# Diurnal Variation of Serum Chondroitin Sulfate WF6 and Hyaluronic Acid in the Healthy, Traumatic Knee and the Osteoarthritic Knee

Dumnoensun Pruksakorn MD, PhD*,
Sirichai Leuvitoonvechakij MD*, Peraphan Pothacharoen PhD**, Cheewa Mungmee MD*,
Pakpoom Poomcharoen MD*, Taninit Leerapun MD*, Jayanton Patumanond MD, PhD***

** Department of Orthopaedics, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand*
*** Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand*
**** Clinical Epidemiology Unit, Department of Community Medicine, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand*

***Objective:*** *An understanding of diurnal change is one of the important milestones for either biomarker validation or therapeutic level monitoring. The present study determines the most suitable period during the day for serum chondroitin sulfate WF6 (CS-WF6) and hyaluronic acid (HA) collection, and identifies the possible factors which affect the estimated putative half-life of serum CS-WF6 and hyaluronic acid (HA).*
***Material and Method:*** *Forty-nine volunteers were enrolled in the present study, 22 healthy, 14 with anterior cruciate ligament (ACL) injury, and 13 volunteers with osteoarthritis (OA). Blood sample collection was carried out every four hours starting at 18.00 hours for 24 hours, with additional samples taken at 07:00 and 08:00 hours. Serum CS-WF6, HA levels were determined by an ELISA-based assay.*
***Results:*** *The serum CS-WF6 level was significantly different between the normal and both pathological conditions. The serum HA level was significantly different in every condition. There was no diurnal pattern of serum CS-WF6 and HA during the 24 hour period. An estimated putative half-life of serum CS-WF6 and HA was 4.32±2.63 and 4.10±2.34, respectively. The maximum CS-WF6, creatinine clearance (CrCl) level and body mass index (BMI) were not related to the changes of the WF6 half-life. The higher maximum HA and CrCl level related to the longer half-life of serum HA level, p = 0.008 and p = 0.001, respectively.*
***Conclusion:*** *There was no diurnal pattern of serum CS-WF6 and HA due to the present study approach. Two hours after awakening in official time would be the suitable for serum CS-WF6. Two hours after awakening and after meals were suitable times for serum HA collection.*

***Keywords:*** *Diurnal variation, Chondroitin sulfate, Hyaluronic acid, Half-life, Proteoglycan*

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Chondroitin sulfate (CS) and hyaluronic acid (HA) are the major components of hyaline cartilage. Both have the potential to be used as cartilage biomarkers in diagnostic and monitoring roles, furthermore their components also have a therapeutic potential in terms of regulating the drugs modifying osteoarthritis[1,2]. Serum chondroitin sulfate (WF6) and HA assay is a test which is specific to chondroitin-6-sulfate on the aggrecan, and hyaluronic molecule, respectively. Both serum biomarkers have been studied to increase the validity for monitoring the metabolism of cartilage and joint pathologies[3,4]. When there is a physiological or pathological change in articular cartilage, the CS and HA levels are regulated by the efflux of these components and also their clearance by the liver and kidney[5,6]. Serum WF6 is sensitive enough to detect the smallest 8-mers containing that epitope[7,8], as the potential catabolic marker of hyaline cartilage. A persistently higher level was demonstrated in patients with cartilage injuries than in patients without[9], and a progressively decreasing level was presented when cartilage healed after autologous chondrocyte transplantation[10]. Hyaluronic acid assay can detect as small as 10-12 mers of the hyaluronic molecule[11]. The higher serum hyaluronic acid levels are related to the more severe radiographic changes in osteoarthritis patients[3].

***Correspondence to:***
*Pruksakorn D, Department of Orthopaedics, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand.*
*Phone: 053-945-544, Fax: 053-946-442*
*E-mail: dumnoensun@hotmail.com*

Hyaluronic acid has also been studied for the relationship between circulation levels and the severity and activities of inflammatory arthritis[12].

An understanding of pharmacokinetic change is one of the important milestones for either biomarker validity or therapeutic level monitoring. Therefore, the diurnal patterns of serum WF6 and HA must be addressed before proceeding to other clinical applications. The time and course of serum collection under well controlled conditions was performed in the present study. The objectives of the present study were as follows:

1) To determine the most suitable period during the day for sample collection.

2) To investigate whether the serum WF6 and HA under controlled conditions of normal, ACL injury and osteoarthritis participants present a common diurnal pattern.

3) To identify possible factors which affect the estimated putative half-life of the serum WF6 and HA.

## Material and Method

### *Participants*

Forty-nine volunteers were enrolled onto this study; 22 healthy volunteers between 20 and 25 years of age, 14 patients with anterior cruciate ligament injury who were scheduled for ACL reconstruction, and 13 patients with advanced stage osteoarthritis of the knee who were scheduled for total knee arthroplasty. All volunteers were free from other inflammatory joint diseases, renal, liver, endocrine system and other chronic disorders. Informed consents were signed before enrollment onto this study, and the protocols were approved by The Research Ethics Committee, Faculty of Medicine, Chiang Mai University.

### *Description of the activity, and sample collective protocol*

All volunteers were advised to practice a sedentary life, and avoid marathon running, contact sports, and any other strenuous activities one week prior to admission. Volunteers were admitted to the hospital research unit eight hours (early morning on day 1) prior to the first sample collection. Controlled daily living conditions were in place for volunteers limiting leisure activities to watching television or reading. Meals of the same composition were served at regular periods: 07:00-7:30 a.m. for breakfast; 12:00-12:30 a.m. for lunch and 17:00-17:30 for dinner. The wake up time was 06:00 and sleep time at 22:00. Blood sample collection was performed at 18:00 p.m. (immediately after dinner), 22:00 p.m. (before sleep), 02:00, 06:00 (before arising from bed), 07:00 a.m. (after morning daily activities), 08:00 a.m. (immediately after breakfast), 10:00 a.m. (after recreation activities) and 14:00 p.m. (two hours after lunch). The blood samples were centrifuged at 6,500 g for ten minutes. The supernatants were stored at -20°C until assayed.

### *Biomarker assays*

Serum chondroitin sulfate epitope was determined by a competitive ELISA with a mAb WF6 Standard (shark cartilage aggrecan at a concentration of 19-10,000 ng/mL) and five fold serum was diluted in 6% (w/v) BSA in TE buffer (0.1 M Tris HCl, pH 7.4 containing 0.15 M NaCl, 0.1% Tween 20 and 0.1% BSA)[7,8]. Then an equal volume of WF6 (cell culture supernatant, 1:200 dilution) was added to the 1.5 mL plastic tube. They were further incubated at 37°C for one hour, and then added to the microplate, which was pre-coated with shark aggrecan ($A_1$ fraction). The plates were incubated at 37°C for one hour, and the wells were then washed followed by the addition of 100 µL of peroxidase-conjugated anti-mouse IgM (1:2,000). The bound conjugate was detected by adding 100 µL ortho-phenylenediamine (o-PD). The reaction was stopped after ten minutes with 50 µL/well of 4M $H_2SO_4$, and absorbance was determined using a microplate reader at 492/690 nm.

Serum HA was determined by ELISA-based assay for HA using biotinylated HA-binding proteins (HABPs). Serum or standard HA (Healon$^R$) at 19-10,000 ng/ml in 6% (w/v) BSA-PBS pH 7.4 were added to 1.5 mL plastic tubes containing biotinylated HABPs (1:200 in 0.05 M Tris-HCl buffer, pH 8.6). The tubes were incubated at room temperature for one hour, and then samples were added to the microplate, which was pre-coated with 100 mg/mL umbilical cord HA and blocked with 1% BSA (150 µL/well). The plate was then incubated at room temperature for one hour. The wells were then washed and 100 µL/well of peroxidase-conjugated antibiotin antibody was added. The plate was incubated at room temperature for another hour. The detection of conjugated antibody was carried out with o-PD substrate[11].

All samples were repeated in triplicate and the average of the three values was used in each case for data analysis. The concentration of biomarkers in sera were calculated by reference to a standard curve.

***Statistical analysis***

The serum WF6/HA concentration from every time point within 24 hours for each condition was presented as mean ± SD, and the different level of each condition was analyzed using the Kruskal-Wallis test. Serum WF6/HA concentrations from each time point were presented as median ± SE. In normal, ACL injury and osteoarthritis individuals, the different serum level of eight time points from their grand mean of each condition were analyzed using regression analysis.

Diurnal change of each condition is subjected to a cubic regression formula in line with the hypothesis that each condition has a bimodal pattern. The putative graph pattern was created and analyzed whether the pattern conformed to the following formula:

Cubic pattern: Serum WF6/HA = $a + x + x^2 + x^3$

Square pattern: Serum WF6/HA = $a + x + x^2$

(a = constant, and x = time variables).

The serum biomarker's half-life (putative half-life) of each individual volunteer was calculated using the following formula:

Putative half-life = /Time of the highest value-of the lowest values) / 2/

The possible factors related to the half-life of the biomarkers including body mass index (BMI), creatinin clearance, maximum value were analyzed by multivariable regression analysis. All statistical methods were analyzed using STATA version 11, the level of significance level was determined at $p<0.05$.

## Results

***Serum WF6 and HA from each condition***

The mean (SD) of the serum WF6 level in normal, ACL injury and osteoarthritis conditions were 92.20 (59.76), 107.55 (57.07) and 133.38 (83.88), respectively. There was a significant difference between serum samples from normal and ACL injury patients ($p = 0.002$) and osteoarthritis patients ($p<0.001$). The mean (SD) of serum HA level in patients with normal, ACL injury and osteoarthritis conditions were 46.86 (15.87), 70.51 (21.36) and 206.29 (74.89), respectively. There was a significant difference between normal, ACL injury and osteoarthritis patients ($p<0.001$). There was also a significant difference between ACL injury and osteoarthritis patients ($p<0.001$) (Fig. 1).

***Diurnal variation of serum WF6 and HA***

The graph displays the diurnal changes in the serum WF6 (Fig. 2A) and HA (Fig. 2B) levels during the 24 hours for all groups, normal, ACL injury and osteoarthritis. The serum WF6 level was significantly low at 10:00 p.m. in normal patients ($p = 0.043$). There was no statistical difference at each time point of serum WF6 between ACL injury and osteoarthritis conditions. The cubic graph pattern was present in normal conditions ($p = 0.024$), but it was not shown in ACL injury and osteoarthritis conditions, $p = 0.71$, $p = 0.94$, respectively.

There was no statistical difference in the serum HA level at each time point from normal conditions. However, the highest level of serum HA at 08:00 a.m. was observed in knee injury conditions ($p = 0.037$), and the highest level of serum HA at 07:00 a.m. and 14:00 p.m. were observed in osteoarthritis conditions, with $p = 0.044$ and $p = 0.049$, respectively. No cubic graph pattern was seen from serum HA in normal, ACL injury and osteoarthritis conditions, $p = 0.24$, 0.39 and 0.67, respectively. However, the square pattern was presented in osteoarthritis condition, $p = 0.03$ but it was not shown in injury and osteoarthritis conditions, $p = 0.71$, $p = 0.94$, respectively.

***Putative diurnal pattern of serum WF6 and HA by condition***

According to the the biomodal pattern assumption, an estimated putative half-life of serum WF6 and HA was 4.32±2.63 and 4.10±2.34, respectively. The maximum WF6, CrCl level and BMI were not related to the changes in the WF6 half-life.

**Table 1.** Characteristics of volunteers by group

| Group | Healthy | ACL-injury | Osteoarthritis |
|---|---|---|---|
| Number | 22 | 14 | 13 |
| Gender (male:female) | 11:11 | 0:14 | 3:10 |
| Age (median ± range) | 21±1 | 35.5±32 | 72±20 |
| BMI (kg/m$^2$) | 20.85±1.46 | 23.86±3.05 | 25.18±3.38 |
| CrCl (mean ± SD) | 131.64±5.66 | 92.27±17.89 | 47.91±12.06 |

BMI = body mass index; CrCl = creatinine clearance; ACL = anterior cruciate ligament

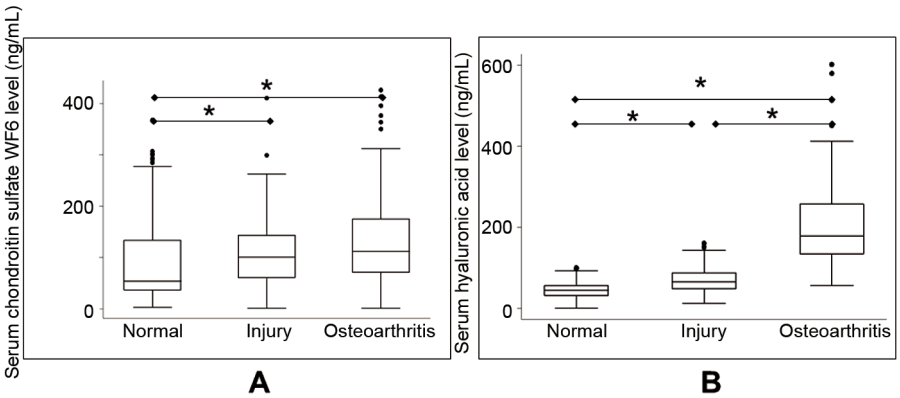


**Fig. 1** The box plot showed median of serum WF6 level of normal, ACL injury and osteoarthritis (A), and serum HA level of normal, injury and osteoarthritis (B).
* Significant difference is $p<0.05$.

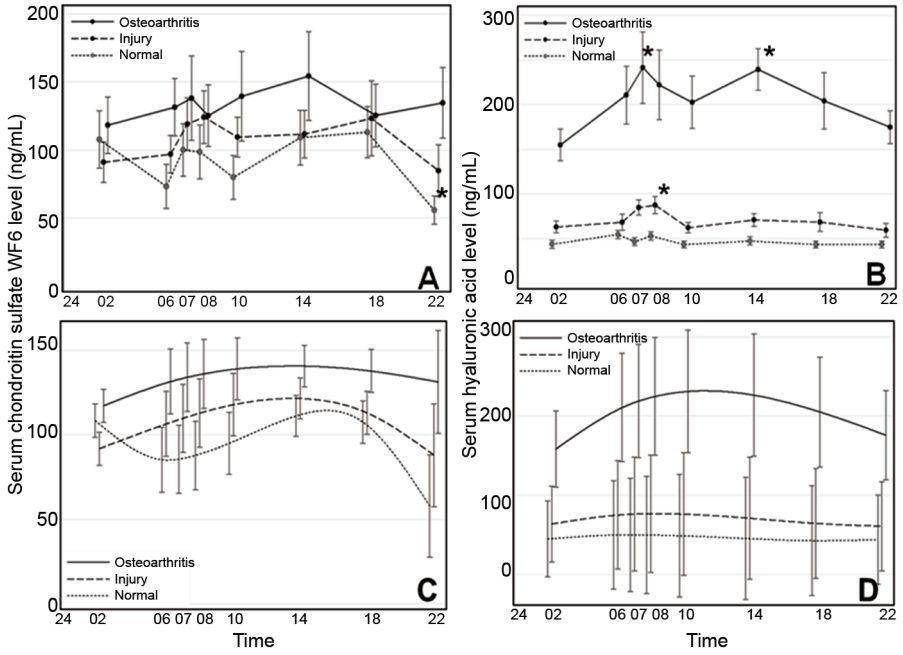


**Fig. 2** The linear graph showed diurnal level of serum WF6 (A), and HA (B) of normal, osteo-arthritis and ACL injury participants which had observations every 4 hours. The putative graph of diurnal variation of WF6 (C), and HA (D).
* Significant difference is $p<0.05$.

The higher maximum HA and CrCl levels were related to a longer half-life of serum HA level, $p = 0.008$ and $p = 0.001$, respectively (Fig. 2C, D).

## Discussion

Serum WF6 level did not show any significant difference at each time point during the 24 hours in the ACL injury and osteoarthritis conditions except for the lowest level at 22:00 p.m. in the healthy group, and there was no identical pattern under controlled activity of the three different conditions. In addition to the pattern of diurnal change, the different load due to changing position during the day was also focused on in this study, particularly an early load after a long period of sleep. Whether the relation of the load intensities, and an efflux of CS components can be perceived from the tested assay.

The serum level variations of other proteoglycans after the load susceptibility to articular cartilage has been studied as the major focus[13]. The

collagen and proteoglycan components were released as early as 24 hours after compression and at various quantities depending upon the degree of loading?[14,15]. Under the range of physiologic or hyper-physiologic strains, an efflux pattern of cartilage oligomeric matrix proteins (COMP) increased in linear proportion to the magnitude of mechanical stress, while chondroitin sulfate (CS), keratin sulfate (KS) and total sulfated-glycosaminoglycan were released in a bimodal pattern[16]. The serum cartilage oligomeric matrix protein (COMP) is a proteoglycan which has been extensively studied for change after load response. The level in healthy participants can be changed by variation in physiological cyclic load[17,18], and is sustainably high for a few days after strenuous physical activity including marathon running and ultra-marathon running[19,20]. The serum WF6 level was increased when subjected to loads during long distance inclinable walking comparing to level walking, but it did not show a significant difference, and the level returned to base-line within 24 hours after activity (manuscript in press).

A rising serum WF6 level was also observed after one or two hours after awakening (07:00 or 08:00 a.m.), the result showed no significant difference when viewing the individual wide range of variation throughout the day. However, serum WF6 showed a distinct difference between normal and pathologic conditions which was much greater than those ranges of variation. Serum WF6 was useful in further identifying the exact cut-off value for metabolism monitoring. Although there was no definite diurnal pattern or significant load susceptibility from physiological activities, practically, the suitable period for sample collection should be avoided in the early morning until at least two hours after awakening, during the sleeping period and for 24 hours after any strenuous load bearing activity.

Similar to the previous observation[21,22], there was no specific diurnal pattern of serum HA in the three different conditions. The change of serum HA level during the day might depend on various factors including lymphatic influx due to posture, muscular contraction during exercise, food intake and possibly from pulmonary lymph flow induced by hyperventilation[22-24]. The serum HA levels were higher at all times in both pathological conditions, osteoarthritis and ACL injury conditions, when compared to those in normal volunteers. An efflux of the HA component from pathological sources plays a major role in maintaining the sustained high level.

Food intake has been a major concern from previous diurnal hyaluronic acid studies as the peak level was one hour after meals[22]. The present study also showed a similar pattern with an affect which was more obvious with a higher baseline level in the osteoarthritis group. The significantly higher level two hours after awakening or one hour after breakfast intake was demonstrated in the ACL injury group. In addition, in the osteoarthritis group the bimodal pattern showed a significant difference, that is, one hour after awakening or immediately after breakfast and two hours after lunch. Food intake does not show any significant affect to serum HA level in subjects in a healthy condition. In order to avoid the effect of food intake on the changes in serum HA level, the suitable time to collect samples would be at least two hours after eating.

The half-life of sera CS and HA presented different values depending on the different approaches. According to the pharmacokinetic study from oral consumption, intravenous injection of CS and intra-articular injection of HA there were indications that the serum level and half-life had significant changes due to the crossover effect. Chondroitin sulfate is rapidly cleared after a 400 mg intravenous injection that has a half-life of 1.56 hours. The longer half-life period was 9.35 and 12.1 hours when given 1,200 mg and 1,600 mg per oral dose, respectively. A much longer half-life was also noted when multiple oral doses were given[6]. A very short half-life (five minutes) was shown after intravenous injection[25], on the other hand, a longer half-life (11-20.8 hours) was presented when injecting HA intra-articularly and monitoring its level in circulation[26]. The kinetic change of the serum biomarker level from a previous study represented the level change due to bolus application in a microgram range. This might not be sensitive enough to represent the minor changes in physiologic or pathologic conditions registered in this study using the nanogram range.

The half-life estimations from this approach are dependent on an efflux from sources, ability of body clearance and unknown variables. A change in the HA half-life is significantly related to the HA component efflux and individual body clearance (Table 2). The higher maximum level and low clearance ability will show a longer alteration period than that in the smaller and higher clearance group. Therefore, the HA comparison between individuals or between different medical conditions might require more consideration of such factors. On the other hand, there

**Table 2.** Multiple regression analysis of serum WF6 and HA half-life with variables

| Variables | Relation coefficient | 95% confidence interval | *p*-value |
|---|---|---|---|
| CrCl | -0.007 | -0.015 to 0.002 | 0.109 |
| BMI | 0.034 | -0.060 to 0.129 | 0.474 |
| Maximum level of WF6 | -0.002 | -0.005 to 0.001 | 0.205 |
| The change of HA half-life | | | |
| CrCl | 0.017 | 0.007 to 0.027 | 0.001* |
| BMI | 0.027 | -0.056 to 0.111 | 0.519 |
| Maximum level of HA | 0.003 | 0.001 to 0.005 | 0.008* |

BMI = body mass index; CrCl = creatinine clearance; HA = hyaluronic acid

was no significant relationship in an alteration of serum WF6 putative half-life to the present study factors.

This information would be helpful in decision making regarding the method of sample collection and grouping volunteers during a validation study. Furthermore, a comparative study of individuals using different time frames and conditions and a comparative study from different types of individual are factors for consideration in future studies.

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**Potential conflicts of interest**

None.

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***การเปลี่ยนแปลงของระดับสาร chondroitin sulfate WF6 และ hyaluronic acid ระหว่างวันในอาสาสมัครที่มีสุขภาพดี ผู้ป่วยข้อเข่าบาดเจ็บ และผู้ป่วยข้อเข่าเสื่อม***

***ดำเนินสันต์ พฤกษากร, ศิริชัย ลือวิฑูรเวชกิจ, พีรพรรณ โปธาเจริญ, ชีวา มั่งมี, ภาคภูมิ ภูมิเจริญ, ธนินนิตย์ ลีรพันธ์, ชยันตร์ธร ปทุมานนท์***

***วัตถุประสงค์:*** *เพื่อศึกษาการเปลี่ยนแปลงในระหว่างวันของระดับสาร chondroitin sulfate WF6 (CS-WF6) และ hyaluronic acid (HA) และประมาณการค่าครึ่งชีวิตของสารดังกล่าวในกระแสเลือด*

***วัสดุและวิธีการ:*** *อาสาสมัครสี่สิบเก้าคนได้เข้าร่วมในการศึกษาเป็นผู้มีสุขภาพดียี่สิบสองคน ผู้ป่วยเอ็นไขว้หน้าขาดสิบสี่ราย และผู้ป่วยโรคข้อเสื่อมสิบสามราย อาสาสมัครได้รับการเจาะเลือดทุกสี่ชั่วโมงเป็นเวลายี่สิบสี่ชั่วโมง และเก็บเพิ่มเติมที่เวลาเจ็ดและแปดนาฬิกาตอนเช้า ตัวอย่างเลือดจะถูกเก็บและวิเคราะห์ระดับ CS-WF6 และ HA พร้อมกัน*

***ผลการศึกษา:*** *ระดับ CS-WF6 ของอาสาสมัครสุขภาพดีมีความแตกต่างจากอาสาสมัครที่มีพยาธิสภาพอย่างมีนัยสำคัญ ในขณะที่ระดับ HA มีระดับแตกต่างกันอย่างมีนัยสำคัญของทั้งสามกลุ่มอาสาสมัครจากการศึกษา ไม่มีการเปลี่ยนแปลงของระดับสารในระหว่างยี่สิบสี่ชั่วโมงของสารทั้งสองชนิด การประมาณค่าครึ่งชีวิตของระดับ CS-WF6 และ HA ประมาณ 4.32±2.63 และ 4.10±2.34 ชั่วโมง ตามลำดับ โดยระดับค่าสูงสุดของ CS-WF6 ระดับ creatinine clearance และ body mass index ไม่แสดงความสัมพันธ์กับการเปลี่ยนแปลงของค่าครึ่งชีวิตของ CS-WF6 ในขณะที่โดยระดับค่าสูงสุดของ HA ระดับ creatinine clearance แสดงความสัมพันธ์ในเชิงบวกกับการเปลี่ยนแปลงของค่าครึ่งชีวิตของ HA อย่างมีนัยสำคัญ $p = 0.008$ และ $p = 0.001$ ตามลำดับ*

***สรุป:*** *จากผลการศึกษาไม่พบการเปลี่ยนแปลงระหว่างวันของระดับสาร CS-WF6 และ HA ช่วงเวลาที่เหมาะสมในการเจาะเลือดตรวจระดับ CS-WF6 คือ ช่วงเวลากลางวันโดยนับจากสองชั่วโมงหลังลุกจากที่นอน ช่วงเวลาที่เหมาะสมในการเจาะเลือดตรวจระดับสาร HA คือสองชั่วโมงหลังตื่นนอนและสองชั่วโมงหลังมื้ออาหาร*