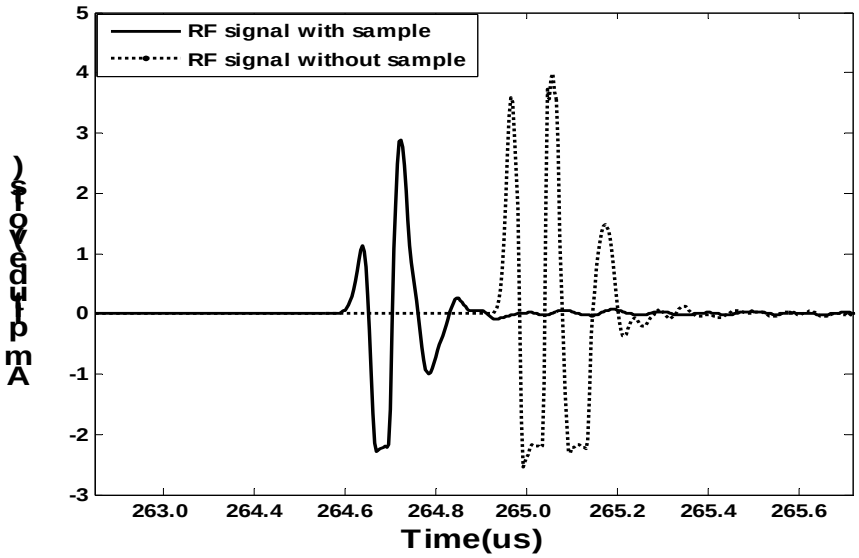


Figure 2.2 Amplitude difference of reflected signal from perfect reflector with and without sample

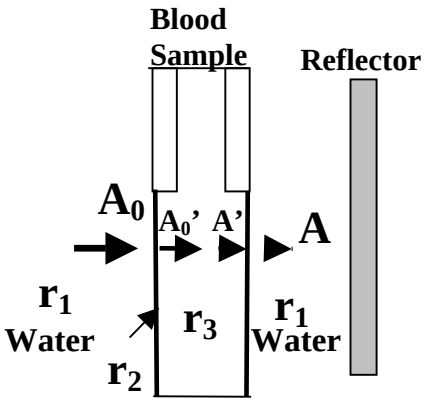


Figure 2.3 Through transmission technique

When a layer of finite thickness is formed between two media, an ultrasonic wave normally incident on the interface from medium 1 generates the reflected waves and transmitted waves into the layer. The transmitted wave into the layer is reflected again at the rear interface and the transmitted into medium 3. In this case, the reflection coefficient R is expressed as follows

$$R = \frac{(1-r_1/r_2)\cos k_2 L + j(r_2/r_3 - r_1/r_2)\sin k_2 L}{(1+r_1/r_3)\cos k_2 L + j(r_2/r_3 + r_1/r_2)\sin k_2 L} \tag{2.16}$$

The intensity transmission coefficient, T_I is represented as follows:

$$T_I = 1 - |R|^2 \tag{2.17}$$

$$T_I = \frac{4}{2+(r_3/r_1+r_1/r_3)\cos^2 k_2 L+(r_2^2/r_1 r_3+r_1 r_3/r_2^2)\sin^2 k_2 L} \tag{2.18}$$

Here

$$k_2 = \frac{2\pi f}{C_2} = \frac{2\pi f \rho_2}{r_2} \tag{2.19}$$

$$T_{total} = \frac{4}{2+(r_3/r_1+r_1/r_3)\cos^2(2\pi f \rho_2 L/r_2)+[r_2^2/(r_1 r_3)+(r_1 r_3)/r_2^2]\sin^2(2\pi f \rho_2 L/r_2)} \tag{2.20}$$

, where T_{total} is the total amplitude transmission coefficient through both bounded wrap layers. r_1, r_2, and r_3 are the acoustic impedance of water, the OHP film and the blood, respectively. f is frequency, ρ_2 is the OHP film density and L is the OHP film thickness.

Chapter III

Materials and Method

3.1 Introduction

The physical properties of blood and plasma will be briefly described in this chapter. There are various approaches for the measurement of sound speed and attenuation coefficients of whole blood and plasma during coagulation. The standard pulse echo method has been used for a long time to measure the quantitative backscattering, includes sound speed and attenuation coefficient of whole blood during coagulation as a function of time or frequency (Shung et al. 1984; Voleisis et al. 2002; Wang et al. 2002). Measurement of whole blood coagulation at real body temperature, at a certain hematocrit and natural process without any chemicals, whole process of plasma coagulation with /without heparin in terms of sound speed and attenuation coefficient has been proposed. The pulse echo method was discussed in this chapter.

3.2 Properties of Blood

3.2.1 General

Blood is the fluid of life, transports oxygen from the lungs to body tissue and carbon dioxide from body tissue to the lungs. Blood is the fluid of growth, transporting nourishment from digestion and hormones from glands throughout the body. Blood is the fluid of health, transports disease fighting substances to the tissue and waste to the kidneys. It is mainly composed of about ~ 45 % of red blood cells (erythrocytes) and ~ 55% of viscous fluid

called plasma with small amount of white blood cells (leukocytes) and platelets (thrombocytes). Plasma is basically a saline solution of three major proteins including fibrinogen, globulin and albumin. The volume of red blood cells in the whole blood is defined as hematocrit and it is expressed in percentage.

Plasma contains clotting factors such as factor VIII, which is used in the treatment of hemophilia, albumin used for the treatment of surgical shock or severe burns, and Immunoglobulins, for protection of the body against infectious diseases and the treatment of immunodeficency. However, it must be noted that blood plasma consists of more than 90 % water. Albumin (the major component), Immunoglobulins, fibrinogens and other components used in the coagulation process. Other components are also important but the fractions are small, like lipids less then 1 gram per 100 milliliters.

3.2.2 Blood and plasma samples

Experiments were conducted using 7 ml of whole blood and 2 ml of plasma sample of ten individual healthy volunteers, without any history of bleeding or thrombosis. The blood was drawn from healthy volunteers in 20 ml syringe and a 7ml of the blood was immediately transferred to the container for measuring the acoustic properties of whole blood. The other 9 ml was placed into 1 ml of 3.8% sodium citrate solution for preparation of plasma. Before preparing plasma sample hematocrit was measured using a centrifuge (HA-200, Hanil Science Industrial). In the laboratory, plasma from blood was prepared by centrifugation of blood for 15 min at about 3500 RCF (relative centrifugal force) using a (Hanshin Medical Co. Ltd., Model-AT-650) centrifuge and than plasma was stored in -70°C until assayed.

3.2.3 Chemicals and other materials

Activated partial thromboplastin reagent aPTT (ellagic+ bovine phospholipids) and calcium chloride ($CaCl_2$) of 0.02M were obtained from an international reagent corporation (Japan).Heparin was obtained from a commercial pharmacy. The blood sample container was designed using acryl material of dimensions 10 cm long x 2 cm wide x 2 cm thick and in that 2 cm wide and 4 cm drill was made in the front and back side of sample container and sealed with OHP film of 100 micron thick for acoustic window as shown in Fig 3.1

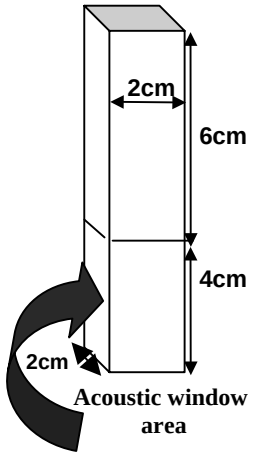


Figure 3.1 Sample container with an acoustic window

3.3 Measurement procedure

Measurements were made with the experimental configuration depicted in Fig 3.2. The experiments were performed in a degassed water tank at temperature of 37.0±0.2°C. A temperature controller with a liquid circulator was used for keeping temperature constant, and temperature was continuously monitored by an immersed thermometer.

First, the acoustic impedance and sound speed of OHP film were computed using a three-layer transmission technique (Kingsler and Frey), film density and thickness were obtained from manufacturer. The attenuation coefficient of water was assumed to be negligible (Madsen et al. 1999). The sound speed and attenuation coefficient of the blood and plasma samples were determined by a pulse echo method using a Pulser/receiver (*Panametrics 5800 PR, CA, USA*). Blood sample was placed in the focal zone of a 5 MHz transducer (*Panametrics V326*) with a diameter of 9.5 mm and the sample was positioned perpendicular to the direction of propagation of ultrasound. Ultrasonic pulses were periodically transmitted at a pulse repetition frequency of 500 Hz. The distance between the transducer and the front surface of the sample was approximately 13 cm was maintained to minimize diffraction error. A magnetic stirrer at the bottom of the tank prevented the blood settling. The reflected acoustic signals from the front and back surface of the sample container and a steel reflector were digitized at a sampling rate of 250 Megasamples/s and recorded in a LeCroy (*LC547AL USA*) digital oscilloscope. The sound speed and attenuation of blood and plasma samples were determined by comparing the reflected signals from the reflector with and without the sample in place.

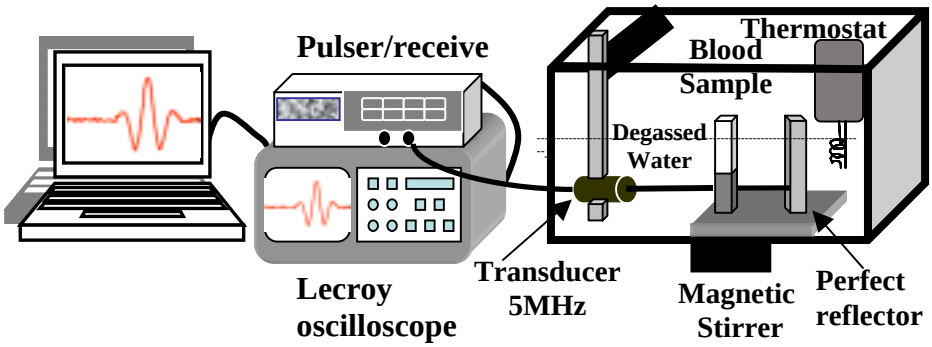


Figure 3.2 Ultrasonic measurement system

After coagulation, mass and volume of the coagulated blood or plasma sample were measured to find the density and acoustic impedance of blood or plasma. The experimental data were collected every 4 seconds over 65 minutes in blood coagulation and over 45 minutes in plasma coagulation. Collected data were analyzed using MATLAB® . Moving average was taken on every 15 data points of the stored data to smoothen the profiles of blood and plasma.

Chapter IV

Measurement of acoustic properties of whole blood during coagulation

4.1 Introduction

Previous measurements of sound speed and attenuation on whole blood coagulation have shown that coagulation process may be affected by temperature, hematocrit, and thrombin or calcium chloride (Shung et al. 1984; Ossant et al. 2004; Huang et al. 2005). This observation can be interpreted that the sound speed and attenuation coefficient of human blood coagulation at real body temperature could be monitored in real time at a certain hematocrit and natural process without any chemicals.

The investigation of acoustic properties from whole blood during coagulation as a function of time at body temperature of 37°C is of interest because coagulation process depends on temperature. Since natural blood coagulation occurs without any chemicals, no chemicals are added. For these reasons, the physiological changes of blood coagulation in terms of sound speed and attenuation were investigated from whole blood after exposed to the air without adding any chemicals. The sound speed and attenuation coefficient of whole blood coagulation was measured at a frequency of 5 MHz unfocused transducer at 37°C.

4.2 Blood preparation

Experiments were conducted using 7 ml of whole blood of ten individual healthy volunteers, without any history of bleeding or thrombosis. The blood was drawn from a forearm vein of a healthy volunteer using a syringe needle. Blood from a syringe was immediately transferred to the sample container for measuring the acoustic properties of whole blood. For each sample, all experiment set up was arranged before drawing the blood.

4.3 Method for whole blood coagulation measurement

Experimental set up was shown in Fig 3.2. The test sample container was placed perpendicular to the direction of propagation of ultrasound. The temperature of water bath was adjusted to 37°C. The distance between the face of the transducer and the sample was adjusted. The correct level was initially determined using amplitude of the first echo from the front face of sample container and perfect reflector was maximized on the oscilloscope. Time was allowed for the temperature to be stabilized. The blood was drawn on site by an experienced nurse through a 10 ml syringe from a healthy volunteer at the test place. It was immediately transferred to the sample container within few seconds. Broadband pulses were transmitted at a pulse repetition frequency of 500 Hz. The reflected signals from the blood sample and a steel reflector were digitized at a sampling rate of 250 MHz. Data were collected in every 4 seconds over 65 minutes. The data were analyzed using Matlab software. The whole process was shown in block diagram as shown in Fig.4.1.

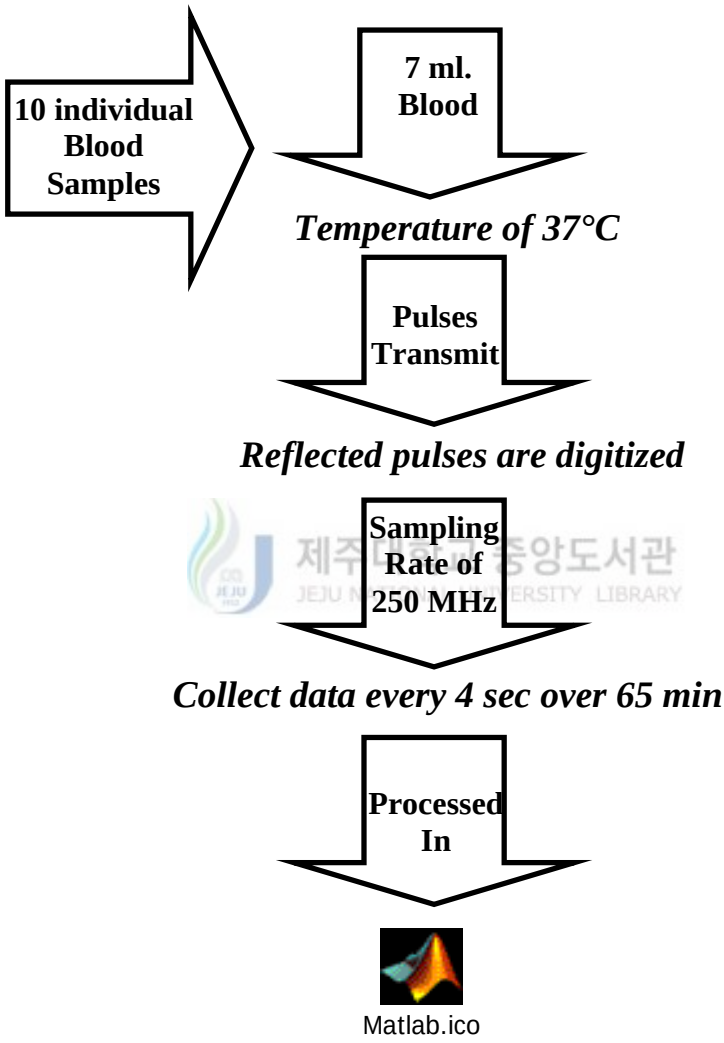


Figure 4.1 Block diagram of whole blood coagulation process

4.4 Results

The temporal variations of sound speed and attenuation coefficient during blood coagulation at 37°C are presented in Figs. 4.2,4.4,4.5 & 4.7.The ten curves (A, B, C, D, E, F, G, H, I, J) with different markers in the figures represent sound speed and attenuation coefficient variations of ten individuals blood samples during coagulation. Out of ten blood samples, eight samples showed the same trend of curves and the other two curves show different pattern. These two samples are not consistent with the other results. These variations may be not from the measurement errors but from the differences of blood itself, since the attenuation coefficients are similar to the ones from the other blood samples. It requires further investigation. Sound speed and attenuation variations from two samples were shown separately in Figs. 4.4 & 4.7 respectively. In this research, only ***eight*** blood samples were considered for further explanation. The average sound speed of eight blood samples over an hour during coagulation was increased from 1585 m/s to 1596 m/s as shown in Fig 4.3. Lines and error bars with open circles in the Fig. 4.3 represent the average and the standard deviation of eight sample measurements, respectively. These results were in good agreement with the previous ones (Shung et al. 1984; Voleisis et al. 2002). Table 1 showed the average sound speed and attenuation coefficient of whole blood during coagulation and the results of the other groups were given for comparison.

The curves represent the peculiar stages of blood coagulation of volunteers in Fig. 4.2. At the beginning stage of coagulation process up to 5

minutes, sound speed of whole blood sharply increased. The slope became less steep after 5 minutes, and further changed at around 27 minutes. After that time, sound speed from *seven* blood samples was not changed much. The other one showed the different pattern from the other *seven* samples, though the sound speed variations are similar.

Attenuation coefficient of eight individual blood samples during coagulation was shown in Fig. 4.5 and their patterns are different from the ones of sound speed. Average attenuation coefficient of eight blood samples was increased from 2.5 dB/cm to 4.3 dB/cm at 5 MHz as shown in Fig 4.4. Lines and error bars with open circles in the figures represent the average and the standard deviation of eight sample measurements, respectively. At the beginning stage of coagulation process, the attenuation of eight blood samples was decreased or remained the same up to about 12 minutes, while the sound speed was increased. At that time it showed minimum attenuation of 2 dB/cm (Fig 4.6). After 12 min, it increased almost linearly with time over 65 minutes.

Parameters / Acoustic properties	Whole blood at 37^0C	Previous researchers	
		K.K.Shung et al.	A.Volesis et al.
		Whole blood at 23^0C	Whole blood at 37^0C
Sound speed (m/s)	1585-1596	1556-1590	1585-1600
Attenuation coefficient (dB/cm)	2.5-4.3	1.7 - 4.0	———

Table 1 Average sound speed and attenuation from eight samples during blood and plasma coagulation and comparison with the previous groups

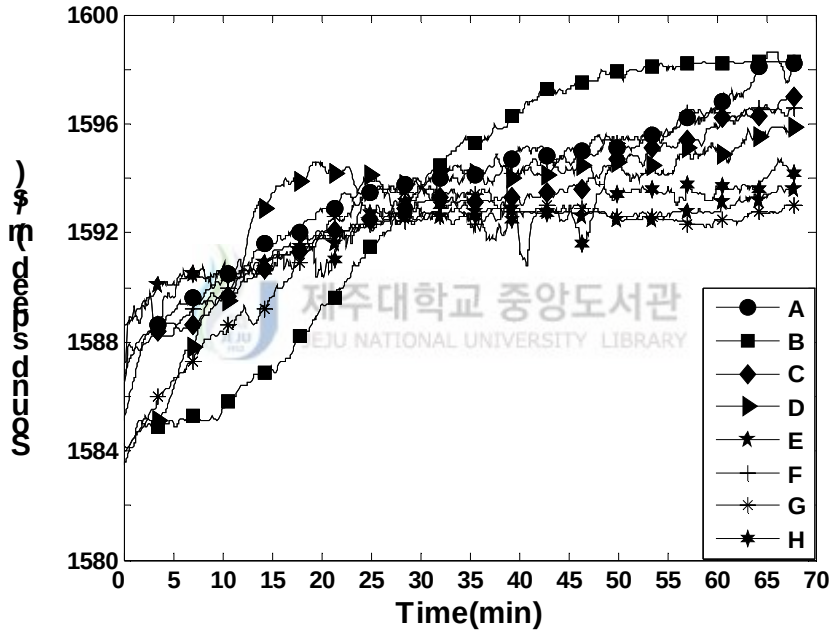


Figure 4.2 Sound speed variations of eight individuals during blood coagulation

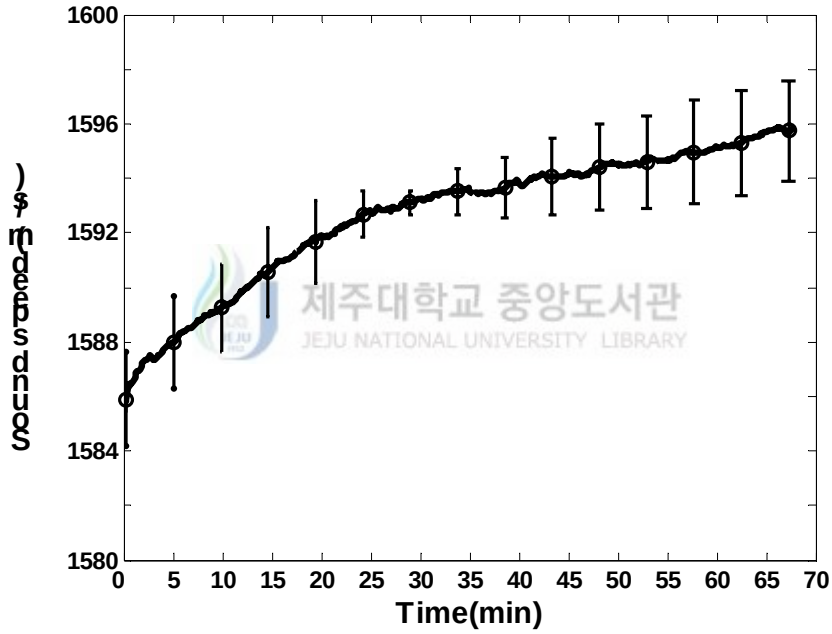


Figure 4.3 Average sound speed variation of eight individuals during blood coagulation

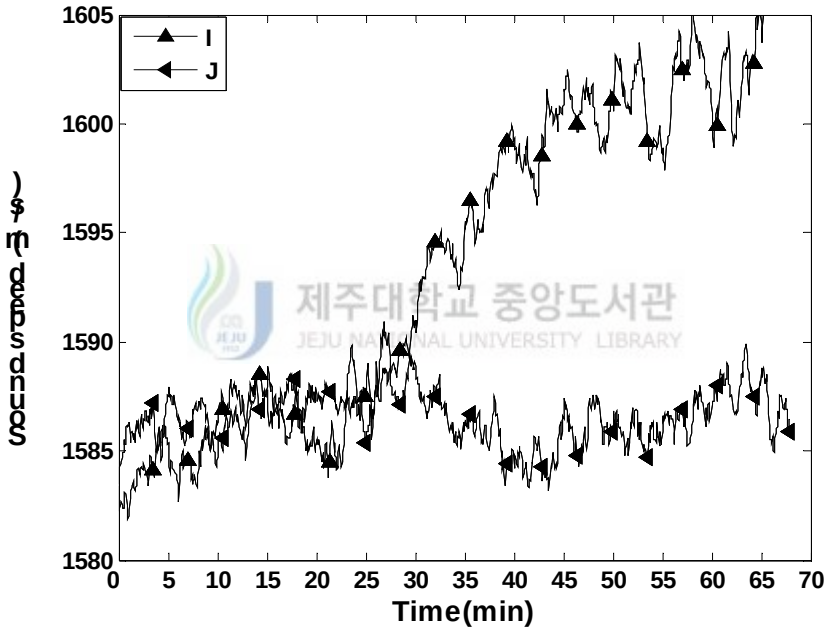


Figure 4.4 Sound speed variation of nine and ten individual during blood coagulation

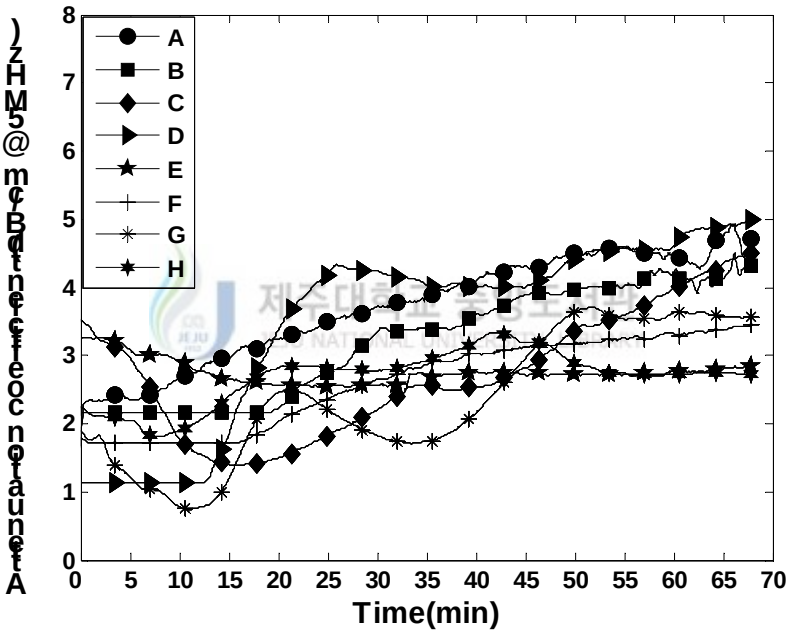


Figure 4.5 Attenuation coefficient variation of eight individuals during blood coagulation

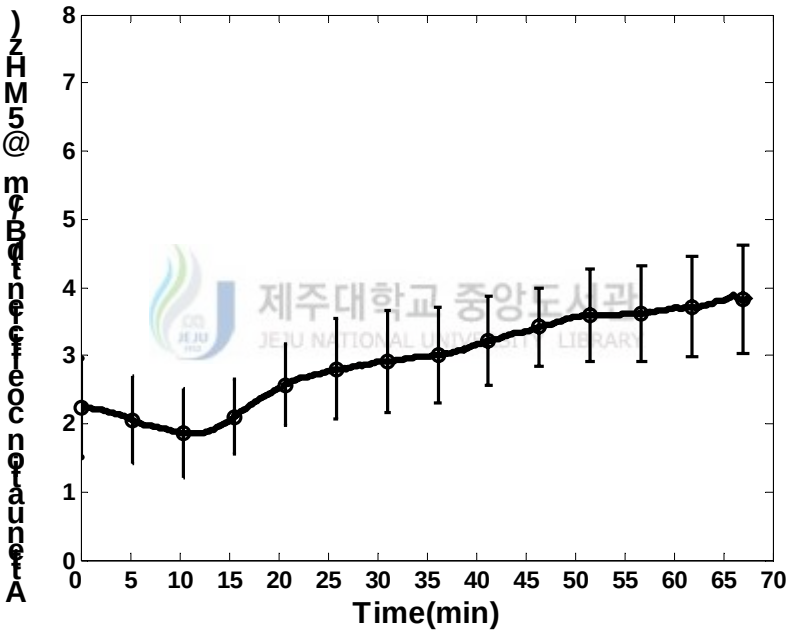


Figure 4.6 Average attenuation coefficient variation of eight individuals during blood coagulation

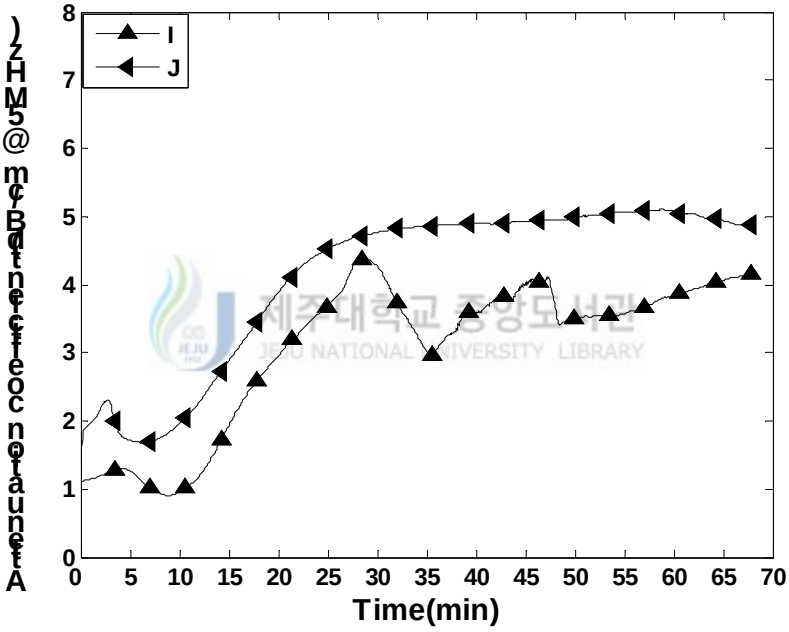


Figure 4.7 Attenuation variation of nine and ten individual during blood coagulation

4.5 Discussion

Whole blood coagulation processes were investigated in terms of acoustic properties. Sound speed and attenuation coefficient were increased during blood and plasma coagulation processes, but they changed with the different patterns. Sound speed variation changed in three phases. At first, sound speed increased sharply until about 5 minutes, followed by a gradual increase until 27 minutes and then slow change toward a steady state situation. The variation of sound speed seemed to correspond to the three physiological phases of coagulation, the enzymatic phase, the propagation phase, and the termination phase. However attenuation variation did not show the three phases. Attenuation was stable or decreased until 12 minutes and then increased linearly with time.

In the first phase, sound speed of eight blood samples started at different values but increased rapidly up to 2 m/s within 5 minutes (Fig. 4.2). At this phase, soft and gel type coagulum was formed as prothrombin converted into thrombin in both intrinsic and extrinsic coagulation pathways. After that the sound speed increased progressively, while attenuation was stable or decreased until 12 minutes. The changes of attenuation coefficient are from the changes in acoustic impedance of the clotted blood, mainly because of an increase in the reflection and the scattered energy losses (Shung et al. 1984). At this phase the coagulated blood was in elastic medium and the energy loss in elastic medium is less. The slope of sound speed and attenuation is dependent on the strength of the coagulum, which may signifies the beginning of proper coagulation because of the bulk of thrombin formation and the reaction with fibrinogen (Mann 2002). In the second phase, the sound speed showed a gradual increase until 27 minutes and sound speed of eight samples was nearly equal to the same value

(~1593 m/s) at about 27 minute which was indicated in small error bar in Fig.4.3. Attenuation was increased linearly from 2~4.5 dB/cm during this stage because the viscosity of blood increased (Wang et al. 2000; Wang and Tsui 2004). The increase of attenuation and sound speed of eight blood samples may indicate the possibility of solid fibrin polymer formation from fibrinogen (Shung et al. 1984).

The third phase of whole blood coagulation seemed to start after formation of solid fibrin, i.e. after 27 minute of coagulation. Average sound speed was increased 3 m/s smoothly until 65 minutes in whole blood. Seven curves showed almost the similar patterns of variations except one showing higher sound speed, while attenuation kept increasing until 65 minutes. This phase may be because of activation of protein C with cofactor protein S and propagation of reactions with thrombomodulin within the blood sample (Mann 1992; Comp 1982).

4.6 Conclusion

The effects of blood coagulation on the acoustic properties from ten volunteers were investigated. It was found that sound speed and attenuation of blood increased during coagulation. The variation of sound speed during whole blood coagulation showed the three distinguishable changes of slope with respect to time, and these three different variations may be comparable to the physiological phases (enzymatic, propagation and termination phases) of blood and plasma coagulation. The processes of whole blood coagulation at nearly body temperature were complicated and sound speed and attenuation fluctuations are relatively high.

Chapter V

Measurement of acoustic properties of plasma during coagulation

5.1 Introduction

In this chapter, the whole process of plasma coagulation was measured in terms of sound speed and attenuation coefficient as a function of time. Ultrasonic backscatter signals from spherical glass particles (Alves and Machado 1991) and ultrasonic shear wave method (Alves and Machado 1994) have been employed to obtain the plasma clotting time. Ultrasonic measurements of the whole process of plasma coagulation with aPTT were not yet reported. The structural changes of whole process of plasma coagulation with aPTT as function of time were measured as temporal changes of sound speed and attenuation this research.

5.2 Plasma preparation

Experiment was performed using 2 ml of plasma of ten individual healthy volunteers, without any history of bleeding or thrombosis. 6 ml of blood was drawn from a forearm vein of a healthy volunteer using a syringe needle. After discarding the first milliliter, 90% volume of blood was collected in a polystyrene tubes containing 10% volume of 3.8% trisodium citrate. The blood was centrifuged for 15 min at about 3500 RCF (relative centrifugal force) using a centrifuge (Hanshin Medical Co. Ltd., Model-AT-650) and then plasma was separated and stored in -70°C until assayed.

5.3 Method for plasma coagulation measurement

Experiment was performed with plasma samples of ten individuals. The experimental set up was shown in Fig. 3.2 and the same as used in the measurement of the blood coagulation, as explained in chapter 4. For all plasma sample measurements, 2 ml of plasma was placed into a sample container using a pipette and incubated at 37±0.2°C for not longer than 5 minutes to avoid loss of factors V and VII. Then 2 ml of aPTT was added and thoroughly mixed by using a magnetic stirrer. After incubation for 5 minutes to reach thermal equilibrium, 2 ml of pre-incubated calcium chloride (0.02M) solutions was added for initiation of coagulation. Ultrasonic broadband pulses were transmitted at a pulse repetition frequency of 500 Hz. The reflected signals from the plasma sample and a steel reflector were digitized at a sampling rate of 250 MHz. Data were collected in every 4 seconds over 45 minutes. Post-processing was done in Matlab software. The block diagram of whole process of plasma coagulation was as shown in Fig. 5.1.

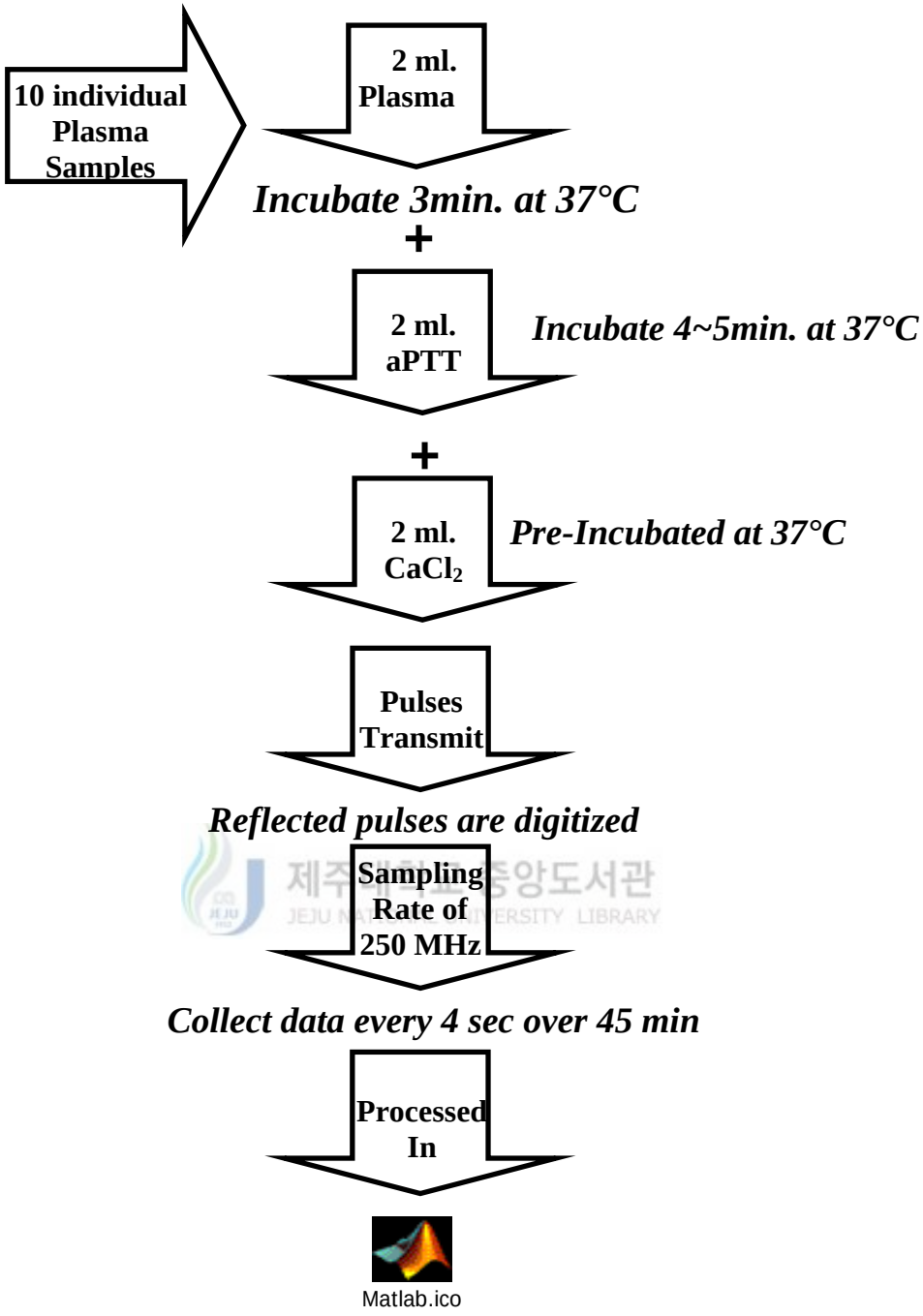


Figure 5.1 Block diagram of plasma coagulation process

5.4 Results

Sound speed and attenuation variations of ten individuals during plasma coagulation at 37^0C are presented in Figs 5.2, 5.3, 5.5 & 5.7. The curves (A, B, C, D, E, F, G, H, I, J) with different markers in the figures represent the structural changes of ten plasma samples during coagulation. In case of plasma, eight samples out of ten plasma samples, showed the same trend of curves and the other two curves showed the different pattern. These two samples variations are not consistent with the other result. . These variations may be not from the measurement errors but from the differences of plasma itself, since one sample sound speed and the other sample attenuation coefficient are similar to the ones from the other plasma samples. It requires further investigation. Sound speed and attenuation variations of two samples were shown separately in figures 5.4 & 5.7 respectively. In this research, only ***eight*** plasma samples were considered for further explanation. The average sound speed of *eight* plasma samples over 45 min of coagulation was increased from 1535 m/s to 1542 m/s which was shown in Fig 5.3. Lines and error bars in the figures represent the average and the standard deviation of eight samples, respectively. The plasma coagulation process is significantly faster compared to the blood coagulation. At initial phase the sound speed was increased rapidly over about 5 minutes. Between 5 to 27 minutes, it increased slowly and after that it remained the same. Sound variations among different plasma samples are smaller excluding one sample (A) of higher sound speed.

The variation of ten individuals attenuation coefficient of plasma during coagulation is shown in Figs. 5.5 & 5.7. Figure 5.6 shows the average and the standard deviation values of the relative attenuation coefficient of plasma during coagulation as a function of time. Average attenuation

coefficient of eight plasma samples was increased form 1.2 dB/cm to 3.1 dB/cm. Attenuation coefficient was linearly increased over 45 minutes except at an early stage of a few minutes. The variation among the different samples was small, it was indicating clearly by small error bars (Fig. 5.6). It was increased linearly even after the sound speed was not changed.

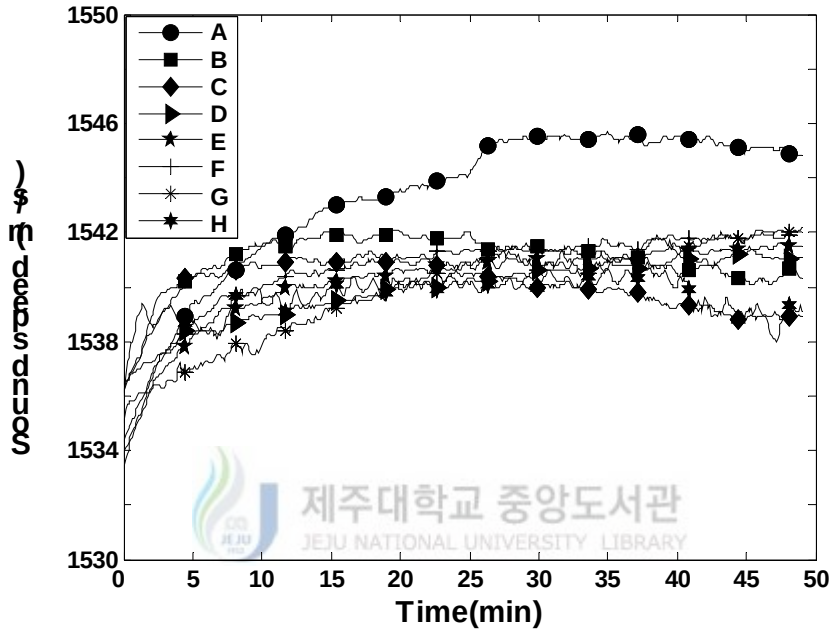


Figure 5.2 Sound speed variation of eight individuals during plasma coagulation

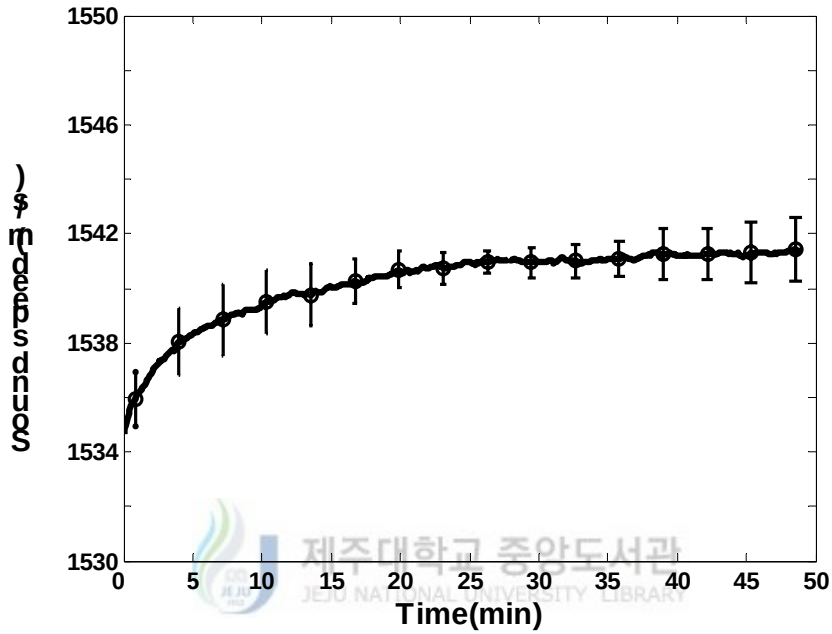


Figure 5.3 Average sound speed variation of eight individuals during plasma coagulation

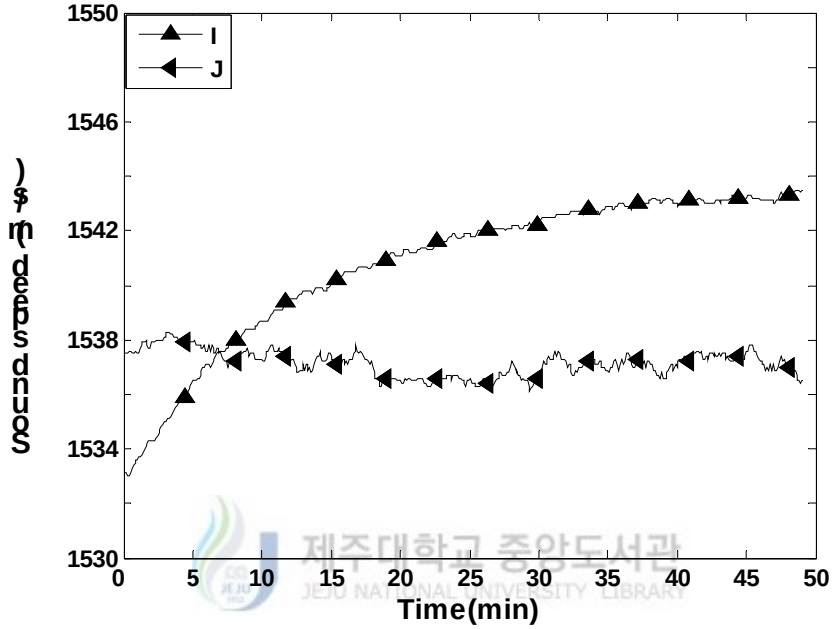


Figure 5.4 Sound speed variation of nine and ten individual during plasma coagulation

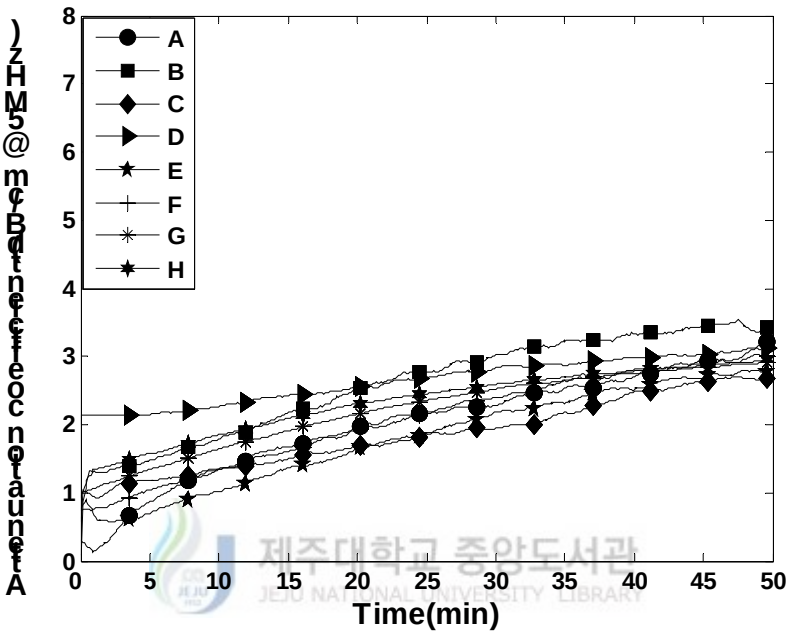


Figure 5.5 Attenuation coefficient variation of eight individuals during plasma coagulation

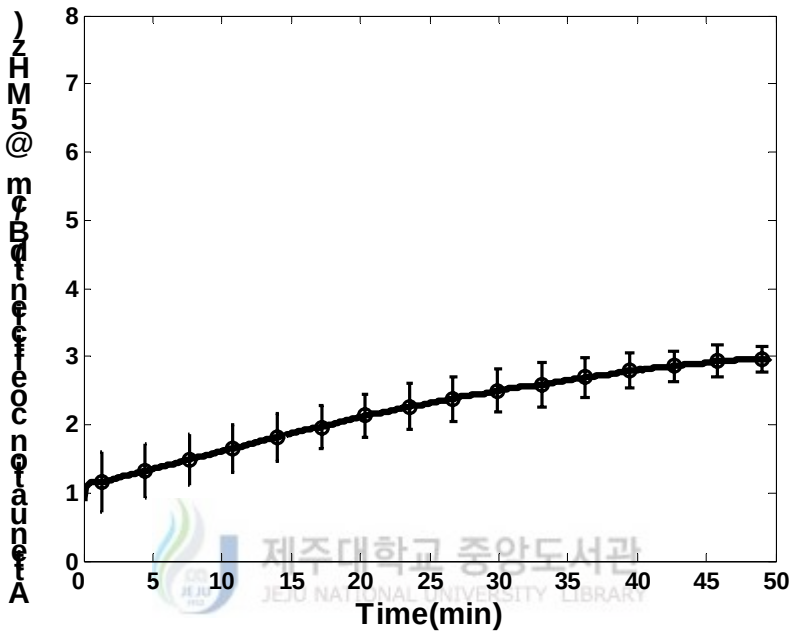


Figure 5.6 Average attenuation coefficient variation of eight individuals during plasma coagulation

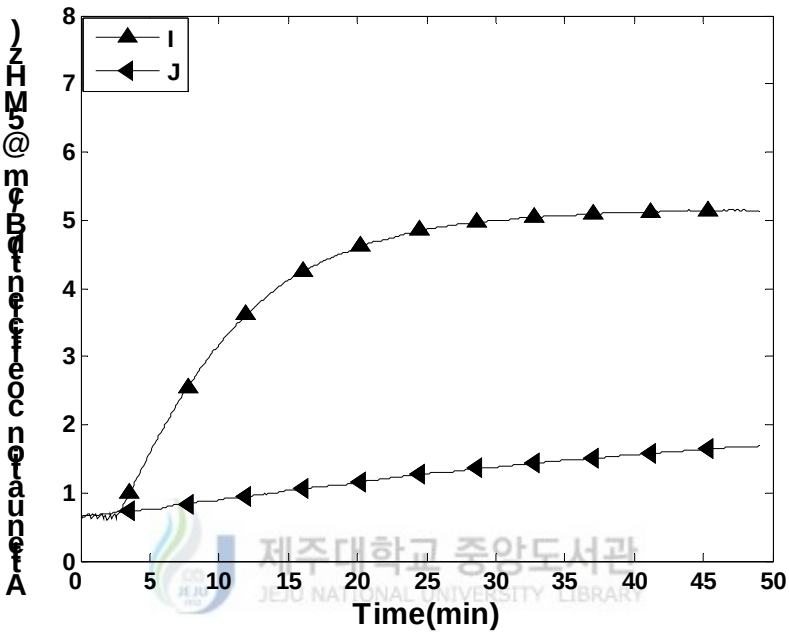


Figure 5.7 Attenuation variation of nine and ten individual during plasma coagulation

5.5 Discussion

Sound speed of plasma also showed three phases of coagulation. Plasma coagulation process was fast compared to whole blood and not much varied among the samples. In the first phase, sound speed and attenuation of eight individuals are different from each other and sound speed was increased rapidly up to around 4 m/s within 5 minutes in Fig. 5.2. However, attenuation was decreased or stable, probably because sound speed depends on bulk modulus and density. For plasma coagulation, initial enzymatic reactions during intrinsic and extrinsic pathways made the coagulum soft and stable. In second phase, the sound speed increased slowly for 7 subjects (except curve "A" in Fig. 5.2). Sound speed reached the same value (~1540m/s) at about 27 minute but attenuation increased linearly from 1 dB/cm to 3.1 dB/cm over 45 minutes. The slow increase of sound speed and linear increase of attenuation may indicate the possibility of thrombin reaction with fibrinogen and formation of fibrin.

The third phase of plasma coagulation seemed to start after formation of solid fibrin, i.e. after 27 minute of coagulation; sound speed was increased smoothly in 45 minutes in plasma. Seven curves showed almost the similar patterns of variations except one showing higher sound speed, while attenuation kept increasing linearly even after sound speed reaches to the same value. This phase may be because of activation of protein C with cofactor protein S and propagation of reactions with thrombomodulin within the blood sample (Mann 1992; Comp 1982).

Comparing Fig.4.2 with Fig.5.2, the responses of sound speed of blood and plasma were different during formation of clot. Blood coagulation is more complex and slower than plasma coagulation. From Figs. 5.2 and 5.5, sound speed of plasma changed faster only at the initial stage of the clotting

process, while attenuation was increased linearly with time even after sound speed was not changed. Simultaneous monitoring of sound speed and attenuation may give a better understanding of the process of coagulation. Sound speed and attenuation curves of J & I shown in Figs. 5.4 & 5.7, respectively revealed that variations are different from the other curves because the rate of conversion of fibrinogen to the insoluble product fibrin or activation of blood coagulation factors may be different from the others. Attenuation coefficient of plasma was increased almost linearly as shown in Fig. 5.6. For plasma coagulation, an amount of aPTT and calcium chloride at the body temperature have impacts on determination of the rate of coagulation. Coagulation process is also dependant on the temperature and the coagulation factors of blood, so all experiments have been performed in the body temperature. For plasma coagulation, clinical laboratories usually calibrate the procedures and equipments for coagulation test, and aPTT is usually in the range of 24-32 sec. Deviation from the aPTT time indicates that some coagulation factors malfunctioned in the process. It needs to be resolved in the future and may give more information about blood clotting process. More works are needed in order to understand the mechanisms of the change of sound speed and attenuation during the coagulation of plasma. Measurements in a wide frequency range and as a function of temperature with more samples may give additional information on coagulation process of blood and plasma.

5.6 Conclusion

The effects of plasma coagulation on the acoustic properties from ten volunteers were investigated. It was found that sound speed and attenuation of plasma increased during coagulation. Unlike the aPTT time alone, the monitoring of sound speed and attenuation during whole process of plasma coagulation using ultrasound may give additional information. The variation of sound speed during plasma coagulation also showed the three distinguishable changes of slope with respect to time, and these three different variations may be comparable to the physiological phases of plasma coagulation. Attenuation of plasma increased linearly even after the sound speed was not changed. It may be because of some factors are malfunctioned with in the sample.

Chapter VI

Measurement of acoustic properties of plasma during coagulation with Heparin

6.1 Introduction

Plasma coagulation with an anti-coagulant was studied in this chapter. Commercially available brand of heparin was selected to study the effects of specific factors and process of coagulation. It has been found that heparin is an effective antithrombotic agent that reduces morbidity and mortality among the patients with deep vein thrombosis, pulmonary embolism and also prevents treatment of cardiovascular disorders. Patients are commonly administered by heparin to minimize the risks associated with surgical procedures. The activated partial thromboplastin time (aPTT) and the activated clotting time (ACT) are the most extensively applied measurements of the heparin effect. Accurate evaluation of the effect of heparin is crucial because of the heparin responses vary greatly among the individuals. A monitoring system of whole process of coagulation must be established.

A coagulation analyzer was frequently used to quantify the perfusion of heparin as a function of aPTT or ACT time format in clinical tests (Cheng et al.1998, Hepel et al.1999). It provides results within minutes so that rapid adjustment of heparin therapy can be made. However, these instruments are unable to monitor the whole process of heparin therapy in diagnostics. Therefore, ultrasonic method was used to measure sound speed and attenuation coefficient variations in plasma during coagulation with heparin.

The physical properties of plasma coagulation with heparin have been studied in terms of sound speed and attenuation as a function of time, the effects of plasma coagulation without and with heparin have also been investigated. The measurements verified that the sound speed and attenuation variations are significantly affected by heparin during coagulation process.

6.2 Method for plasma coagulation with heparin measurement

Experiment was performed with plasma samples of five individuals. The experimental set up was shown in Fig. 3.2 and the same as used in the measurement of the blood coagulation, as explained in chapter 4. For all plasma coagulation with heparin measurements, 0.9 ml of plasma was placed in to the sample container using 1 ml capacity pipette and incubated at 37±0.2°C for not longer than 5 minutes to avoid loss of factors V and VII. Then 0.1 ml of 100 μg/ml concentrated heparin was added using 20~200 μl capacity pipette. After 1 minute 1 ml of aPTT was added and this mixture was thoroughly mixed by using a magnetic stirrer and incubated for 5 minutes to reach thermal equilibrium. After that 1 ml of pre-incubated calcium chloride (0.02M) solutions was added for initiation of coagulation. Ultrasonic broadband pulses are transmitted at a pulse repetition frequency of 500 Hz. The reflected signals from plasma sample and steel reflector were digitized at a sampling rate of 100 MHz using NI high speed digital oscilloscope (PCI-5122, National instrument, Austin, TX, USA). Data were collected every 4 seconds over 30 min. collected data were analyzed using LABVIEW software to obtain plasma profiles in real time. The block diagram of whole process was as shown in Fig 6.1.

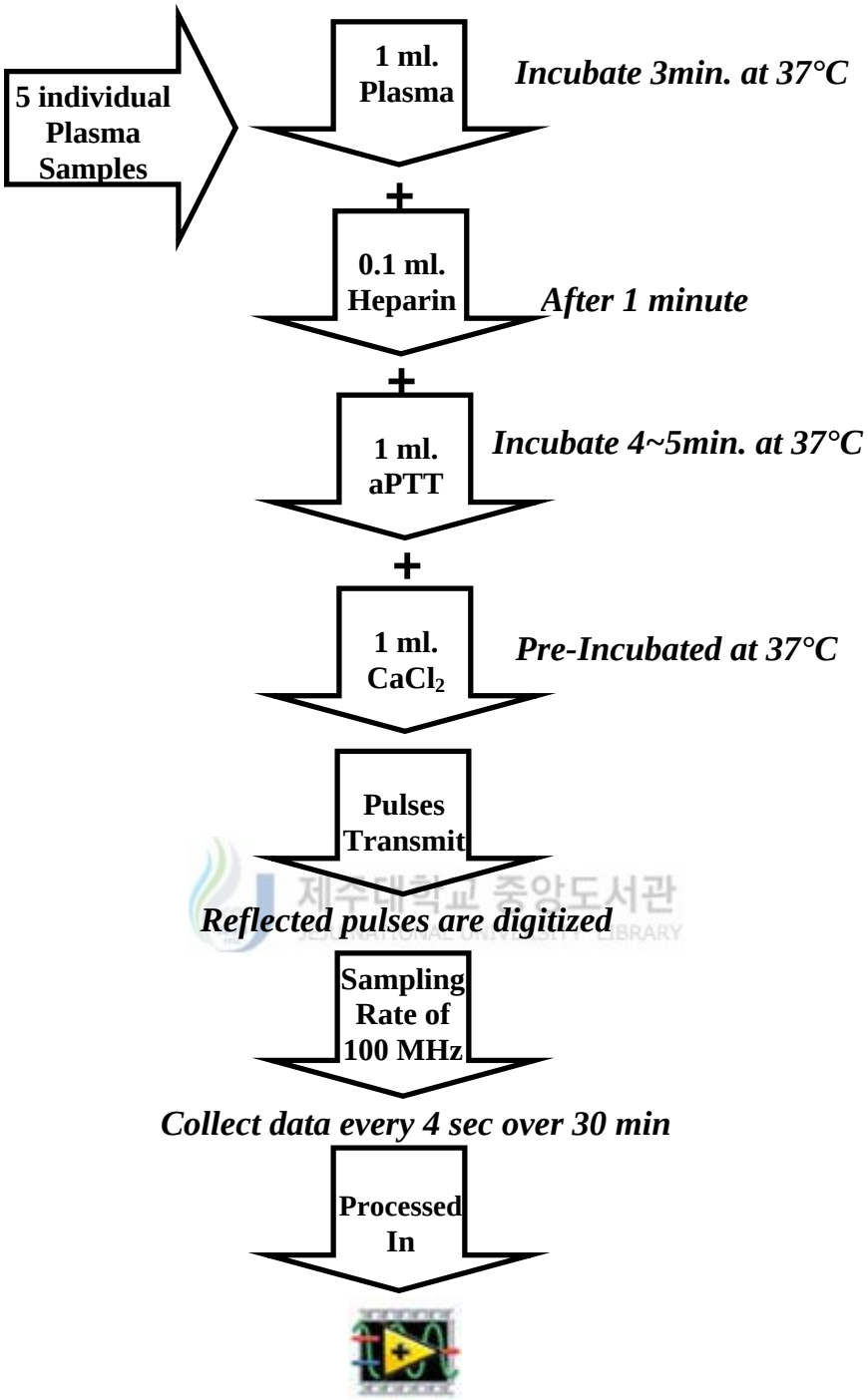


Figure 6.1 Block diagram of plasma coagulation with heparin process

6.3 Results

Sound speed and attenuation coefficient variations of five individuals during plasma coagulation at 37°C are presented in Figs 6.2 & 6.4. The curves (A, B, C, D, and E) with different markers in the figures represent the structural changes of five plasma samples with heparin during coagulation. The average sound speed of five plasma samples was increased 1 m/s at initial stage of 5 minutes after that it reached to steady state. The peak value of sound speed was showed after 3 minutes of coagulation (Fig. 6.3), after that it slightly decreased and reached to steady state at about 5 min. From 5 minutes to over 10 minutes the sound speed was not changed as shown in Fig. 6.2. Lines and error bars with open circles in Fig. 6.3 represent the average and the standard deviation of five measurements, respectively. Sound speed variations among different plasma samples are smaller.

Attenuation coefficient of plasma coagulation with heparin slightly increased for first few seconds after that it was observed to be stable (fig 6.4). Fig 6.5 shows the mean and the standard deviation value of relative attenuation coefficient parameters of five plasma samples measurements, respectively. Attenuation coefficient of plasma coagulation with heparin did not show any significant variation.

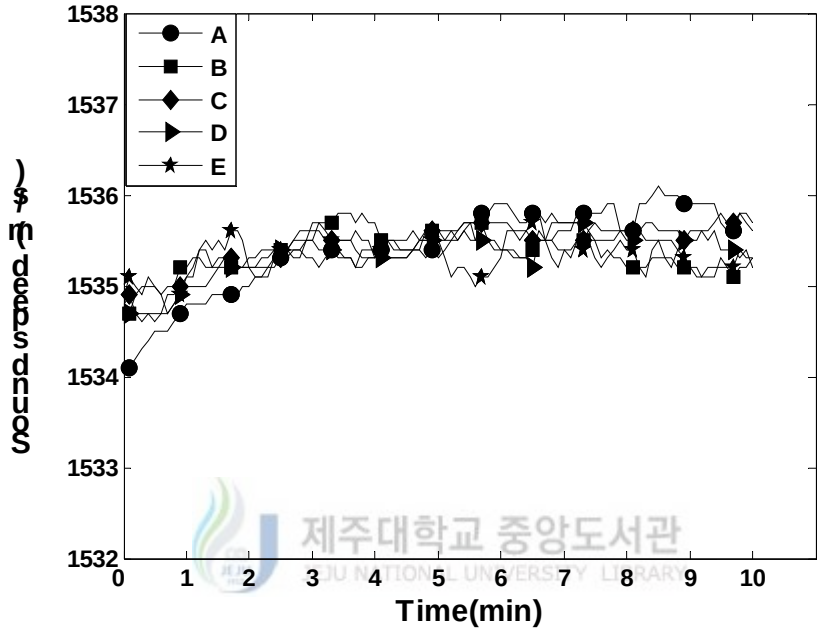


Figure 6.2 Sound speed variation of five individuals during plasma coagulation with heparin

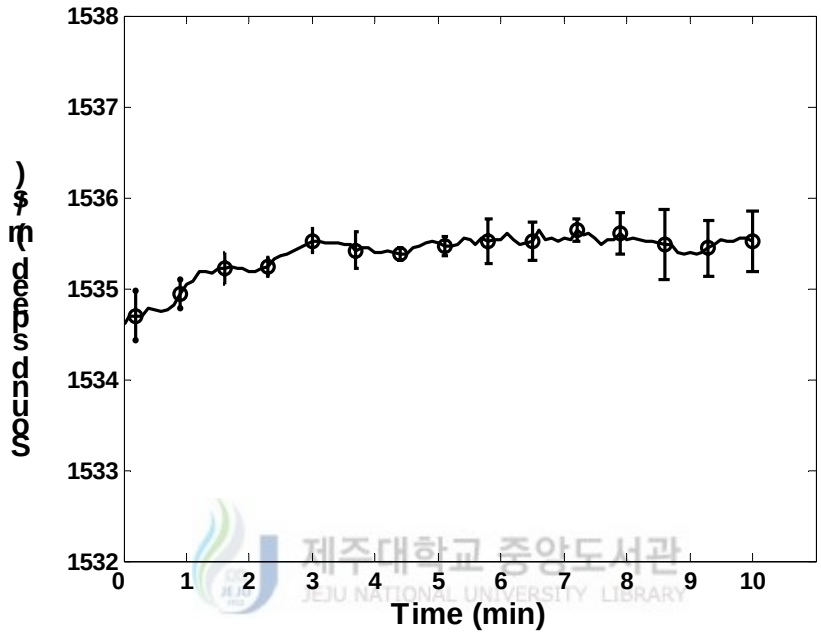


Figure 6.3 Average sound speed variation of eight individuals during plasma coagulation with heparin

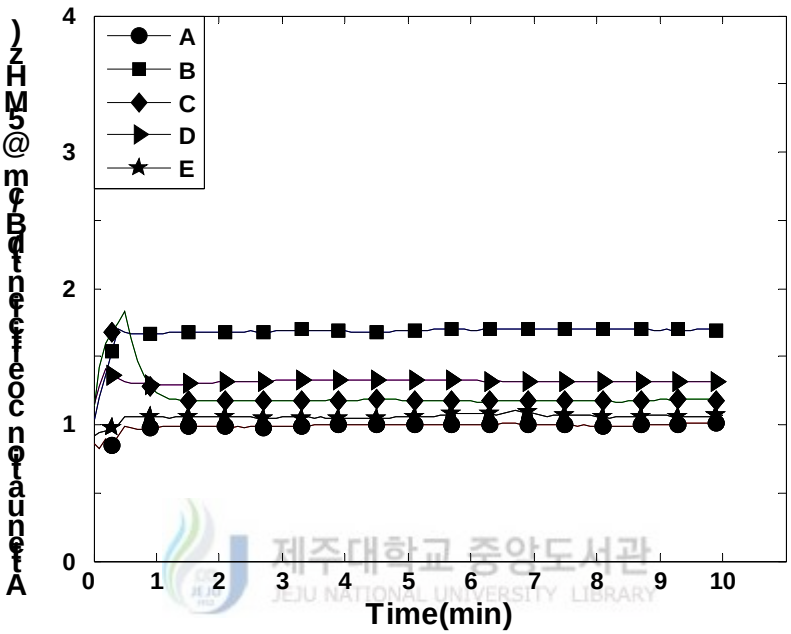


Figure 6.4 Attenuation coefficient variation of five individuals during plasma coagulation with heparin

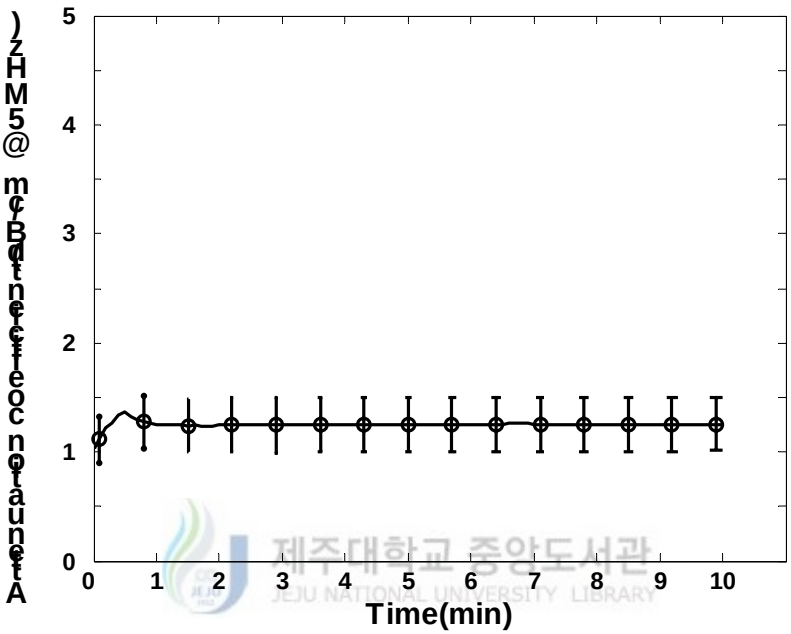


Figure 6.5 Average attenuation coefficient variation of five individuals during plasma coagulation with heparin

6.4 Discussion

The present study measured the acoustic properties of plasma coagulation with heparin and then investigated anticoagulant activity of heparin *in vitro* with aPTT as a function of time. Figs. 6.2 & 5.2 represent the behaviour of sound speed variations of plasma coagulation with and without heparin. Sound speed variations of plasma with and without heparin assays during coagulation were different from each other, but their patterns were similar. In case of plasma without heparin contained assay showed higher sound speed nearly 6 m/s and also attenuation increased linearly from 1.25 dB/cm to 3.1 dB/cm over 45 minutes. In case of plasma with induced heparin showed at initial stage of sound speed increased only 1 m/s until 5 minutes (Fig. 4) after that sound speed fall down to a relatively low value and then attained steady state. Attenuation also not changed much except beginning of few seconds.

These methods were compared in determining the heparin anticoagulant activity. In case of plasma coagulation without heparin, sound speed kept increased over the observation time. Where as, the sound speed would gradually dropped to relatively low value at about 300 seconds in case of heparinized plasma. Apparently, in vitro, sound speed of plasma with heparin disappears faster than that of without heparin. In general, it would take 15 minutes for normal plasma coagulation, owing to a series of cascade enzymatic reactions, to take place.

In fact, the time of decrease in sound speed responses was extremely dependent on heparin concentration. Obviously, heparin anticoagulant activity works at initial phase of coagulation according to physiological process. It could be concluded that the effects of heparin can work only at initial enzymatic phase of plasma coagulation. Simultaneous monitoring of

sound speed and attenuation of plasma coagulation with and without anti-coagulant may give a better understanding of anticoagulant activity of anticoagulant. Comparing Fig.6.2 with Fig.5.2, the responses of sound speed of plasma with and without heparin were different during formation of clot. Plasma coagulation with heparin is harder and slower than plasma coagulation without any heparin. From Figs. 5.6 and 6.5, plasma without heparin assay indicating that attenuation coefficient was increased linearly with time even after sound speed remained the same for plasma without heparin. However, attenuation coefficient was not changed throughout the process in heparinized plasma. It could be concluded that heparin may affect the malfunctioning factors with in the sample.

6.5 Conclusion

The application of ultrasonic pulse echo method to study the plasma coagulation with an anticoagulant, such as heparin has been demonstrated. It was the first time has been used to measure the plasma coagulation with heparin using ultrasound. Sound speed variation had an apparent step ladder curves that was distinctly suitable for determination the coagulation time. Sound speed and attenuation variations of plasma without and with anticoagulant are different from each other. Plasma coagulation with heparin showed only at initial phase of reactions, sound speed and attenuation fluctuations were lower compared to plasma coagulation without heparin. Monitoring of plasma coagulation without and with heparin may give more information of anticoagulant activity of an anticoagulant. These results might help a better understanding of coagulation process, which may be applied in various techniques for development of anticoagulant and evaluation of the drugs.

Chapter VII

Conclusions and Future work

7.1 Conclusions

The effects of blood and plasma coagulation on the acoustic properties from ten volunteers were investigated. Further plasma coagulation with heparin as an anticoagulant was also investigated. It was found that sound speed and attenuation of blood and plasma increased during coagulation. Unlike the aPTT time alone, the monitoring of sound speed and attenuation during whole process of blood and plasma coagulation using ultrasound may give additional information. The variation of sound speed during whole blood and plasma coagulation showed the three distinguishable changes of slope with respect to time, and these three different variations may be comparable to the physiological phases (Enzymatic, Propagation and Termination phases) of blood and plasma coagulation. The processes of whole blood coagulation at nearly body temperature with natural process were complicated and sound speed and attenuation fluctuations are high compared to plasma coagulation. Sound speed and attenuation variations of whole blood and plasma are different from each other. Sound speed and attenuation of whole blood were increased over 60 minutes, but sound speed of plasma rapidly increased in the first stage and then remained similar while attenuation increased linearly over 45 minutes.

Sound speed and attenuation variations of plasma without and with an anticoagulant are different. Plasma coagulation with heparin changed only at initial phase of reactions and sound speed and attenuation fluctuations are

smaller compared to plasma coagulation without heparin. Finally plasma coagulation with an anti-coagulant such as heparin was found helpful to study the effects of the anti-coagulant on specific factors and/or processes of coagulation. These results might help a better understanding of coagulation processes, which may be applied in various techniques for development of anticoagulants and drugs.

7.2 Future work

Extending this work to measure the backscattering from whole blood during coagulation is required, since it may give more information to understand the phases of coagulation. In addition to precise measurements of the backscattering from blood during coagulation, B-mode or harmonic imaging may be directly applied to measure the blood coagulation inside of blood vessel in real time. Combining the sound speed and attenuation with scattering measurements would yield to implement the diagnosis and therapy in cardiac surgery process, deep vein thrombosis and pulmonary embolisms.

Another approach is to measure the anti-coagulant activity of heparin or other anticoagulants may help to implement anticoagulants and development of the drugs. In appendix, the preliminary experiments with other anticoagulants from seaweed were presented.

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APPENDIX

Measurement of acoustic properties of plasma during coagulation with brown and red algae

A.1 Introduction

Heparin and Warfarin are the sole anticoagulants used to prevent thrombotic disorders and treatment of cardiovascular disorders. Heparin and its derivatives have low molecular weight, although an effective antithrombotic agent that reduces morbidity and morality among patients with deep vine thrombosis and pulmonary embolism. However, it cannot be administered orally and is therefore difficult to maintain the extended therapy (Daniel et al. 1999). Warfarin is an orally bioavailable anticoagulant that inhibits production of vitamin k dependent coagulation factors. However the anticoagulant agent delays onset and offset of action. Therefore it takes several days to reach therapeutic anticoagulant levels, requires frequent monitoring of anticoagulation and is subject to many drug interactions (Daniel et al. 1999). Due to these limitations, it is needed to develop new anticoagulants that exhibit rapid and predictable onset and offset kinetics.

Anticoagulant properties of marine algae have been extensively studied for last 60 years. Anticoagulants activity of marine algal polysaccharide has potential ability to prolong blood coagulation time. This is due to their hemi-ester helphate groups in their sugar residues (Shanmugam and Mody 2000). Therefore, seaweeds are good alternative sources for anti-coagulant drug production. In last decades, seaweeds or their extracts from red alga and brown alga have been studied (Shanmugam and Mody 2000) as novel

sources which have been reported to possess bioactivity of potential medicinal value.

In this study, an ultrasonic method was introduced for monitoring the change of whole process of plasma coagulation with anticoagulant from brown and red algae, but did not investigate whole process and anti-coagulant activity.

A.2 Materials and Method

Two kinds of anticoagulants such as red alge and brown alga were obtained from marine brown algae, ***Ecklonia cava***. This ***E.cava*** was collected from sea shores of Jeju Island in Korea and processed (Yashantha 2004) in Marine biology laboratory at Cheju National University.

Experiment was performed with plasma samples of five individuals. The experimental set up was shown in Fig. 3.2 and the same as used in the measurement of the plasma coagulation with heparin, as explained in chapter 6. For all plasma coagulation with red alge or brown alge measurements, 0.9 ml of plasma was placed in to sample container using 1 ml capacity pipette and incubated at 37±0.2°C for not longer than 5 minutes to avoid loss of factors V and VII, and then 0.1 ml of 100 μg/ml concentrated red algae or brown algae was added using 20~200 μl capacity pipette. After 1 minute 1 ml of aPTT was added and this mixture was thoroughly mixed by using a magnetic stirrer and incubated for 5 minutes to reach thermal equilibrium . After that 1 ml of pre-incubated calcium chloride (0.02M) solutions was added for initiation of coagulation.

A.3 Results and Discussion

Sound speed and attenuation variations of five individuals during plasma coagulation with brown and red algae compounds at 37°C are presented in Figs 8.1, 8.3, 8.5 & 8.7, respectively. The curves (A, B, C, D, E,) with different markers in the figures represent the variations of five individuals plasma samples during coagulation. Lines and error bars in the Figs.8.2, 8.4, 8.6 & 8.8 represent the average and the standard deviation of sound speed and attenuation of five individuals plasma coagulation with brown algae and red algae, respectively.

Sound speed variation of five individuals during plasma coagulation with brown algae was increased nearly 1 m/s until 7 min after that it seams to be stable. There is not much variation among the samples as seen in the small error bars in Fig. 8.2. Attenuation coefficient was observed to be stable during coagulation.

Sound speed of five plasma samples during with red algae did not show any significant variations. The sound speed seems to be increased during first 2 min (Fig.8.6) but variation of the samples is pretty big and the results were not consistent to identify differences among the samples. Sound speed during plasma coagulation with red algae was fluctuated too much compared to plasma coagulation with brown algae. However, attenuation coefficient was similar to the one with brown algae (Fig.8.7).

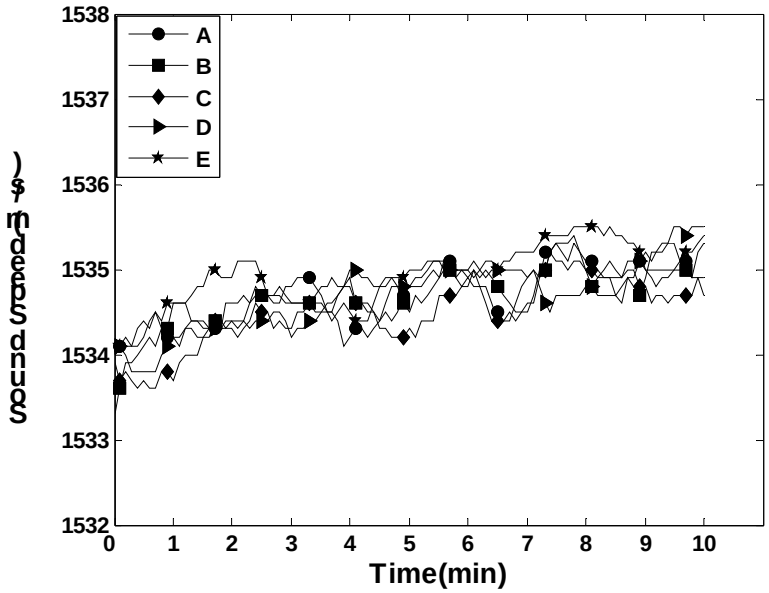

Figure 8.1 Sound speed variation of five individuals in plasma with brown algae during coagulation

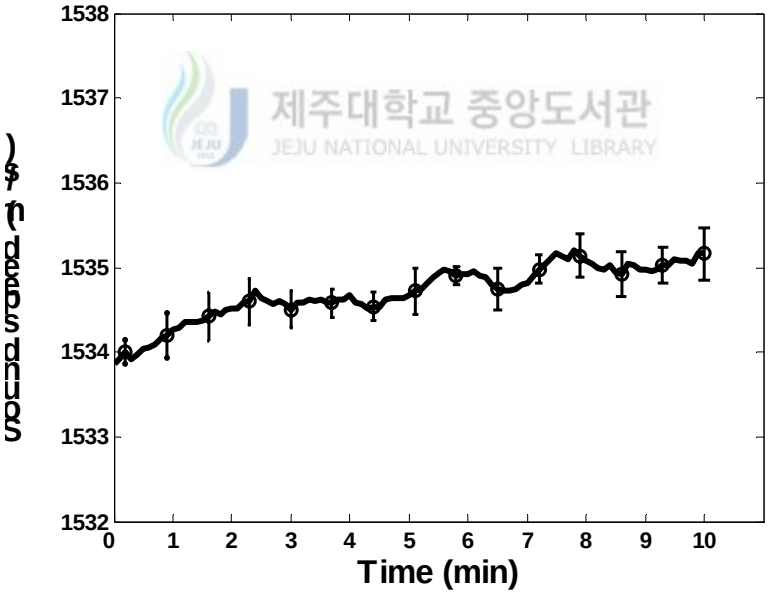

Figure 8.2 Average sound speed variation of five individuals during plasma coagulation with brown algae

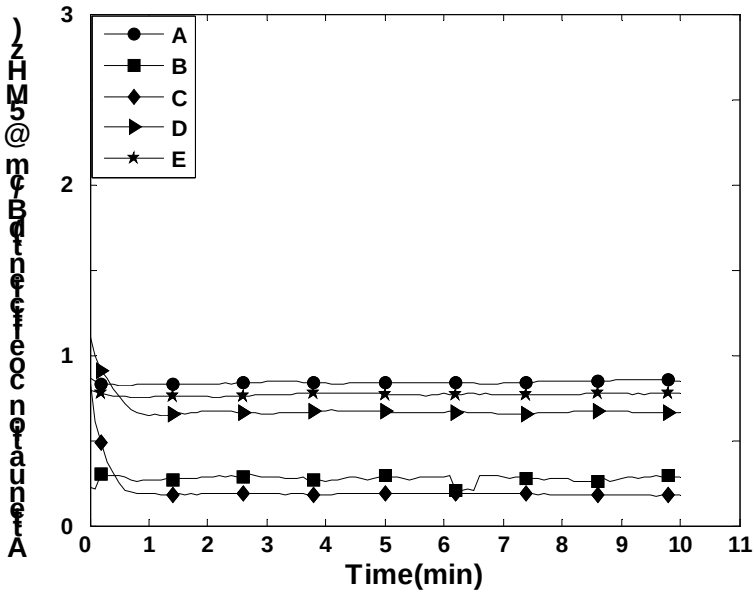

Figure 8.3 Attenuation variation of five individuals during plasma coagulation with brown algae

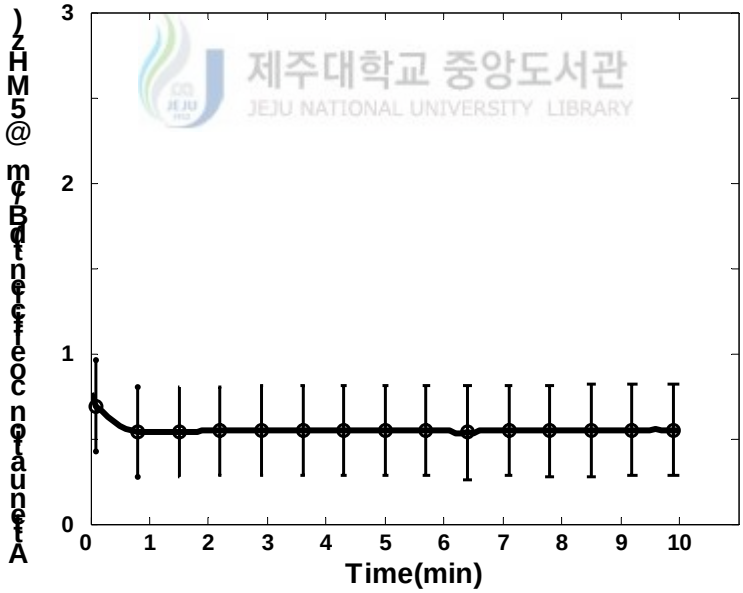

Figure 8.4 Average attenuation variation during plasma coagulation with brown algae

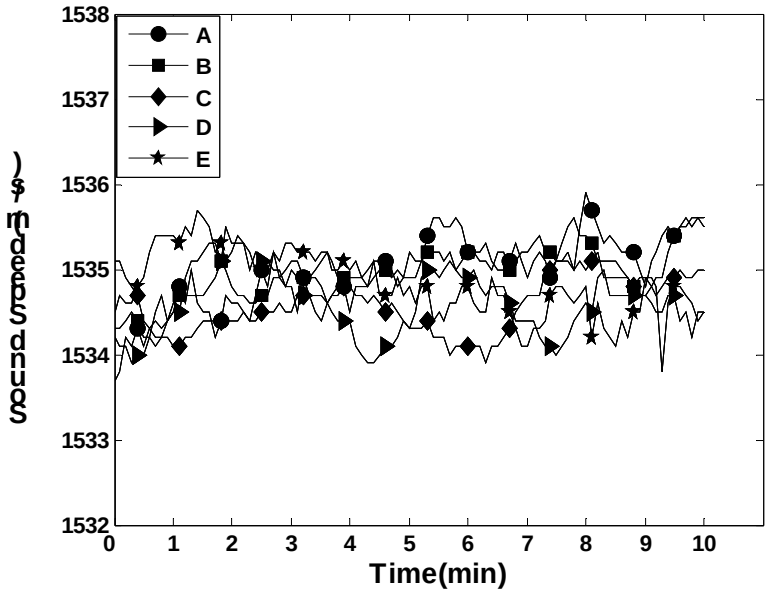

Figure 8.5 Sound speed variation of five individuals during plasma coagulation with red algae

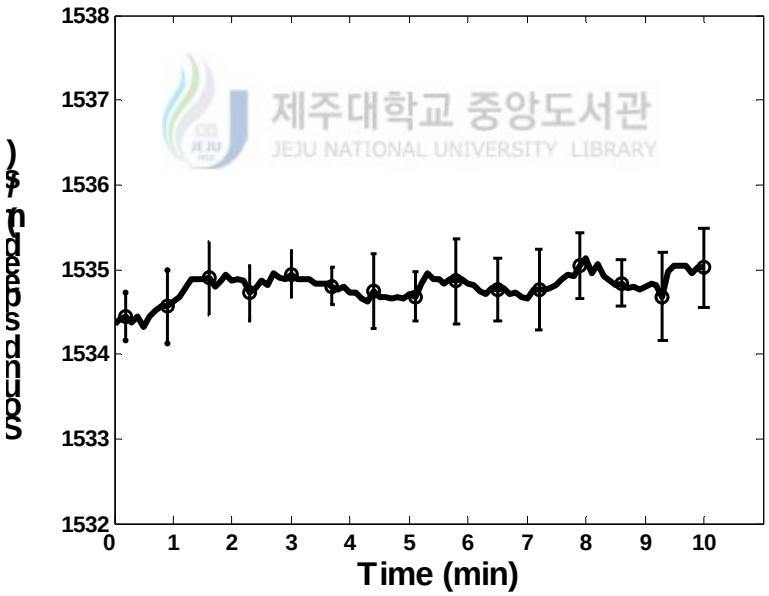

Figure 8.6 Average sound speed variation of five individuals during plasma coagulation with red algae

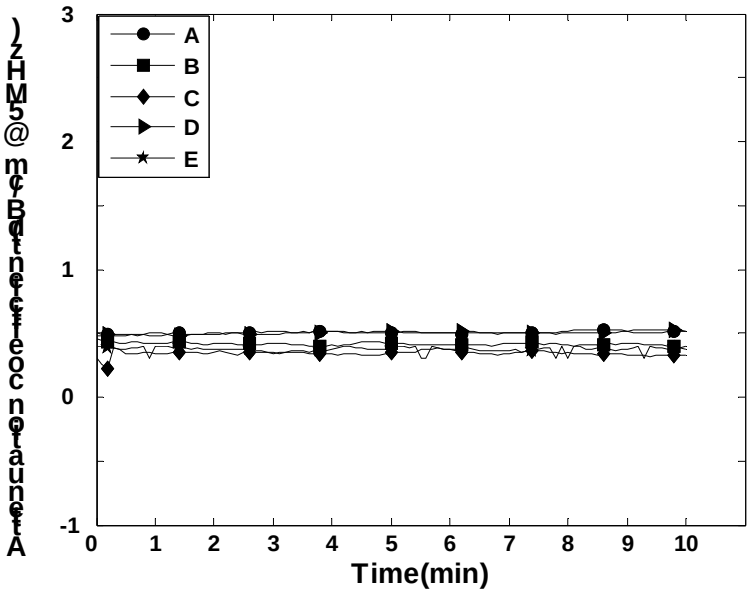

Figure 8.7 Attenuation variation of five individuals during plasma coagulation with red algae

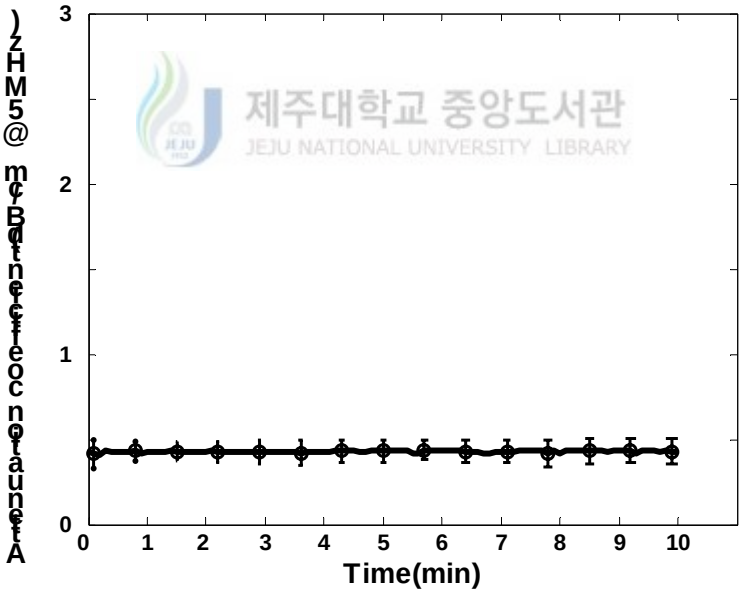

Figure 8.8 Average attenuation of five individuals during plasma coagulation with red algae

According to these results it is difficult to understand the brown algae and red algae activity to any specific factors or process. These results were similar to results of plasma coagulation with heparin, as explained in chapter 6.

A.4 Conclusion

The application of ultrasonic pulse echo method to study the plasma coagulation with anticoagulants from brown and red algae has been demonstrated. It was the first time has been used to measure variations of plasma coagulation with brown and red algae using ultrasound. The responses of sound speed variation may not be consistent to find specific process of coagulation or prolonged clotting time. One finding was that the attenuation coefficient was stable in both cases during coagulation. Sound speed fluctuations were big among the samples compared to plasma coagulation with heparin. These results were may not be enough to explain the anti coagulant activity of brown and red algae and it is required to study further.

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