การพัฒนาวิธีไฮเพอร์ฟอร์แมนซ์ลิควิดโครมาโทกราฟีสำหรับตรวจหาปริมาณแอลฟา-โทโคฟีรอลในพลาสมาของคนไทยในกลุ่มสูบบุหรี่และกลุ่มไม่ได้สูบบุหรี่

นางสาวแคทลียา นิรังสรรค์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD DEVELOPMENT FOR DETERMINING PLASMA α-TOCOPHEROL IN THAI SMOKERS AND NON-SMOKERS

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การวิเคราะห์แอลฟา-โทโคฟีรอลในพลาสมาอย่างง่ายและรวดเร็วได้ถูกพัฒนาขึ้นโดยใช้วิธีไฮเพอร์ฟอร์แมนซ์ลิควิดโครมาโทกราฟี พลาสมาโปรตีนถูกแยกออกด้วยสารละลายผสมระหว่างแอซีโตไนไตรล์กับไอโซโพรพานอล โดยใช้ตัวอย่างพลาสมาเพียง 100 ไมโครลิตรและมีแอลฟา-โทโคเฟอริล อะซิเตตเป็นสารมาตรฐานภายใน เมทานอลถูกใช้เป็นโมบายเฟสในการแยกสารและวัดปริมาณด้วยดีเทคเตอร์อัลตราไวโอเลตที่ความยาวคลื่น 292 นาโนเมตร แอลฟา-โทโคฟีรอลและสารมาตรฐานภายในถูกชะออกมาจากคอลัมน์ที่เวลา 7.1 และ 8.3 นาที ตามลำดับ ความสัมพันธ์ระหว่างความเข้มข้นของแอลฟา-โทโคฟีรอลกับสัญญาณจากเครื่องมือวิเคราะห์เป็นเส้นตรงอยู่ในช่วง 0-30 ไมโครกรัมต่อมิลลิลิตร โดยค่าต่ำสุดที่สามารถวิเคราะห์ได้เท่ากับ 0.72 ไมโครกรัมต่อมิลลิลิตร ความถูกต้องและความแม่นยำของวิธีวิเคราะห์ทั้งภายในวันเดียวกันและต่างวันกัน ซึ่งแสดงในค่าของ %bias และ %RSD มีค่าน้อยกว่า 15% และ 10% ตามลำดับ วิธีวิเคราะห์ที่ได้มีความจำเพาะเจาะจงไม่ถูกรบกวนด้วยสารอื่นในร่างกาย แอลฟา-โทโคฟีรอลในพลาสมามีความคงตัว ณ อุณหภูมิห้องได้นาน 9 ชั่วโมง ขณะที่เก็บที่อุณหภูมิ –47$\pm$1 องศาเซลเซียส ได้ 15 วัน ทั้งนี้แอลฟา-โทโคฟีรอลในตัวอย่างพลาสมายังคงตัวแม้ผ่านขั้นตอนการแช่แข็งและละลายถึง 3 รอบ สารละลายตัวอย่างคงตัวในเครื่องฉีดตัวอย่างอัตโนมัติที่ 4 องศาเซลเซียส ได้ นาน 6 ชั่วโมง การนำวิธีวิเคราะห์ที่พัฒนาขึ้นนี้ไปใช้วิเคราะห์ตัวอย่างพลาสมาจากอาสาสมัครชายไทย 58 คน โดยแบ่งเป็นกลุ่มสูบบุหรี่ 30 คน และกลุ่มไม่สูบบุหรี่ 28 คน ได้ค่าเฉลี่ยของแอลฟา-โทโคฟีรอล ในพลาสมาของกลุ่มสูบบุหรี่ เท่ากับ 12.23 ไมโครกรัมต่อมิลลิลิตร และในกลุ่มไม่สูบบุหรี่ มีค่าเท่ากับ 12.16 ไมโครกรัมต่อมิลลิลิตร ซึ่งไม่มีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติ (p = 0.931) แต่ถ้าแปรผลในรูปอัตราส่วนของความเข้มข้นของแอลฟา-โทโคฟีรอลต่อไตรกลีเซอร์ไรด์ หรือแอลฟา-โทโคฟีรอลต่อไตรกลีเซอร์ไรด์รวมกับคลอเลสเตอรอล พบความแตกต่างของค่าความเข้มข้นระหว่างกลุ่มสูบบุหรี่และกลุ่มไม่สูบบุหรี่ อย่างมีนัยสำคัญทางสถิติโดย p = 0.010 และ 0.034 ตามลำดับ ดังนั้นเป็นการพิสูจน์ว่าวิธีวิเคราะห์ที่พัฒนาขึ้นสามารถใช้วิเคราะห์หาปริมาณแอลฟา-โทโคฟีรอลในพลาสมาของคนได้ การใช้ระดับแอลฟา-โทโคฟีรอลในร่างกายเป็นดัชนีชี้วัดสภาวะการสูบบุหรี่ ควรนำค่าคลอเรสเตอรอลและไตรกลีเซอร์ไรด์มาใช้ด้วย

ภาควิชา เภสัชเคมี ลายมือชื่อนิสิต .....

สาขาวิชา เภสัชเคมี ลายมือชื่ออาจารย์ที่ปรึกษา .....

ปีการศึกษา 2546 ลายมือชื่ออาจารย์ที่ปรึกษาร่วม -

4376558933 : MAJOR PHARMACEUTICAL CHEMISTRY
KEY WORD : METHOD DEVELOPMENT / DEPROTEINIZATION / PLASMA α-TOCOPHEROL / SMOKER / HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

KATTHALEEYA NIRUNGSAN : HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD DEVELOPMENT FOR DETERMINING PLASMA α-TOCOPHEROL IN THAI SMOKERS AND NON-SMOKERS. THESIS ADVISOR : ASSO. PROF. PHENSRI THONGNOPNUA, Ph.D., 89 pp. ISBN 974-17-4303-3

A simple and rapid reversed-phase high-performance liquid chromatographic method was developed to determine α-tocopherol in plasma. The mixture of acetonitrile and isopropanol was used to deproteinize plasma protein in only 100 μl sample prior to HPLC analysis having α-tocopheryl acetate as an internal standard (IS). The mobile phase composed of methanol and the effluent was quantitated at 292 nm. α-tocopherol and IS were eluted at 7.1 and 8.3 min, respectively. The concentrations of α-tocopherol were linear related to response in the range of 0-30 μg/ml with the lowest limit of quantitation of 0.72 μg/ml. The intra-day and inter-day accuracy and precision in term of %bias and %RSD were less than 15% and 10%, respectively. No endogenous interference was detected, indicating the specificity of the method. Plasma sample could be withstood at room temperature for 9 hours but at –47±1 °C for 15 days without any detectable deterioration. Plasma sample could be restored within three freeze-thaw cycles and the processed analyte was still stable within autosampler at 4 °C till 6 hours. To analyze plasma samples from fifty-eight Thai males of both smoker (n=30) or non-smoker (n=28) volunteers using the developed method, the mean value of endogenous α-tocopherol in plasma was nonstatistically significant (p = 0.931) with the determined valued of 12.23 μg/ml for smokers and 12.16 μg/ml for non-smokers. In interpreting concentration ratio of α-tocopherol to triglyceride or to cholesterol plus triglyceride between smokers and non-smokers, the statistically significant difference was observed with the p-value of 0.010 and 0.034, respectively. Thus, the developed method was successfully proven to be utilized for endogenous α-tocopherol determination in human plasma. In considering endogenous α-tocopherol level as the indicator for smoking status, the cholesterol and triglyceride value would also play the role.

Department Pharmaceutical chemistry Student's signature .....

Field of study Pharmaceutical chemistry Advisor's signature .....

Academic year 2003 Co-advisor's signature -

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LIST OF ABBREVIATIONS

%	percent
μg	microgram
μl	microliter
^{o}C	degree Celsius
conc	concentration
g	gram
hrs	hour
IS	internal standard
LLOQ	lower limit of quantification
mg	milligram
min	minute
ml	milliliter
nm	nanometer
PA	peak area
PAR	peak area ratio
PH	peak height
PHR	peak height ratio
R^2	coefficient of determination
RSD	relative standard deviation
SD	standard deviation
SE	standard error
UV	ultraviolet

CHAPTER I

INTRODUCTION

Vitamin E is a major antioxidant micronutrient in lipid phase. In the last few years there has been growing clinical interest in the relationship between vitamin E and several degenerative human health conditions associated with oxidative reaction such as cardiovascular disease, cancer and cataract, etc (Vendemiale, 1999; "Vitamin E", 2001). In addition, some epidemiological studies indicated the inverse relationship between plasma/serum vitamin E level and coronary heart disease (Duthie, 2000). Reduced plasma vitamin E levels have also been reported in the acute pancreatitis (Curran, 2000), cervical intraepithelial neoplasia (Palan, 1996) and various types of cancer (Skulchan, 1987). Therefore, it is interesting whether plasma vitamin E level in people could relate to their high risk in receiving free radical for the awareness of some diseases.

Structure and nomenclature of vitamin E

In 1922, Evans and Bishop discovered the new nutritional factor in wheat germ oil. It was essential for the development of fetus in pregnant rat. Initially, it was called "factor X", and later called vitamin E or tocopherol. Vitamin E is a collective name for tocopherols and tocotrienols. They are biosynthesized only by plants. Tocopherols are found in leaves, seeds and grains. Tocotrienols do not occur in green tissues but rather in the bran and germ of plants. Tocopherols consist of four forms including α-, β-, γ- and δ-tocopherols. Tocotrienols also consist of four forms including α-, β-, γ- and δ-tocotrienol.

Structure of each tocopherol consists of chromanol ring and phytyl side chain (Figure 1). This structure consists of three chiral carbon atoms at position 2, 4' and 8'; so, eight stereoisomers (RRR, RRS, RSS, RSR, SSS, SSR, SRR and SRS configurations) can be obtained but the stereoisomer occurred in plants is only the RRR configuration. In regard to stereoisomers of vitamin E, the RS system is recommended by the IUPAC to

name the isomeric form. For example, the mixture of eight stereoisomers of α-tocopherol is called 2RS, 4'RS, 8'RS-α-tocopherol and one stereoisomer occurred in plants is called 2R, 4'R, 8'R-α-tocopherol. However, four tocopherols (Figure 1) are usually called by common name. Common names are defined as α-, β-, γ- and δ-tocopherol. For structure of each tocotrienol, it consists of chromanol ring and unsaturated side chain (Figure 2). The common names of four tocotrienols are defined in the same series as those four tocopherols.

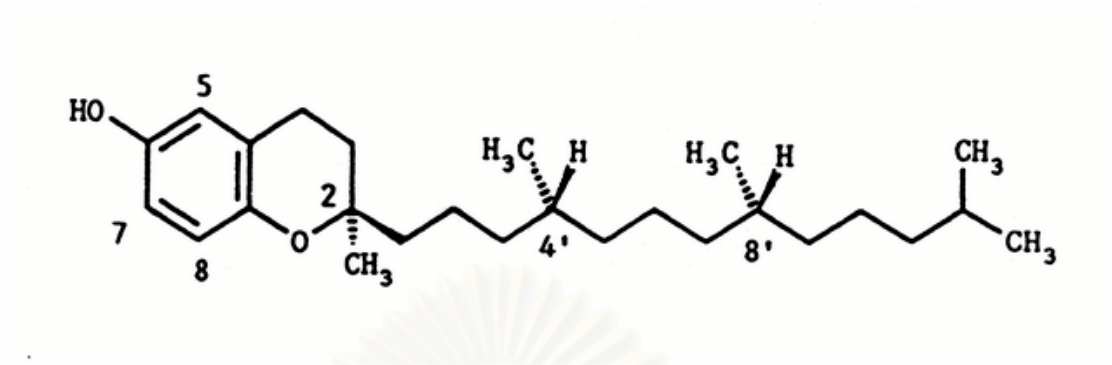
RRR-α-tocopherol is the most active form. The structure that represents the activity includes (1) trimethyl group in chromanol ring, (2) nonpolar phytyl side chain, and (3) 2R configuration.

Sources of vitamin E

Vitamin E could not be biosynthesized in animal and human, it must be supplied only from the diet. The major source of vitamin E is plant oil such as sunflower and wheat germ oil. Vitamin E is also found in nut, fruits, vegetables, pork, chicken, beef, fish, milk, egg and butter. Vegetable oils contain four tocopherols and four tocotrienols in varying proportions. For example, wheat germ oil mainly consists of α- and β-tocopherol, while γ- and δ-tocopherols is found in soybean oil. For palm oil, vitamin E is detected as α-, γ-tocopherols and δ-tocotrienol. Vitamin E in meat and dairy products are mostly α-tocopherol.

Only RRR-α-tocopherol and all-*rac*-α-tocopherol are commercially usable. RRR-α-tocopherol (d-α-tocopherol) could be obtained from natural sources by molecular distillation and methylation of the mixture of α-, β-, γ- and δ-tocopherols, or by hydrogenation of α-tocotrienol. RRR-α-tocopherol obtained from these processes is identical to the stereoisomer found in plants.

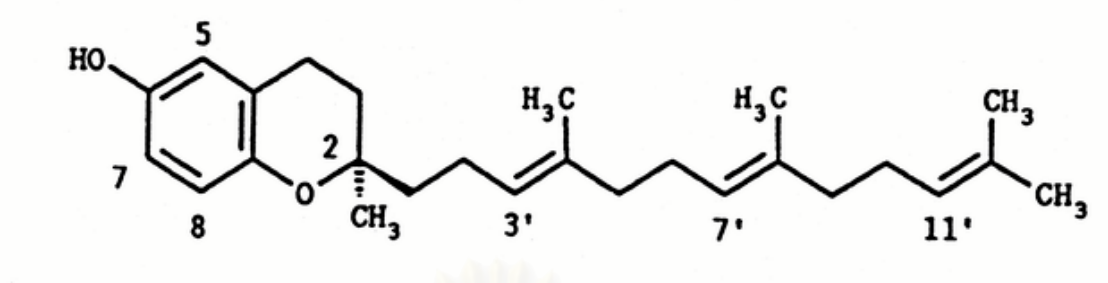
All-*rac*-α-tocopherol (dl-α-tocopherol) could only be obtained by chemical synthesis, such the product composes of the mixture of eight stereoisomers of α-tocopherol in equal proportion. All-*rac*-α-tocopherol is generally synthesized by the condensation of trimethyl hydroquinone with racemic isophytol.



Tocopherols (2R4'R8'R configuration)

Figure 1. Structure and nomenclature of four tocopherols

Position of methyl groups in the structure	Chemical name	Common name (abbreviation)
5,7,8	2,5,7,8-tetramethyl-2-(4', 8', 12'-trimethyltridecyl)-6-chromanol	α-tocopherol (α-T)
5,8	2,5,8-trimethyl-2-(4', 8', 12'-trimethyltridecyl)-6-chromanol	β-tocopherol (β-T)
7,8	2,7,8-trimethyl-2-(4', 8', 12'-trimethyltridecyl)-6-chromanol	γ-tocopherol (γ-T)
8	2,8-dimethyl-2-(4', 8', 12'-trimethyltridecyl)-6-chromanol	δ-tocopherol (δ-T)



Tocotrienols

Figure 2. Structure and nomenclature of four tocotrienols

Position of methyl groups in the structure	Chemical name	Common name (abbreviation)
5,7,8	2,5,7,8-tetramethyl-2-(trimethyltridecyl-3', 7', 11'-triene)-6-chromanol	α-tocotrienol (α-T3)
5,8	2,5,8-tetramethyl-2-(trimethyltridecyl-3', 7', 11'-triene)-6-chromanol	β-tocotrienol (β-T3)
7,8	2,7,8-tetramethyl-2-(trimethyltridecyl-3', 7', 11'-triene)-6-chromanol	γ-tocotrienol (γ-T3)
8	2,8-tetramethyl-2-(trimethyltridecyl-3', 7', 11'-triene)-6-chromanol	δ-tocotrienol (δ-T3)

The free phenolic hydroxyl group in α-tocopherol is slowly oxidized by atomic oxygen. The oxidation reaction could be accelerated by light, heat and alkali or iron and copper salts. RRR-α-tocopherol and all-*rac*-α-tocopherol are therefore in the forms of acetate, succinate or nicotinate ester. This esterification of the free phenolic hydroxyl group could prevent the oxidation. Nonetheless, the acetate ester is widely used in pharmaceuticals, infant formulas, fish feeds and cosmetics whereas the other esters are used in the other applications.

The biological activity among the different commercial vitamin E forms is different. In general, the biopotency of vitamin E in pharmaceuticals, dietaries and cosmetics is labeled in International Unit (IU). International Unit (IU) defines the biological activity of vitamin E from rat fetal resorption test. One International Unit (IU) is defined as the activity of 1 mg of all-*rac*-α-tocopheryl acetate. In addition, the National Research Council defines biological activity of vitamin E in term of RRR-α-tocopherol equivalence. The biological activity of commercial vitamin E forms is shown in Table 1.

Table 1. Biological activity of commercial vitamin E forms (Duthie, 2000)

Vitamin E forms	Biologic activities	
	IU/mg	Compared to RRR-α-T(%)
RRR-α-tocopherol	1.49	100
all-*rac*-α-tocopherol	1.10	74
RRR-α-tocopheryl acetate	1.36	91
all-*rac*-α-tocopheryl acetate	1.00	67

Absorption and distribution of vitamin E in human

Vitamin E could be absorbed by intestinal mucosa following oral administration either from food or supplement. For vitamin E ester, it has to be metabolized by pancreatic esterase before absorption. By the assistance of bile salts, the absorbed vitamin E could form micelle called chylomicron that consists of

triglycerides, free and esterified cholesterols, phospholipids and apolipoproteins before secreting into lymph and blood stream. Only 15-45% of the uptake vitamin E can be absorbed, no matter whether forms the vitamin E is. Vitamin E in plasma is reported to be α-tocopherol approximately 88%, whereas β- and γ-tocopherol are only 2%, 10%, respectively and virtually no tocotrienols (Traber and Serbinova, 1999; Nelis, 2000). Thus as, α-tocopherol is the major detectable form of vitamin E, the mostly mentioned of vitamin E is only α-tocopherol.

The distribution of α-tocopherol following absorption is clarified. Most of α-tocopherol is transported to liver. The hepatic α-tocopherol transfer protein (α-TT) incorporate α-tocopherol with VLDL (very low density lipoproteins). Since RRR-α-tocopherol is the favorable form for α-TT, almost 80% of vitamin E in VLDL are RRR-α-tocopherol. α-tocopherol in VLDL can then be secreted into blood circulation before distribution to tissues. Plasma lipoproteins including HDL (high density lipoproteins) and LDL (low density lipoproteins) are the carrier for this distribution. The half-life of vitamin E in plasma is approximately 48 hours.

α-tocopherol could be maintained in human body by equilibrating plasma α-tocopherol with the tissues. Erythrocytes, liver and spleen are tissues that could be rapidly equilibrated with α-tocopherol while heart, muscle, spinal cord and brain are the slow equilibrating tissues. Brain has the slowest turnover time (Traber, 1999). Therefore, the change of α-tocopherol level in plasma can higher reflected its change in erythrocytes, liver and spleen than that in heart, muscle, spinal cord and brain.

<u>Function of α-tocopherol in human</u>

α-tocopherol is a potent peroxyl radical scavenger and especially protects PUFAs (polyunsaturated fatty acids) within phospholipid in biological membranes and in plasma lipoproteins. In biological membranes, α-tocopherol exerts in the bilayer with hydrophobic side chain for every 2,000 phospholipid molecules. The possible position of α-tocopherol in the membranes is shown in Figure 3. In plasma lipoproteins, α-tocopherol is located in the core center of the molecules.

α-tocopherol functions *in vivo* as a chain breaking antioxidant that prevents propagation of free radical damage. When lipid hydroperoxide are oxidized to peroxyl radicals ($ROO^{\cdot}$), these react 1,000 times faster with α-tocopherol (α-T-OH) than with PUFAs (RH). Thus, the presence of α-tocopherol could protect PUFAs by phenolic hydroxyl group of α-tocopherol (α-T) reacts with an organic peroxyl radical ($ROO^{\cdot}$) to form organic hydroperoxide (ROOH) and the α-tocopheroxyl radical ($\alpha\text{-T-}O^{\cdot}$) (Traber, 1999). In the absence of α-tocopherol, peroxyl radical can react with the other PUFAs; so, oxidation chain reaction is occurred and damaging the tissues.

In the presence of α-T

$$ROO^{\cdot} + \alpha\text{-T - OH} \longrightarrow ROOH + \alpha\text{-T -}O^{\cdot}$$

In the absence of α-T

$$ROO^{\cdot} + RH \longrightarrow ROOH + R^{\cdot}$$

$$R^{\cdot} + O_2 \longrightarrow ROO^{\cdot}$$

The α-tocopheroxyl radical ($\alpha\text{-T-}O^{\cdot}$) reacts with vitamin C (or other reductants serving as hydrogen donors, AH) and returning to α-tocopherol (α-T -OH).

$$\alpha\text{-T -}O^{\cdot} + AH \longrightarrow \alpha\text{-T -OH} + A^{\cdot}$$

Biologically important hydrogen donors which have been demonstrated *in vitro* for regenerating α-tocopherol from α-tocopheroxyl radical, are ascorbate (vitamin C) and thiol especially glutathione. Subsequently, the vitamin C and thiyl radicals can be reduced via metabolic process. The phenomenon has led to the idea of "α-tocopherol recycling" (Traber, 1999)

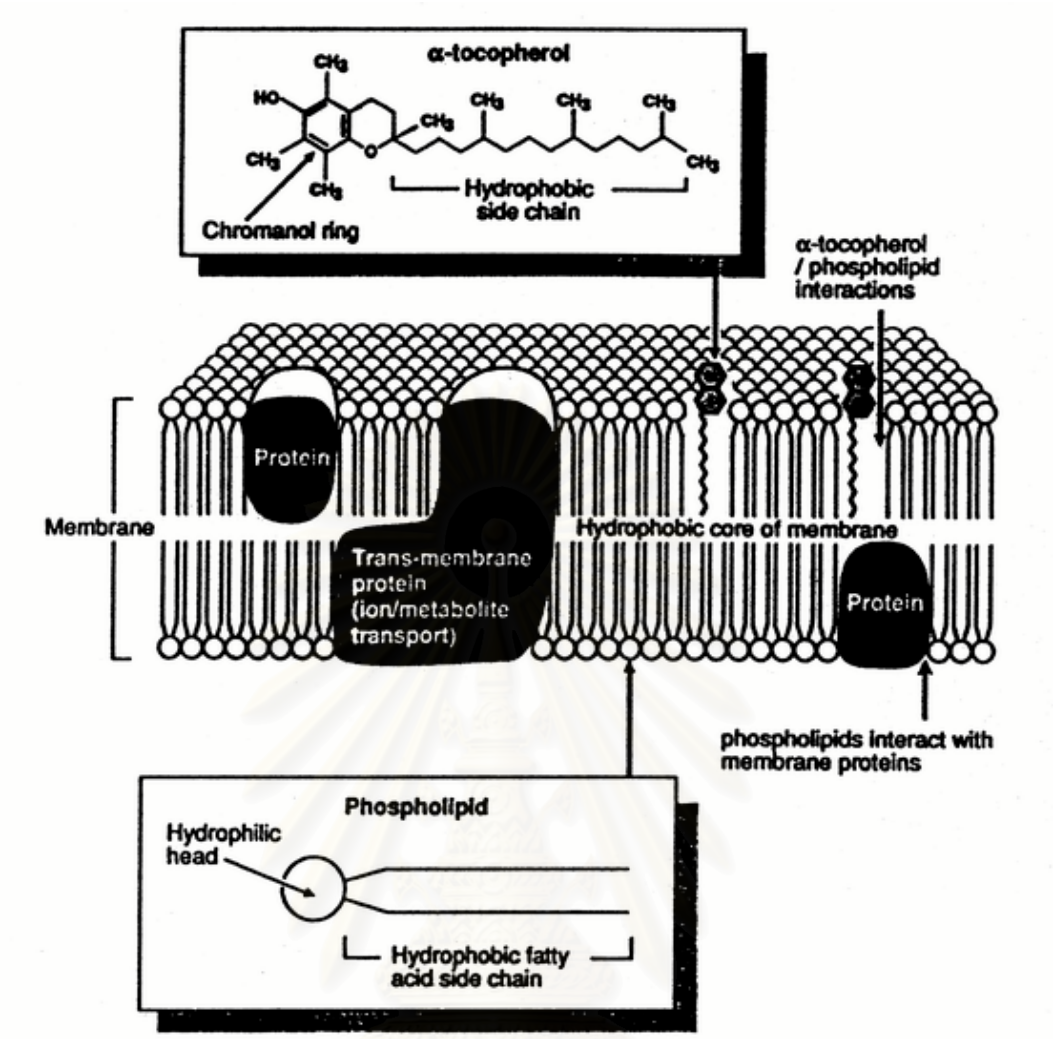


Figure 3. Representation of the lipid bilayer of a cell membrane, showing the possible position of the α-tocopherol molecule (Duthie, 2000)

Decomposition products and metabolites of α-tocopherol

The reaction of α-tocopherol with peroxyl radical *in vivo* can produce oxidative decomposition products including α-tocopherolquinone (TQ). It is further reduced to α-tocopherolhydroquinone (THQ), which is secreted into bile and feces as glucuronide conjugates. The other oxidative urinary metabolites include α-tocopheronic acid and 2,5,7,8-tetramethyl-2-(2'-carboxyethyl)-6-hydroxychroman (α-CEHC). However, less than 1% of the absorbed α-tocopherol is excreted in the urine. α-Tocopherolquinone can also be formed by *in vitro* oxidation of α-tocopherol. The pathway leading to excretion of α-tocopherol metabolites are shown in Figure 4.

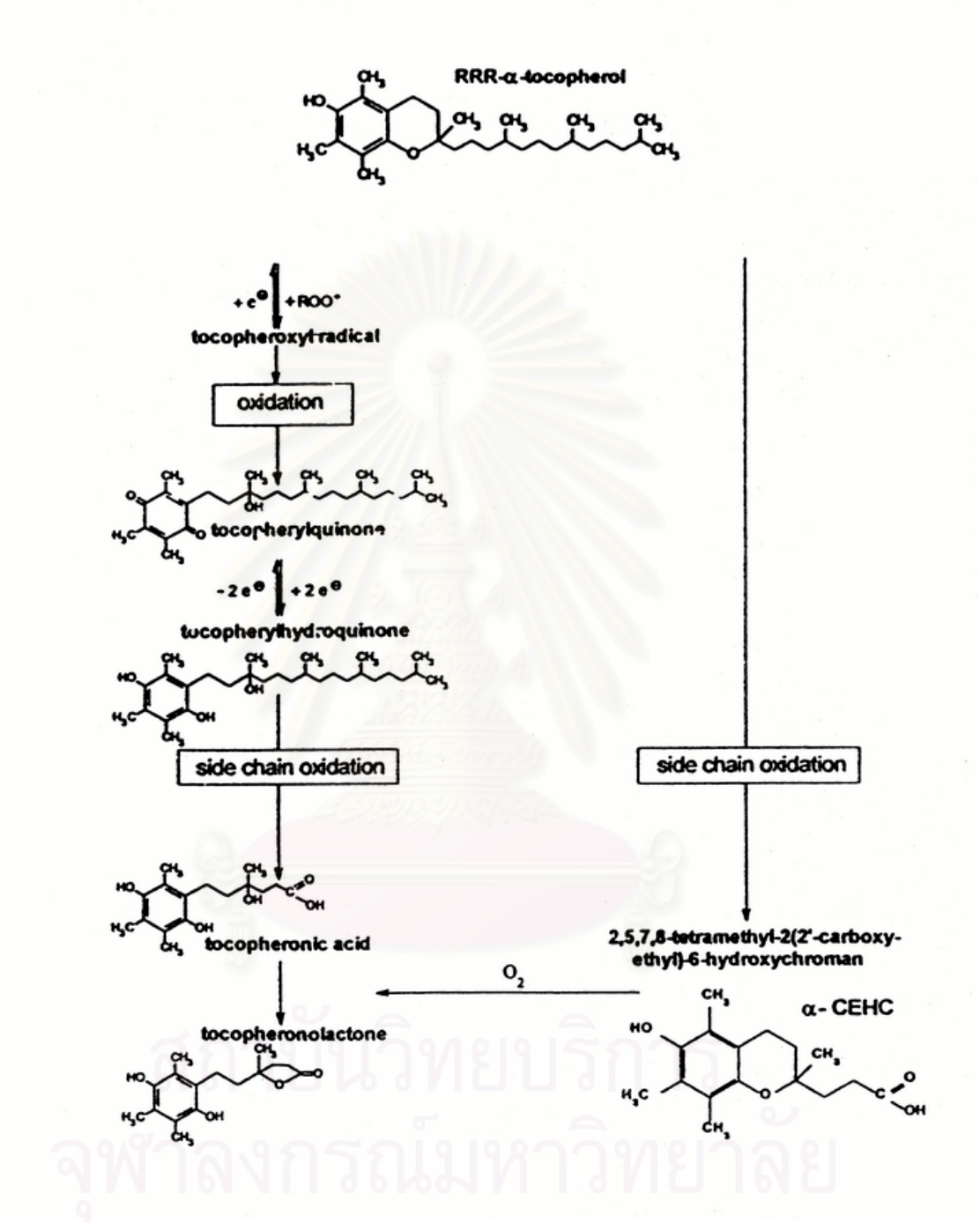


Figure 4. Metabolite pathway of α-tocopherol (Schultz, 1995)

Status of α-tocopherol level in human

The storage site for α-tocopherol in human is still unknown. However, more than 90% of α-tocopherol in the body is located in oil droplets of adipose tissues. The other major deposit sites are liver and muscle (Traber, 1999). Normal level of α-tocopherol, called endogenous α-tocopherol, in human plasma is about 10 μg/ml. If the level is less than 5 μg/ml, hemolysis of erythrocytes can be occurred (Machlin, 1991). If the supplement pill is taken, the level of α-tocopherol in plasma can be maximized to 3-4 times of the normal level. The mechanism could still not clarified. The recommended daily allowance (RDA) for α-tocopherol is 8 mg of α-tocopherol equivalence for women and 10 mg of α-tocopherol equivalence for men. Generally, α-tocopherol from daily food is enough for normal human, deficiency have never been observed. Nonetheless, the deficiency of α-tocopherol could be resulted from genetic abnormalities and various fat malabsorption syndromes. The genetic abnormalities are caused by the deficiency of α-tocopherol transfer protein (α-TT) that is important for incorporating α-tocopherol into VLDL or the deficiency of apolipoprotein B which is a protein in α-tocopherol transport lipoproteins (chylomicron, VLDL, LDL and HDL). For fat malabsorption syndrome, the disorder of pancreatic and bile secretions are commonly detected.

A routine assessment of vitamin E status in human is not required but only justified for risk groups (e.g. premature, genetic disorder and fat malabsorption syndrome, etc.) and as part of studies on the chemoprevention of diseases involving oxidative stress such as cancer, atherosclerosis, and cataract. The most common biological matrix for the determination of α-tocopherol is plasma or serum. However, using plasma or serum α-tocopherol as an indicator of the vitamin E status has not been concluded because strong correlation between α-tocopherol concentration and those of cholesterol and triglyceride in plasma; therefore, α-tocopherol/cholesterol ratio or α-tocopherol/triglyceride ratio or α-tocopherol/(cholesterol plus triglyceride) ratio can be used as an indicator for vitamin E status.

The analysis of α-tocopherol in human plasma

A variety of techniques have been reported for plasma α-tocopherol determination, these include GC-MS (Kock, 1997 and Melchert, 2000), HPLC (Chou, 1985; MacCrehan, 1987; Seta,1990; Bortolotti, 1993; Gonzalez-Corbella, 1994; Teissier, 1996; Cooper, 1997; Göbel, 1997; Lane, 1997; Sommerberg, 1997; Talwar, 1998; Julianto, 1999; Gemino, 2001; Taibi, 2002 and Anderson, 2003) and capillary electrochromatography (CEC) (Fanali, 2002). The GC-MS needs the chemical derivatization of sample prior to analysis that is inconvenience and time-consuming. Two GC-MS methods (Kock, 1997 and Melchert, 2000) used SPE for preparing plasma sample. Kock (1997) used C18 cartridge, whereas Melchert (2000) used two cartridges including C18 and then, Si cartridges.

For CEC, the new technique that based on both chromatographic and electrophoretic principles. Various tocopherol forms that contain similar structures could be separated within only short duration. However this method used hexane as the extracting solvent and needed 2 ml of plasma sample.

Although HPLC is more practically used than GC and CEC, most of them required the tedious liquid-liquid extraction for sample preparation. The accuracy of the method was limited by the loss of sample due to the occurrence of oxidation reaction during evaporation of the extracting solvent.

To avoid these shortcomings, plasma deproteinization methods for sample preparation have also been experimented (Lee, 1992; Teissier, 1996; Cooper, 1997; Julianto, 1999 and Taibi, 2002). Many disadvantages due to sensitivity limitation (Teissier, 1996), incomplete deproteinization (Gemino, 2001), complexation reaction (Lee, 1992; Cooper, 1997; Julianto, 1999 and Taibi, 2002) were detected. In spite of these observations, plasma deproteinization is still the challenging method in preparing plasma sample prier to HPLC analysis because of its convenience, simple and less time consuming. Therefore, it is the intention of this study in developing the new HPLC method for plasma α-tocopherol determination.

Objectives of the study

1. To develop a simple and rapid HPLC method for determining endogenous plasma α-tocopherol
2. To apply the developed method for determining endogenous plasma α-tocopherol in Thai smokers and non-smokers

The significance of this study

1. The new HPLC method that was simple and rapid in determining endogenous plasma α-tocopherol using plasma deproteinization was developed.
2. The developed method could be utilized for endogenous α-tocopherol determination in human plasma. The relationship between the endogenous α-tocopherol level and smoking status was determined and proven as its importance for various groups of people especially who have high risk to receive free radical.

CHAPTER II

MATERIALS AND METHODS

Materials

1. Chemical reagents

   1.1 Reference standard

      1.1.1 α-tocopherol, Lot. No. 32K1243, purity 99%; Sigma, St. Louis, USA

      1.1.2 α-tocopheryl acetate, Lot. No. 30K1172, purity 97.9%; Sigma, St. Louis, USA

   1.2 The other chemical reagents

      1.2.1 Methanol, HPLC grade; E. Merck, Damstadt, Germany

      1.2.2 Acetonitrile, HPLC grade; Lab Scan Analytical Science, Thailand

      1.2.3 Isopropanol, AR grade; Riedel-deHaen, Germany

      1.2.4 Ethanol, AR grade; E. Merck, Damstadt, Germany

2. Human plasma

   Human plasma was generously supplied from the Plasma Division, Thai Red Cross Society, Thailand.

3. Apparatus

   3.1 High Performance Liquid Chromatography, HPLC, TSP including P4000 gradient pump, AS3000 autosampler, UV2000 UV detector, Degasser, system software PC1000; Bioanalytical system , USA

   3.2 Ultraviolet spectrophotometer; spectronic 3000 array; Milton Roy, Florida, USA

3.3 Centrifuge, EBA 20; Hettich, Germany

3.4 Microcentrifuge, Z 230 MA; Berthold Hermle, Germany

3.5 Vortex mixer, Vortex-Genie; Scientific, Germany

3.6 HPLC column

3.6.1 μBondapak® C18, (300 x 3.9 mm, i.d.) 10 μm; Waters Associates Pty, Ltd., Massachusetts, USA

3.6.2 Guard column, (20 x 2.0 mm, i.d.) packed with Corosil® C18, 37-50 μm; Waters Associates Pty, Ltd., Massachusetts, USA

3.7 Micropipette; Socorex®, USA

4. Preparation of standard solutions

4.1 A stock solution of α-tocopherol (1 mg/ml)

A 10 mg of α-tocopherol was accurately weighed, dissolved and made up to 10 ml volume with methanol. The solution was light protected and used within a week.

4.2 A stock solution of α-tocopheryl acetate (1 mg/ml)

A 10 mg of α-tocopheryl acetate was accurately weighed, dissolved and made up to 10 ml volume with methanol. The solution was light protected and used within a week.

Methods

To prevent the degradation of α-tocopherol, the whole experiment in this study was performed in the room that illustrated with yellow light.

Four distinguished processes were performed in this study. They included

1. To develop the analytical method for determining α-tocopherol in human plasma by HPLC
2. To perform the bioanalytical method validation

3. To determine the appropriate calibration method for detection of endogenous α-tocopherol
4. To determine the concentration of endogenous α-tocopherol in Thai smokers and non-smokers

1. To develop the analytical method for determining α-tocopherol in human plasma by HPLC

1.1 HPLC condition

The HPLC condition appropriate for determining α-tocopherol was developed according to physicochemical properties of α-tocopherol (Table 2) in term of the detection wavelength, HPLC column and the composition of mobile phase.

1.1.1 To determine the detection wavelength for HPLC

Procedure

Standard ethanolic α-tocopherol solution (100 μg/ml) was spectrophotometrically scanned between the wavelength of 200 to 400 nm. The absorption wavelength at which α-tocopherol exerts the maximum absorbance was confirmed and used as UV detection wavelength for α-tocopherol in the analytical method development.

1.1.2 HPLC column and the composition of mobile phase

Isocratic reversed-phase technique was performed utilizing the octadecylsilane bonded phase column with methanol as the mobile phase.

Table 2. Physicochemical properties of α-tocopherol and α-tocopheryl acetate (British Pharmacopoeia, 1998)

Name	Molecular formula	M.W.	Appearance	Maximum absorption wavelength of ethanolic α-tocopherol solution	Solubility
α-tocopherol	$C_{29}H_{50}O_2$	430.7	Yellowish brown, viscous oily liquid	292	Practically insoluble in water, freely soluble in acetone, ethanol, ether, methylene chloride and fatty oils
α-tocopheryl acetate	$C_{31}H_{52}O_3$	472.7	Slightly greenish - yellow, viscous oily liquid	284	Practically insoluble in water, freely soluble in acetone, ethanol, ether and fatty oils, soluble in alcohol

1.1.3 Internal standard

Theoretically, internal standard for bioanalytical method should not be the compound that usually found in human body, dietaries or beverages. Neither pharmaceuticals nor its metabolites can be used. Generally, the compound being selected as internal standard should contain similar physicochemical properties to the sample. If possible, the internal standard used should be eluted later than the sample (Smith, 1981).

According to the aforementioned guideline, α-tocopheryl acetate was selected as internal standard for α-tocopherol in this study.

Procedure

Standard ethanolic α-tocopheryl acetate solution (100 μg/ml) was spectrophotometrically scanned as in section 1.1.1. The specific absorbance at the detection wavelength for HPLC analysis from 1.1.1 was determined.

1.2 Sample preparation by deproteinization

Standard plasma was prepared by deproteinization based on dehydration reaction with water-miscible organic solvent.

The single organic solvents were studied including methanol, ethanol, isopropanol and acetonitrile because they were simple and compatible with HPLC system. Then, if the results of single deproteinizing agents were not appropriate, the mixture of deproteinizing agents would be studied. The pattern of the mixture of deproteinizing agents that studied, depended upon the results of single deproteinizing agents.

For selection of the appropriate deproteinizing agent, both appearance and the percentage of recovery were considered. Deproteinizing agent that resulted the dense protein precipitate with easily separated supernatant for directly injection to HPLC addition to the percentage of recovery of 70-100% would be the selection one.

Procedure

Three replications of plasma samples were prepared according to Scheme 1. The standard solution of α-tocopherol and IS was spiked into blank plasma pre- or post-deproteinization. The appearance was observed and the percentage of recovery was calculated according to equation (1).

$$\%\text{recovery} = \frac{\text{PA of } \alpha\text{-tocopherol or IS spiked pre-deproteinization}}{\text{PA of } \alpha\text{-tocopherol or IS spiked post-deproteinization}} \times 100 \quad \ldots\ldots\ldots(1)$$

Scheme 1.

Blank plasma 200 μl in test tube

↓

Added 10 μl standard solution of α-tocopherol (0.2 mg/ml)

↓ vortex mixed

Added 10 μl of IS (0.8 mg/ml)

↓ vortex mixed

Added 800 μl of deproteinizing agent

↓ vortex mixed for 1 minute

Centrifuged at 1,500 x g, 20 minutes

↓

Injected the supernatant to HPLC

HPLC conditions

Analytical column	:	μBondapak® C18, (300 x 3.9 mm, i.d.) 10 μm
Guard column	:	Corosil® C18 (20 x 2.0 mm, i.d.) 37-50 μm
Mobile phase	:	100% of Methanol
Flow rate	:	1 ml/min
Detector wavelength	:	292 nm

2. To perform the bioanalytical method validation

The developed analytical method was validated to ensure the linearity, accuracy, precision, sensitivity, specificity and stability of the method according to the criteria of bioanalytical method validation. (Shah, 1992; Dadgar, 1995; Causon, 1997; Hartmann, 1998 and US. FDA., 2001).

2.1 Linearity

To determine the appropriate chromatographic response for α-tocopherol analysis, the response in term of peak area ratio (PAR) or peak height ratio (PHR) of α-tocopherol to internal standard (IS) was compared. The least variations of the response among three concentrations would be carried out for determining the linearity of the response.

Procedure

A series of standard plasma α-tocopherol concentrations (0, 0.80, 5.0, 10.0, 15.0, 20.0 and 30.0 μg/ ml) were analyzed according to the developed method. Both PAR and PHR of α-tocopherol to IS for each individual analysis were calculated. Meanwhile, the pattern of linear relationship of the selected response was determined. The reproducibility of linearity pattern was confirmed by doing the other three replications analysis of the series of α-tocopherol standard plasma in the concentrations of 0, 0.80, 10.0, 20.0 and 30.0 μg/ml. The standard errors of the slope and the intercept were then calculated according to regression analysis.

2.2 Accuracy and precision

2.2.1 The intra-day accuracy and precision

Procedure

Six replications of spiked α-tocopherol standard plasma in the concentrations of 5.0, 12.0 and 24.0 μg/ml were analyzed according to the developed method along with the series of standard plasma α-tocopherol in the concentrations of 0, 0.80, 5.0, 10.0, 15.0, 20.0 and 30.0 μg/ ml for the calibration curve.

The accuracy of analytical method can be presented in the term of %bias as shown in equation (2).

$$\%bias = [(analyzed\ conc. - added\ conc.)/added\ conc.\ ] \times 100 \quad \ldots\ldots\ldots(2)$$

For the acceptable accuracy, the percentage of bias obtained from the analysis of standard plasma should be within ± 15%.

The precision of analytical method were determined in the term of the percentage of relative standard deviation (%RSD) of α-tocopherol concentration. The method is considered to be precise if the %RSD should not more than 15% for all α-tocopherol concentrations studied.

2.2.2 The inter-day accuracy and precision

Procedure

The aforementioned procedure for intra-day analysis was followed but only one replication of standard plasma spiked α-tocopherol was analyzed on six separately different days. Also, the accuracy and the precision were determined with the same criteria as the intra-day procedure.

2.3 Sensitivity

The sensitivity of an analytical method can be described in the term of the lowest limit of quantification (LLOQ). Since it was the endogenous α-tocopherol that was determined in this study, the LLOQ would then be the lowest concentration of

endogenous α-tocopherol that could still be analyzed with the acceptable accuracy and precision.

Procedure

The endogenous α-tocopherol in blank plasma was analyzed along with the standard plasma for preparing the calibration curve. The concentration of endogenous α-tocopherol was determined from the calibration curve by standard addition method.

The endogenous α-tocopherol in the prepared spiked plasma was then diluted with deproteinizing agent before injecting to HPLC. The acceptable dilution would be confirmed by repeating the analysis of this dilution. The variation of analysis in the term of %bias and %RSD would be within ± 20%.

2.4 Specificity

To determine the specificity of the analytical method, the retention time of α-tocopherol and IS obtained from the analysis of standard solution and spiked IS plasma sample should be identical. In addition, endogenous peak shouldn't interfere with α-tocopherol and internal standard.

2.5 Stability of plasma sample, in-processed analyte and stock solution

The stability of plasma sample, in-processed analyte and stock solution were studied. The stability of plasma sample were determined at room temperature (25°C), storage temperature (-48 °C) and freeze-thaw cycle (25 °C and -48 °C). For in-processed analyte, the stability in autosampler was studied. In addition, the stability of α-tocopherol stock solution and working solution as well as IS stock solution and working solution were determined.

All stability programs were performed in three replications.

2.5.1 Stability of α-tocopherol in plasma sample

It composed of three different conditions as follows:

Stability of α-tocopherol in plasma sample at room temperature

Stability of α-tocopherol in plasma sample at storage temperature

Stability of α-tocopherol in plasma sample under the freeze-thaw cycle

The procedure of these three programs was similar but the condition and time schedule were fixed for appropriateness to each stability program (Table 3).

Procedure

On the day of analysis, calibration curve was prepared including a series of standard plasma α-tocopherol concentrations (0, 10.0, 20.0 and 30.0 μg/ml). Human plasma samples were analyzed following the developed method. The concentration of endogenous α-tocopherol in plasma sample was determined by using the calibration curve. The results were calculated in term of the percentage of recovery that was calculated from the following equation.

$$\% \text{ recovery} = [Ct/Co] \times 100 \qquad \text{.....(3)}$$

Ct was endogenous α-tocopherol concentration at any time point or cycle and Co was endogenous α-tocopherol concentration at zero time.

2.5.2 Stability of in-processed analyte in the autosampler

Procedure

The procedure was similar with the stability of plasma sample but the condition and time schedule were difference as shown in Table 4.

Table 3. The stability study for α-tocopherol in plasma sample

Stability program / Parameters	Room temperature	Storage temperature	Freeze-thaw cycle
Concentration of α-tocopherol (μg/ml)	Endogenous α-tocopherol	Endogenous α-tocopherol	Endogenous α-tocopherol
Temperature (°C)	25	-48	-48 and 25
Stability condition	Lab bench	Freezer	Freezer and room temperature
Stability checking time	0, 3, 6, and 9 hrs.	0, 15, 30 and 60 days	3 cycles with the time range of not less than 12 hrs.

Table 4. The stability study for in-processed analyte in the autosampler

Stability program / Parameters	In-processed analyte in the autosampler
Concentration of α-tocopherol (μg/ml)	Endogenous α-tocopherol
Temperature (°C)	4
Stability condition	autosampler
Stability checking time	0, 6, and 12 hrs.

2.5.3 Stability of stock solution and working solution of α-tocopherol and IS

Procedure

Stability of stock solution

The methanolic solution of α-tocopherol (1.0 mg/ml) and IS (1.0 mg/ml) were kept at -18°C and analyzed at 0, 7, 14, 21 and 28 days. The peak height (PH) was used in determining the percentage of recovery comparing to the zero time as shown in equation (4).

Stability of working solution

The methanolic solution of α-tocopherol (0.30 and 0.10 mg/ml) and IS (0.20 mg/ml) were analyzed at 0, 1, 2, 3 and 7 days. The percentage of recovery was calculated as procedure in stock solution.

$$\% \text{ recovery} = [PHt/PHo] \times 100 \qquad \text{.....(4)}$$

PHt was PH of α-tocopherol or IS at any time point and PHo was PH of α-tocopherol or IS at zero time.

2.5.4 Criteria required for stability study

For the stability of α-tocopherol in plasma and in-processed analyte in autosampler, the stable condition should show the results between 95 and 105 %recovery and for the stability of α-tocopherol and IS in stock and working solution, it should be between 90 and 110 %recovery.

3. To determine the appropriate calibration method for detection of endogenous α-tocopherol

In chromatographic analysis, concentration of analyte could be determined via calibration curve that relates the analytical response to standard analyte concentration. Three patterns of calibration methods can be used, they are the external standard method, the internal standard method, and the standard addition method. The internal standard method is the most commonly used in both GC and HPLC while the external standard method would be selected only that there could not be any appropriate internal standard for the analyte (Synder, 1997 and Harvey, 2000).

Since, this study emphasized the endogenous α-tocopherol in human plasma, it was necessary to use the standard addition method. By the standard addition method, blank plasma containing endogenous α-tocopherol was analyzed according to the developed method along with the same blank plasma that was spiked with the known standard concentration of α-tocopherol. This latter was used as the calibration for endogenous α-tocopherol determination in the former analysis. Also, α-tocopheryl acetate was added as the internal standard for controlling the efficiency of the method.

3.1 The standard addition method for determining plasma α-tocopherol

To determine endogenous α-tocopherol by the standard addition method, both single-point and multiple-point addition were experimented. For single-point standard addition, blank plasma containing endogenous α-tocopherol was spiked with the known concentration of α-tocopherol. For multiple-point standard addition, it was the series of known α-tocopherol concentrations that were spiked into blank plasma. (Harvey, 2000).

The percentage of relative standard deviation (%RSD) for both standard addition methods were compared. The one with the lower %RSD value would be carried on for volunteers study.

Single-point standard addition

Since only one known concentration of α-tocopherol would be spiked into the blank plasma, two concentrations of α-tocopherol (10.0 and 30.0 μg/ml) were tested for its appropriateness as the standard concentration.

Procedure

Nine replications were performed for each standard addition study. For homogeneity of endogenous α-tocopherol, plasma was vortexed 30 seconds before being aliquoted into two Eppendorfs, one for endogenous α-tocopherol analysis, the other for adding standard α-tocopherol before analysis. Endogenous α-tocopherol concentration was calculated from the following equation (Smith, 1981).

$$\text{Concentration of endogenous } \alpha\text{-tocopherol} = \frac{(\text{concentration of spiked standard})(PHR_A)}{PHR_{diff}} \quad \ldots(5)$$

$PHR_{diff} = PHR_B - PHR_A$

PHR_A = peak height ratio of analyte to internal standard obtained from blank plasma sample

PHR_B = peak height ratio of analyte to internal standard obtained from spiked plasma sample

Mutiple-point standard addition

Procedure

Plasma was vortexed 30 seconds to ensure the homogeneity of endogenous α-tocopherol before being aliquoted into three Eppendorfs for each series of calibration curves. The total six series were performed. Each series contained 0, 15.0 and 30.0 μg/ml of spiked α-tocopherol into plasma. They were all analyzed

according to the developed method. The calibration curve was constructed such that the concentration of endogenous α-tocopherol could be determined by either extrapolation or interpolation. (Meier, 2000).

Extrapolation

The calibration curve was plotted between the PHR in Y-axis and known concentration of added α-tocopherol in X-axis. The calibration equation was represented in equation (6). The concentration of endogenous α-tocopherol was determined by replacing PHR = 0 in equation (6) and calculated α-tocopherol concentration by extrapolating the calibration curve through the X-axis (Figure 5A).

$$PHR = a_1 Conc. + b_1 \quad \text{.....(6)}$$

PHR = peak height ratio

Conc. = concentration of α-tocopherol (μg/ml)

a_1 = slope

b_1 = Y-intercept

Interpolation

The calibration curve was plotted between the PHR after subtracting PHR of blank plasma in Y-axis and known concentration of α-tocopherol in X-axis. Then, the concentration of endogenous α-tocopherol was determined by replacing PHR value obtained from blank plasma in equation (7) and calculated α-tocopherol concentration (Figure 5B).

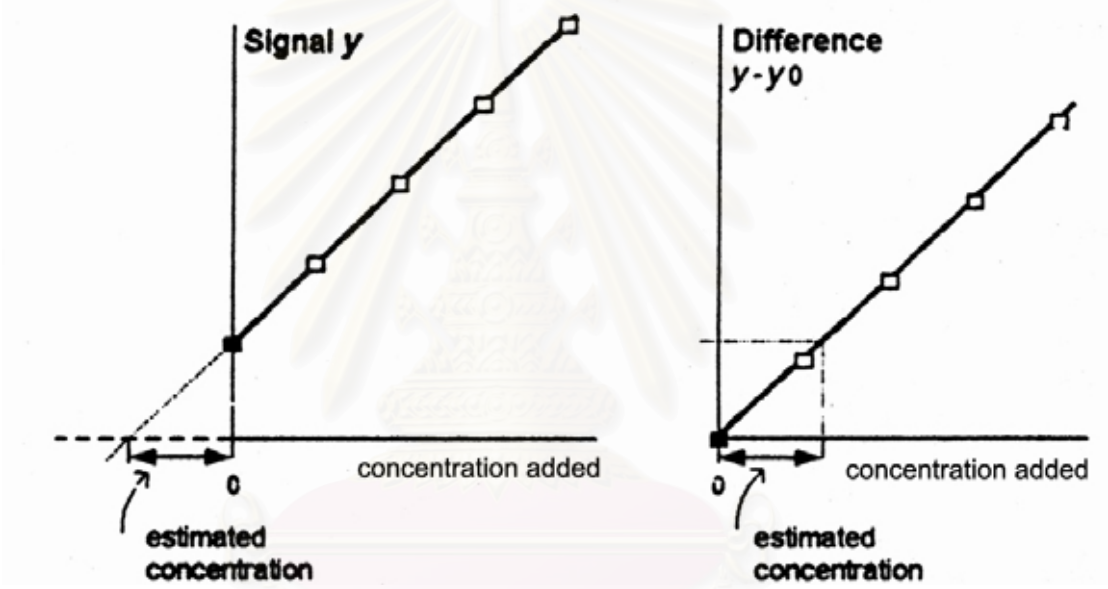
$$PHR' = a_2 Conc. + b_2 \quad \text{.....(7)}$$

PHR' = PHR after subtracting PHR of blank plasma

Conc. = concentration of α-tocopherol (μg/ml)

a_2 = slope

b_2 = Y-intercept



(A) (B)

Figure 5. The standard addition method: Extrapolation (A) and Interpolation (B)

3.2 Matrix effect from endogenous α-tocopherol on application of the standard addition method

In bioanalytical method especially using standard addition method, many compounds in plasma sample may affect the analyzed concentration. This is called the matrix effect. In this study, blank plasma containing endogenous α-tocopherol was used for constructing the calibration curve; therefore, the nonuniformity of endogenous α-tocopherol in blank plasma might cause the relative systemic error of the analyzed concentration. For studying plasma matrix effect, slopes of the standard addition line constructed in plasma and an aqueous calibration line constructed in deproteinizing agent solution were compared. If the matrix did not interfere, both lines were expected to have the same slope (Massart, 1997).

Procedure

The standard plasma containing 0.80, 10.0, 20.0 and 30.0 μg/ml of α-tocopherol were analyzed along with the deproteinizing agent containing the same α-tocopherol concentration. The calibration curve was constructed and the slope of both curves were determined.

4. To determine the concentration of endogenous α-tocopherol in Thai smokers and non-smokers

The developed method was applied for determining endogenous α-tocopherol in plasma obtaining from Thai male healthy volunteers with smoker and non-smoker habit.

Procedure

This part of the study had been already approved by the Ethic Committee at Pharmaceutical Science, Chulalongkorn University (1st October 2002).

Including volunteers

The total sixty volunteers were used in this study. They were equally separated into two groups: one for smoker and the other for non-smoker.

Criteria for including volunteers

Both groups were in the age range of 20-50 years, nonobesity (Body Mass Index; BMI less than 30 kg/m^2) and no systemic disease such as cardiovascular disease, diabetes and cancer. Also, three months before the day of experiment, they should not be on therapy with any particular drug or vitamin supplement especially antioxidant vitamin such as vitamin A, C and E.

The smoker volunteers should smoke average 20 cigarettes/day for at least 6 months before inclusion. For nonsmoker volunteers, they shouldn't smoke any kind of cigarette.

Excluding criteria

Volunteers who didn't pass the physical examination that confirmed healthy status would be excluded.

Random selection of the volunteers was performed by using public poster announcement and interview.

Volunteers who passed the including criteria were selected into the study and would be explained about the detail of experiment and then being requested to sign in the consent form. They also have to fill in the questionnaires about their lifestyle.

Food would be abstained at least 6 hours before the blood sample was drawn. Only 10-15 ml of blood from forearm vein would be withdrawn from each subject on the designed day. Blood sample was immediately centrifuged and plasma was separated. Plasma sample was divided into two parts. One part was kept frozen in light protection for subsequent analysis of endogenous α-tocopherol utilizing the developed method. The other part was used for examining the blood chemistry (complete blood count, glucose, BUN, creatinine, uric acid, cholesterol, triglyceride, SGOT, SGPT, alkaline phosphatase, HDL, LDL) by the laboratory center of faculty of allied health sciences, Chulalongkorn University.

Interpretation of data

To confirm the similarity of characteristics between smokers and non-smokers

Their age, sex and BMI should be similar. In this study, all volunteers were Thai male. Age and BMI were in the criteria range. However, age and BMI were compared in order to confirm the similarity of both groups by using student t-test (2-tailed) at 5% significance level ($\alpha = 0.05$). The calculation was performed by SPSS program version 10.0.

To determine endogenous α-tocopherol in volunteers

To determine endogenous α-tocopherol in plasma, various parameters could be used including α-tocopherol concentration, α-tocopherol/cholesterol ratio, α-tocopherol/triglyceride ratio and α-tocopherol/(cholesterol plus triglyceride) ratio. In this study, these four parameters were determined. Then, the significant difference of each parameter between smokers and non-smokers was determined using statistical student t-test (2-tailed) at 5% significance level ($\alpha = 0.05$). The calculation was performed by SPSS program version 10.0.

CHAPTER III

RESULTS AND DISCUSSION

1. To develop the analytical method for determining α-tocopherol in human plasma by HPLC

1.1 HPLC condition

To determine the detection wavelength for HPLC

α-Tocopherol in ethanol could exhibit the maximum absorption at the wavelength of 292 nm with the specific absorbance (1 μg/ml) to be 7.59 x 10^{-3} (Figure 6). This wavelength was used as the detector wavelength of α-tocopherol for further HPLC method development.

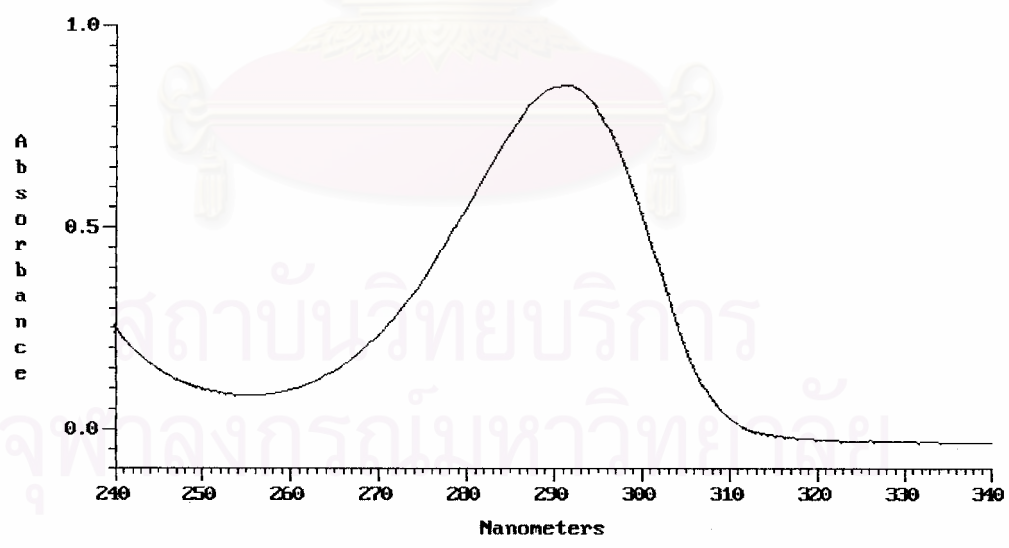


Figure 6. UV-spectrum of ethanolic α-tocopherol solution (112 μg/ml)

The wavelength that α-tocopheryl acetate (IS) in ethanol could exhibit the maximum absorption was 284 nm (Figure 7). At the detection wavelength for HPLC analysis of α-tocopherol (292 nm), IS could exhibit the absorption at the specific absorbance of 1.01 x 10^{-3} (1 μg/ml).

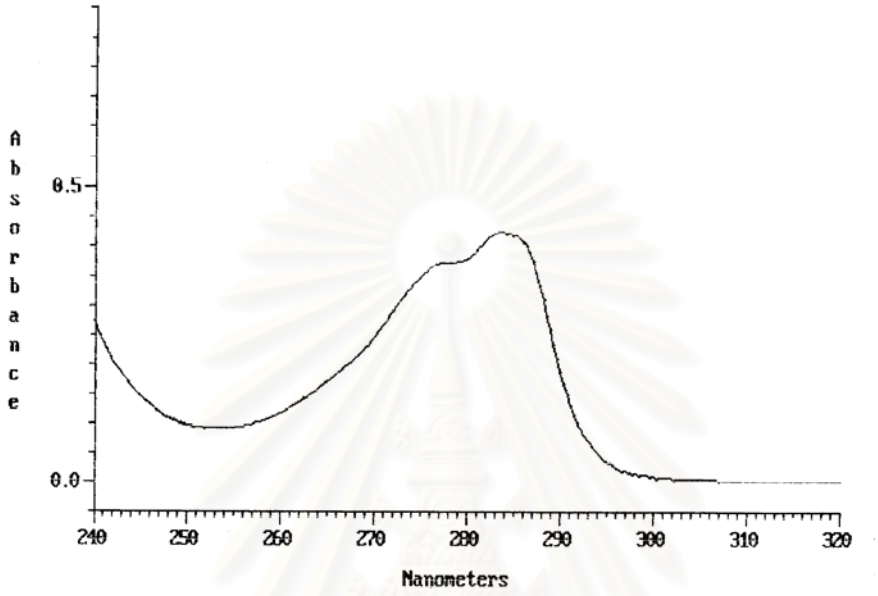


Figure 7. UV-spectrum of ethanolic α-tocopheryl acetate solution (99 μg/ml)

1.2 Sample preparation by deproteinization

Single deproteinizing agent

Each individual deproteinizing agent (methanol, ethanol, isopropanol and acetonitrile) could completely deproteinize the plasma protein at the volume ratio of 4:1 to the plasma. The characteristics of plasma following deproteinization and the efficiency of deproteinization were demonstrated in Table 5. Methanol could slowly deproteinize plasma protein resulting light protein precipitate that hardly to separate and the percentage of average recovery of α-tocopherol and IS were 76.15% and 58.80%, respectively. By using ethanol and isopropanol, protein precipitate was denser and the percentage of average recovery was higher than using methanol. By using ethanol, the percentage of average recovery of α-tocopherol and IS were 80.15% and 70.06%,

respectively while using isopropanol, those of α-tocopherol and IS were 87.93% and 85.94%, respectively. Acetonitrile could rapidly deproteinize plasma protein resulting hard solid mass precipitate in the meantime. Therefore, acetonitrile resulted in the best appearance. However, the percentage of average recovery of α-tocopherol and IS were only 32.64% and 24.12%, respectively. Chromatograms of plasma following deproteinization by methanol, ethanol, isopropanol and acetonitrile were also shown in Figure 8.

For single deproteinizing agents, acetonitrile exhibited good appearance but the percentage of average recovery was poor while the other deproteinizing agents including methanol, ethanol and isopropanol could exhibit the higher recovery but it was difficult to separate the supernatant for HPLC analysis. Therefore, the mixture of deproteinizing agents was studied.

For the pattern of the mixture of deproteinizing agents study, acetonitrile was mixed with the other solvents including methanol, ethanol or isopropanol in order to exhibit the good appearance and also increase the percentage of recovery. The mixture was performed in volume ratio of 1:1.

Table 5. Appearance and efficiency of plasma deproteinization by methanol, ethanol, isopropanol and acetonitrile

Deproteinizing agent	Characteristic of precipitate	Supernatant		%recovery of α-tocopherol (n = 3)			%recovery of IS (n = 3)		
		characteristic	pH	mean	SD	%RSD	mean	SD	%RSD
Methanol	White, very fine and light mass	pale yellow clear solution	7.0	76.15	6.59	8.66	58.80	6.80	11.57
Ethanol	Pale yellow, fine and dense mass	pale yellow clear solution	7.0	80.15	0.96	1.20	70.06	10.44	14.90
Isopropanol	Pale yellow, fine and mass denser than using ethanol	pale yellow clear solution	7.5	87.93	1.15	1.31	85.94	3.24	3.77
Acetonitrile	Yellow,coarse solid mass	pale yellow clear solution	7.0	32.64	3.29	10.08	24.12	7.03	29.13

$$\%\text{recovery} = \frac{\text{PA of } \alpha\text{-tocopherol or IS spiked pre-deproteinization}}{\text{PA of } \alpha\text{-tocopherol or IS spiked post-deproteinization}} \times 100$$

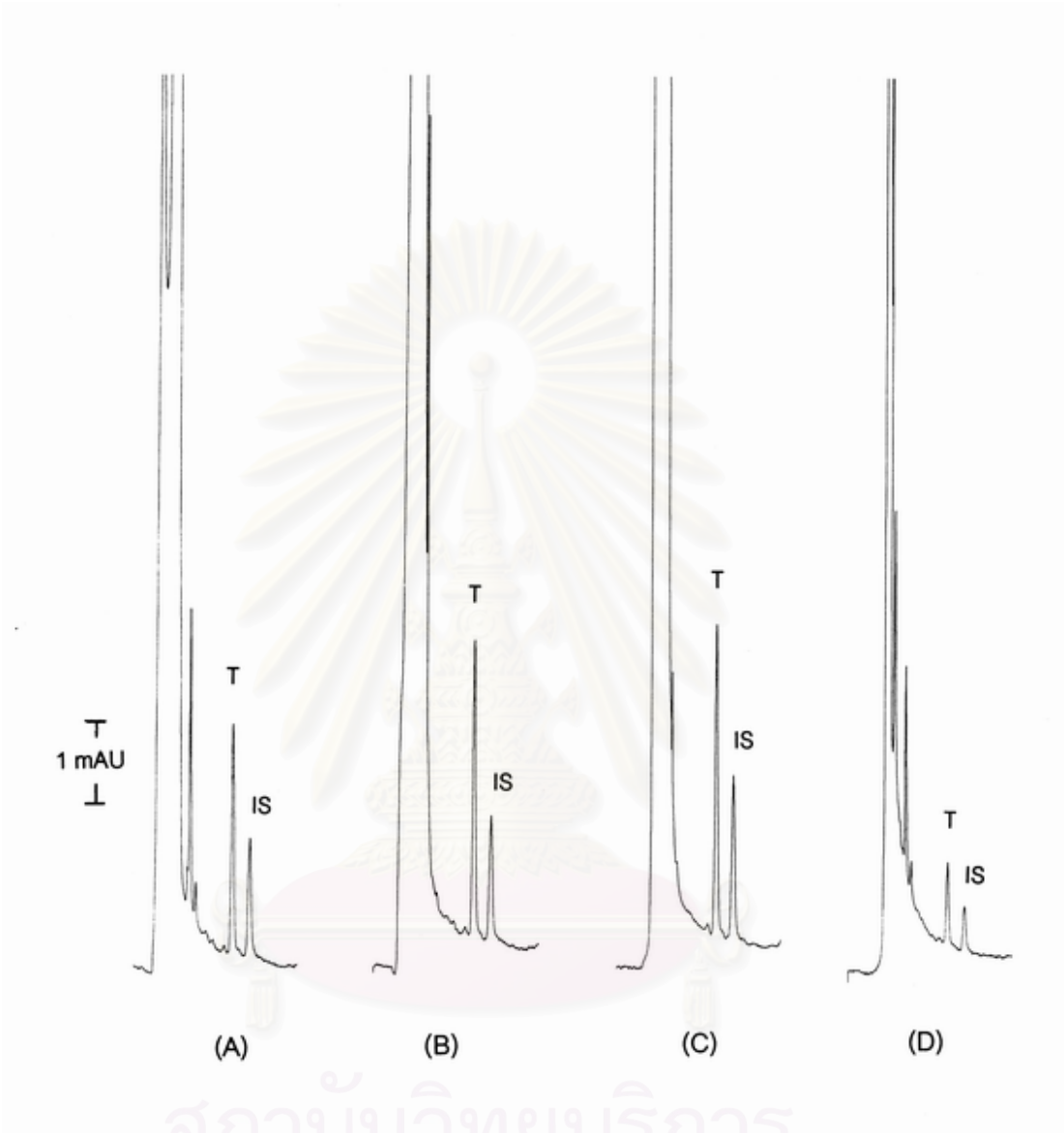


Figure 8. Chromatograms of plasma spiked with α-tocopherol (10 μg/ml) and IS (40 μg/ml) following deproteinization by (A) methanol, (B) ethanol, (C) isopropanol and (D) acetonitrile,
T = α-tocopherol, IS = internal standard

The mixture of deproteinizing agent

As shown in Table 6, the mixture of acetonitrile and methanol couldn't completely deproteinize plasma protein resulting the turbid supernatant that was not appropriate for HPLC analysis. Thus, incorporating acetonitrile to methanol gave the worse appearance than single agent and it was then excluded from the study. Either acetonitrile with ethanol or acetonitrile with isopropanol could completely deproteinize plasma protein in the volume ratio of 4:1 to the plasma. The percentage of average recovery of α-tocopherol and IS following acetonitrile with ethanol were 87.19% and 89.26%, respectively while using acetonitrile with isopropanol, they were 82.54% and 78.80%, respectively. No endogenous interference was observed from chromatograms of both deproteinizing mixtures (Figure 9). Nonetheless, acetonitrile with isopropanol gave the better appearance with easily separated supernatant and less variation in the percentage of recovery for both α-tocopherol and IS (Table 6) Therefore, acetonitrile with isopropanol was selected as the deproteinizing agent for further study.

Table 6. Appearance and efficiency of plasma deproteinization by the mixture of deproteinizing agents

Deproteinizing agent	Characteristic of precipitate	Supernatant		%recovery of α-tocopherol (n = 3)			%recovery of IS (n = 3)		
		characteristic	pH	mean	SD	%RSD	mean	SD	%RSD
Acetonitrile: Methanol (1:1)	pale yellow, fine and light mass	pale yellow and turbid solution	7.0	-	-	-	-	-	-
Acetonitrile: Ethanol (1:1)	pale yellow, fine and dense mass	pale yellow clear solution	7.0	87.19	6.23	7.15	89.26	8.98	10.06
Acetonitrile: Isopropanol (1:1)	pale yellow, fine and mass denser than using acetonitrile: ethanol	pale yellow clear solution	7.5	82.54	3.70	4.48	78.80	6.56	8.33

$$\%\text{recovery} = \frac{\text{PA of } \alpha\text{-tocopherol or IS spiked pre-deproteinization}}{\text{PA of } \alpha\text{-tocopherol or IS spiked post-deproteinization}} \times 100$$

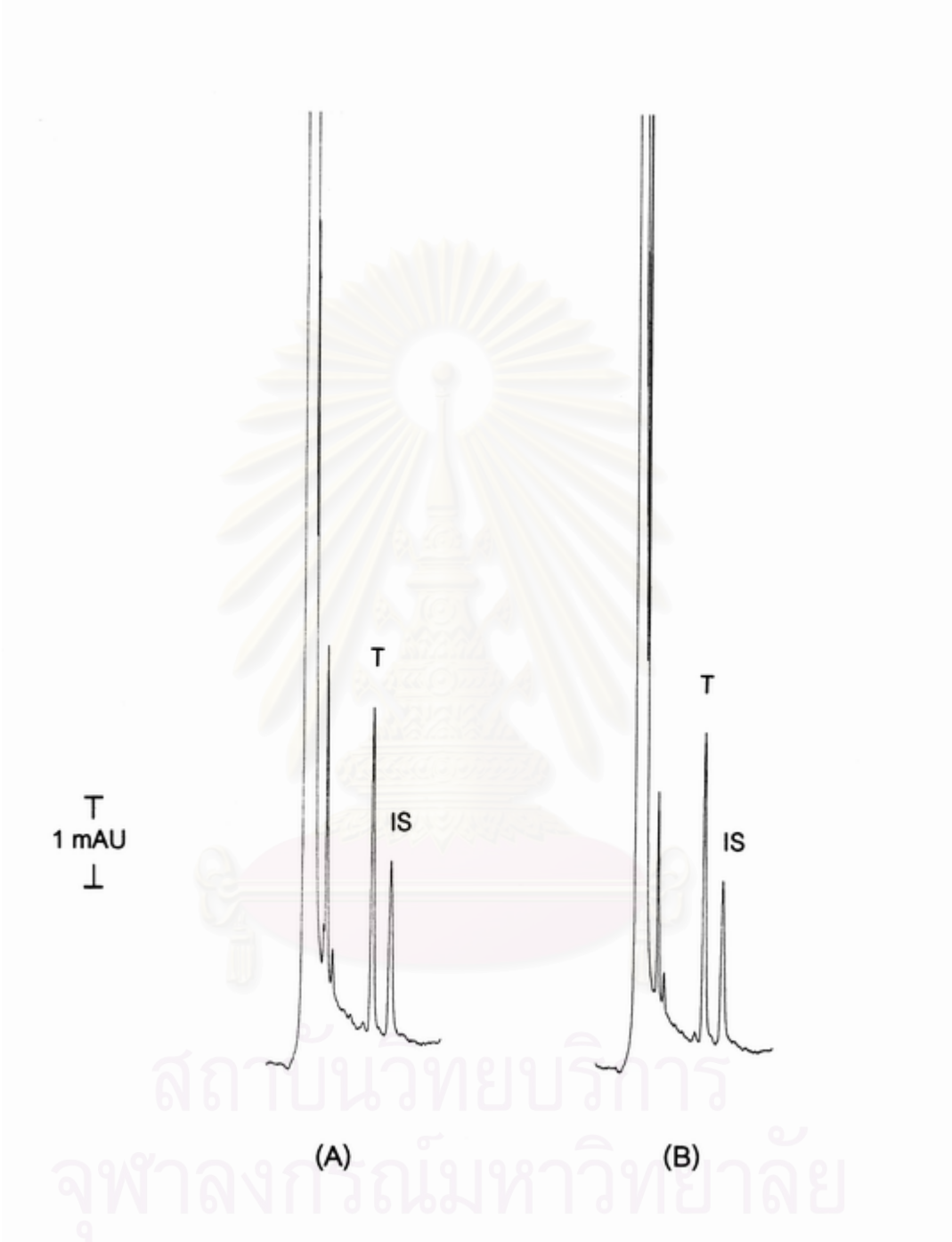


Figure 9. Chromatograms of plasma spiked with α-tocopherol (10 μg/ml) and IS (40 μg/ml) following deproteinization by (A) acetonitrile : ethanol (1:1) and (B) acetonitrile : isopropanol (1:1),

T = α-tocopherol, IS = internal standard

To ensure the best performance of acetonitrile with isopropanol as deproteinizing mixture, the appropriate proportion of both agents were experimented. The volume ratio of acetonitrile to isopropanol of 9:1, 8:2 and 7:3 were used. The selection was still followed the same aforementioned criteria for both appearance and the percentage of recovery. By varying the volume ratio, it was shown in Table 7 that acetonitrile and isopropanol in the ratio of 8:2 and 7:3 indicated quite similar appearance. The percentage of average recovery of α-tocopherol and IS following acetonitrile with isopropanol (8:2) were 97.66% and 83.23%, respectively while using acetonitrile with isopropanol (7:3), they were 85.35% and 82.43%, respectively. However, these were performed only three replications for screening. Therefore, the efficiency of plasma deproteinization by acetonitrile with isopropanol in the volume ratio of 8:2 or 7:3 was confirmed by repeating the selected procedure of either proportion with hepta replications performed in more replications.

As indicated in Table 8, acetonitrile with isopropanol in the volume ratio of 7:3 resulted in the acceptable percentage of average recovery of α-tocopherol and IS (96.72% and 88.44%, respectively), while those from volume ratio of 8:2 resulted in the average recovery of α-tocopherol of 103.5% and IS of 86.61%.

Table 7. Appearance and efficiency of plasma deproteinization by acetonitrile or the mixture of acetonitrile and isopropanol in varied ratio

Deproteinizing agent	Characteristic of precipitate	Supernatant		%recovery of α-tocopherol (n=3)			%recovery of IS (n=3)		
		characteristic	pH	mean	SD	%RSD	mean	SD	%RSD
Acetonitrile	pale yellow solid mass	pale yellow clear solution	7.0	57.09	8.15	14.28	48.99	5.55	11.33
Acetonitrile: isopropanol (9:1)	pale yellow, fine and dense mass	pale yellow clear solution	7.0	98.76	8.62	8.73	68.37	8.70	12.72
Acetonitrile: isopropanol (8:2)	pale yellow, fine and compact mass but looser than ratio 9:1	pale yellow clear solution	7.0	97.66	5.83	5.97	83.23	14.04	16.87
Acetonitrile: isopropanol (7:3)	pale yellow, fine and compact mass but looser than ratio 8:2	pale yellow clear solution	7.0	85.35	4.20	4.92	82.43	6.79	8.23

$$\%\text{recovery} = \frac{\text{PA of } \alpha\text{-tocopherol or IS spiked pre-deproteinization}}{\text{PA of } \alpha\text{-tocopherol or IS spiked post-deproteinization}} \times 100$$

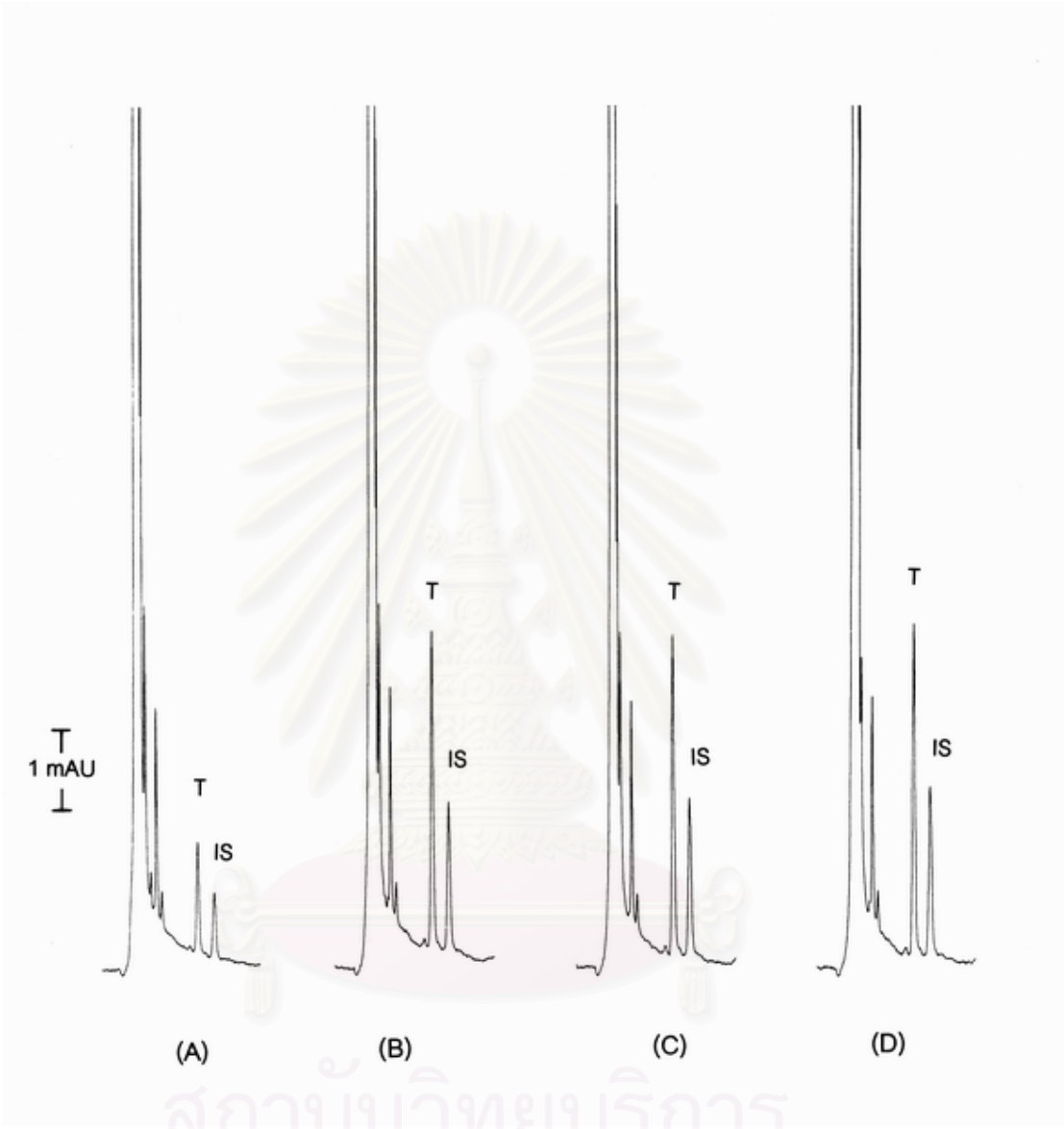


Figure 10. Chromatograms of plasma spiked with α-tocopherol (10 μg/ml) and IS (40 μg/ml) following deproteinization by (A) acetonitrile , (B) acetonitrile : isopropanol (9:1), (C) acetonitrile : isopropanol (8:2) and (D) acetonitrile : isopropanol (7:3),
T = α-tocopherol, IS = internal standard

Table 8. Confirmation of the efficiency of plasma deproteinization by the mixture of acetonitrile and isopropanol in ratio of 8:2 and 7:3.

Deproteinizing agent	%recovery of α-tocopherol (n=7)			%recovery of IS (n=7)		
	mean	SD	%RSD	mean	SD	%RSD
Acetonitrile: isopropanol (8:2)	103.5	8.72	8.43	86.61	3.08	3.55
Acetonitrile: isopropanol (7:3)	96.72	5.13	5.30	88.44	7.56	8.54

$$\%\text{recovery} = \frac{\text{PA of } \alpha\text{-tocopherol or IS spiked pre-deproteinization}}{\text{PA of } \alpha\text{-tocopherol or IS spiked post-deproteinization}} \times 100$$

Therefore, the appropriate deproteinizing agent for plasma α-tocopherol analysis was considered to be acetonitrile with isopropanol in the volume ratio of 7:3 with the final procedure in Scheme 2.

Scheme 2.

Blank plasma 100 μl in Eppendorf

↓

Added 10 μl of standard solution of α-tocopherol

↓ vortex mixed

Added 10 μl of IS (0.2 mg/ml)

↓ vortex mixed

Added 400 μl of acetonitrile:isopropanol (7:3)

↓ vortex mixed for 1 minute

Centrifuged at 15,340 x g, 20 minutes

↓

Aliquot 200 μl of supernatant into insert vial

↓

Injected 50 μl to HPLC analysis

<u>HPLC conditions</u>

Analytical column : μBondapak® C18, (300 x 3.9 mm, i.d.) 10 μm

Guard column : Corosil® C18, (20 x 2.0 mm, i.d.) 37-50 μm

Mobile phase : 100% of Methanol

Flow rate : 1 ml/min

Detector wavelength : 292 nm

Even though, there have been three reports for α-tocopherol analysis utilizing deproteinization (Teissier, 1996; Cooper, 1997 and Julianto, 1999), this developed method was more simple in using only the mixture of acetonitrile and isopropanol. Cooper have to pre-treat the plasma with magnesium chloride and sodium tungstate that could injur the column while the method of Teissier, α-tocopherol have to be determined by fluorescence detector. Julianto also deproteinize plasma sample with the mixture of deproteinizing agent (acetonitrile plus tetrahydrofuran); however, there was no complete validation.

2. To perform the bioanalytical method validation

2.1 Linearity

As indicated in Table 9, the chromatographic response that was selected for this α-tocopherol analysis would be peak height ratio (PHR). The percentage of relative standard deviation (%RSD) for PHR were ranged from 2.20 to 8.25 that was less variable than those from peak area ratio (PAR) (2.68-9.98%RSD).

The relationship between concentration of spiked α-tocopherol and PHR could be explained as the linear pattern with the linear range of 0-30 μg/ml. Figure 11 displayed the represent standard addition calibration curve of α-tocopherol with the regression equation of PHR = 0.45Conc. + 2.89, R^2 = 0.9934. To confirm the linear pattern, the %RSD of the slope, the intercept and R^2 were determined to be 6.84, 2.30 and 0.46, respectively (Table 10).

Table 9. Chromatographic response for standard plasma α-tocopherol analysis

Concentration of spiked α-tocopherol (μg/ml)	PAR (n=6)			PHR (n=6)		
	Mean	SD	%RSD	Mean	SD	%RSD
3.0	3.55	0.10	2.68	4.20	0.09	2.20
12.0	6.87	0.38	5.51	7.97	0.36	4.55
24.0	11.65	1.16	9.98	13.44	1.11	8.25

Figure 11. The represent standard addition calibration curve of α-tocopherol

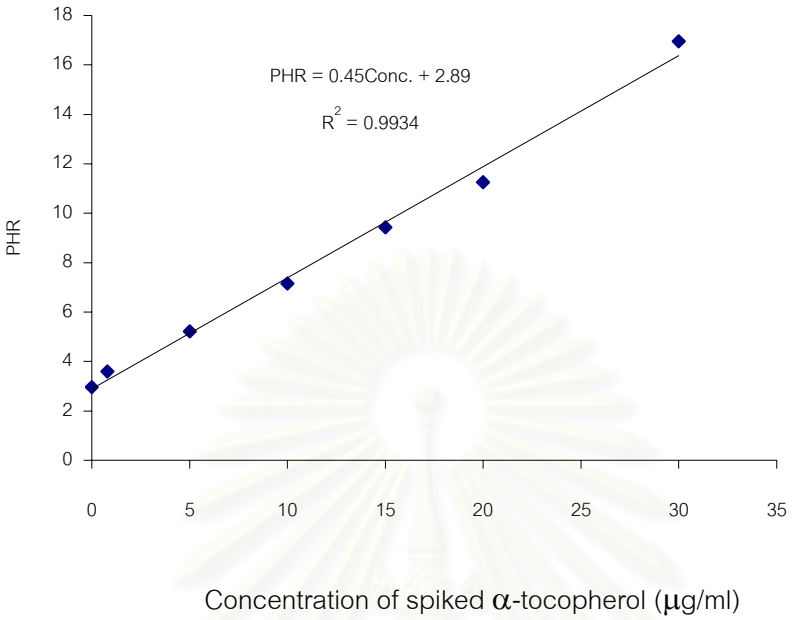


Table 10. Confirmation of linear standard addition calibration curve (0-30 μg/ml)

N	Parameters of calibration curve		
	Slope	Intercept	R^2
1	0.43	3.15	0.9905
2	0.44	3.16	0.9978
3	0.49	3.03	0.9995
Mean	0.45	3.12	0.9907
SD	0.03	0.70	0.0046
%RSD	6.84	2.30	0.46

Since %RSD of the slope, intercept and R^2 were all less than 15%. This confirmed the linearity of the standard addition calibration curve.

2.2 Accuracy and precision

2.2.1 The intra-day accuracy and precision

The percentage of bias in the same day assay of spiked α-tocopherol in plasma were ranged from –14.67 to +11.67 for the three plasma α-tocopherol concentrations covered the concentration range in plasma that possibly found in the body as shown in Table 11. The percentage of relative standard deviation were between 7.36 and 10.04 (Table 12). Therefore, the method for α-tocopherol analysis in plasma was accurate and precise enough for applicable use.

2.2.2 The inter-day accuracy and precision

The percentage of bias at six difference days of spiked α-tocopherol in plasma were ranged from –10.58 to +14.80 and the percentage of relative standard deviation were between 6.69 and 8.43 as displayed in Table 13 and 14, respectively.

Both accuracy and precision of either intra-day or inter-day were within the limit range (± 15% of %bias and within 15% of %RSD); therefore, this analytical method revealed acceptable accuracy and precision.

Table 11. The intra-day accuracy of analysis of α-tocopherol in spiked plasma

Spiked α-tocopherol concentration (μg/ml)	%Bias (n=6).						Mean
	1	2	3	4	5	6	
5.0	-9.20	+1.80	-3.40	+7.60	+11.20	-12.80	-0.80
12.0	-8.50	-10.33	+2.33	+7.92	+4.33	-2.50	-1.12
24.0	+5.75	+11.67	-6.79	-14.67	-2.67	-9.04	-2.62

Table 12. The intra-day precision of analysis of α-tocopherol in spiked plasma

Spiked α-tocopherol concentration (μg/ml)	Analyzed α-tocopherol concentration (n=6)						Mean	SD	%RSD
	1	2	3	4	5	6			
5.0	4.54	5.09	4.83	5.38	5.56	4.36	4.96	0.47	9.48
12.0	10.98	10.76	12.28	12.95	12.52	11.70	11.86	0.87	7.36
24.0	25.38	26.80	22.37	20.48	23.36	21.83	23.37	2.35	10.04

Table 13. The inter-day accuracy of analysis of α-tocopherol in spiked plasma

Spiked α-tocopherol concentration (μg/ml)	%Bias (6 days)						Mean
	1	2	3	4	5	6	
5.0	+10.43	-1.20	+2.40	+14.80	-9.20	+3.40	+3.44
12.0	-10.58	+5.42	+0.25	+4.08	-8.50	-3.33	-2.11
24.0	-6.25	+13.75	+11.92	-0.92	+5.75	-5.62	+3.10

Table 14. The inter-day precision of analysis of α-tocopherol in spiked plasma

Spiked α-tocopherol concentration (μg/ml)	Analyzed α-tocopherol concentration (6 days)						Mean	SD	%RSD
	1	2	3	4	5	6			
5.0	5.52	4.94	5.12	5.74	4.54	5.17	5.17	0.42	8.20
12.0	10.73	12.65	12.03	12.49	10.98	11.60	11.75	0.79	6.69
24.0	22.50	27.30	26.86	23.78	25.38	22.65	24.74	2.09	8.43

2.3 Sensitivity

The lowest limit of quantification (LLOQ) of α-tocopherol in plasma was determined to be 0.72 μg/ml. It was the concentration that PHR could be quantitated. This LLOQ was confirmed as demonstrated in Table 15 that the percentage of bias were ranged from –19.44 to +5.56 and the percentage of relative standard deviation was 9.72. This lowest plasma concentration of α-tocopherol would be the lowest concentration in the calibration curve for α-tocopherol analysis. However, the lowest concentration in the calibration curve was 0.80 μg/ml for simple preparation.

Based on the same technique as deproteinization and then HPLC analysis, the sensitivity of this developed method was better than Cooper (1997) that the value of LLOQ was 1 μg/ml but it was not better than method of Julianto (1999) that the value of LLOQ was 0.42 μg/ml. However, the sensitivity of this developed method was enough to detect the concentration of endogenous α-tocopherol in human plasma.

2.4 Specificity

As shown in Figure 12, the specificity of the analytical method was clearly presented in which α-tocopherol in standard solution and endogenous α-tocopherol in blank plasma gave the identical retention time at approximately of 7.1 min while the retention time of α-tocopheryl acetate (IS) in standard solution was also similar to that in blank plasma spiked with IS (approximately 8.3 min). The presented chromatograms showed that both α-tocopherol and α-tocopheryl acetate were well resolved from each other without any detectable interference.

Table 15. The lower limit of quantification (LLOQ) for α-tocopherol in spiked plasma

Concentration of α-tocopherol (μg/ml)	N	Analyzed concentration of α-tocopherol (μg/ml)	%Bias
0.72	1	0.76	+5.56
	2	0.58	-19.44
	3	0.69	-4.17
	4	0.68	-5.56
	5	0.65	-9.72
Mean		0.67	-6.67
SD		0.07	
%RSD		9.72	

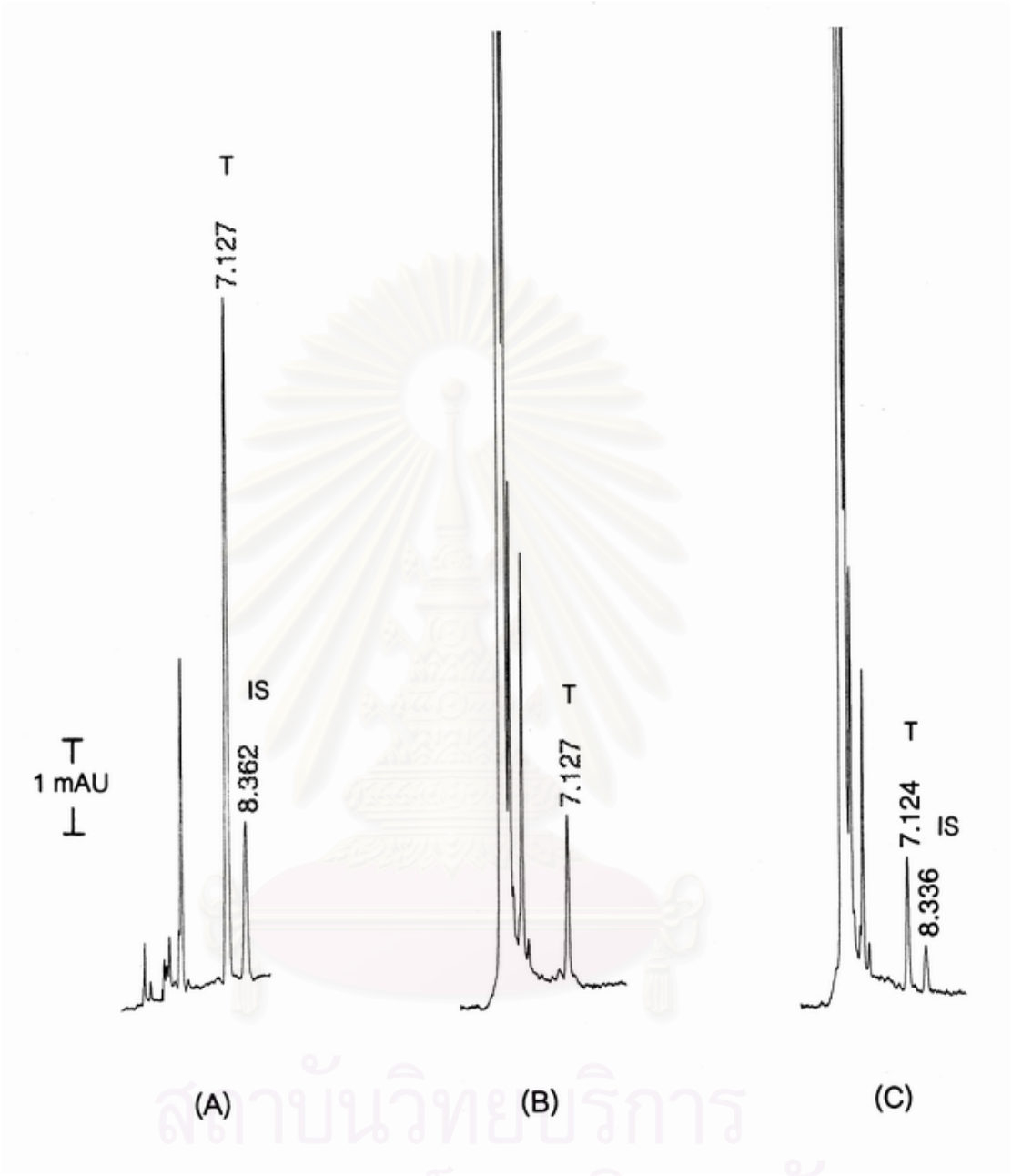


Figure 12. Chromatograms of α-tocopherol and IS when using methanol as mobile phase :

(A) Standard solution of α-tocopherol (5 μg/ml) and α-tocopheryl acetate (10 μg/ml),

(B) Blank plasma with endogenous α-tocopherol, and

(C) Spiked plasma sample with IS 20 μg/ml plasma,

T = α-tocopherol, IS = internal standard

2.5 Stability of plasma sample, in-processed analyte and stock solution

2.5.1 Stability of α-tocopherol in plasma sample

Stability of α-tocopherol in plasma sample at room temperature (23±2 °C)

As shown in Table 16, the percentage of average recovery of α-tocopherol in plasma sample at room temperature (23±2 °C) within 3, 6 and 9 hours. were ranged from 95.34 to 96.18 comparing to zero time concentration. The percentage of relative standard deviation confirmed the variations of analysis were ranged from 4.28 to 6.25. Therefore, α-tocopherol in plasma was stable at room temperature for 9 hours during the process of analysis.

Stability of α-tocopherol in plasma sample at storage temperature (-47±1 °C)

α-tocopherol in storage plasma at frozen temperature of -47±1 °C could be stable with the percentage of average recovery of 100.4% within 15 days as shown in Table 17.

Stability of α-tocopherol in plasma sample under the freeze-thaw cycle

As demonstrated in Table 18, α-tocopherol in plasma could still be stable even after three cycles of freeze and thaw. The percentage of average recovery of α-tocopherol from the first to third cycle were determined to be 97.53, 101.1 and 102.2, respectively. Thus, plasma sample could be used and restored in the frozen temperature within three times.

Table 16. Stability of α-tocopherol in plasma at room temperature (23±2 °C)

Storage time at room temperature (hrs.)	N1		N2		N3		Mean of % recoveries	SD	%RSD
	α-toco-pherol conc. (μg/ml)	% recovery	α-toco-pherol conc. (μg/ml)	% recovery	α-toco-pherol conc. (μg/ml)	% recovery			
0	6.46	100.0	6.15	100.0	6.47	100.0	100.0	0.00	0.00
3	5.95	92.11	6.34	103.1	6.04	93.35	96.18	6.01	6.25
6	6.57	101.7	5.69	92.52	5.94	91.81	95.34	5.52	5.79
9	6.37	98.61	6.00	97.56	5.89	91.04	95.74	4.10	4.28

$$\% \text{ recovery} = \frac{\text{concentration at determined time} \times 100}{\text{concentration at zero time}}$$

Table 17. Stability of α-tocopherol in plasma stored at -47±1 °C

Storage time in freezer (days.)	N1		N2		N3		Mean of % recoveries	SD	%RSD
	α-toco-pherol conc. (μg/ml)	% recovery	α-toco-pherol conc. (μg/ml)	% recovery	α-toco-pherol conc. (μg/ml)	% recovery			
0	5.95	100.0	6.22	100.0	6.01	100.0	100.0	0.00	0.00
15	6.27	105.4	5.82	93.57	6.15	102.3	100.4	6.13	6.10
30	5.39	90.59	5.86	94.21	5.81	96.67	93.82	3.06	3.26
60	6.77	113.8	6.93	111.4	6.94	115.5	113.6	2.04	1.80

$$\% \text{ recovery} = \frac{\text{concentration at determined time} \times 100}{\text{concentration at zero time}}$$

Table 18. Stability of α-tocopherol in plasma under the freeze-thaw cycle

Number of freeze and thaw cycle	N1		N2		N3		Mean of % recoveries	SD	%RSD
	α-toco-pherol conc. (μg/ml)	% recovery	α-toco-pherol conc. (μg/ml)	% recovery	α-toco-pherol conc. (μg/ml)	% recovery			
zero time	6.11	100.0	6.38	100.0	5.79	100.0	100.0	0.00	0.00
1	5.59	91.49	6.56	102.8	5.69	98.27	97.53	5.70	5.85
2	6.13	100.3	6.42	100.6	5.92	102.2	101.1	1.03	1.02
3	6.11	100.0	6.64	104.1	5.93	102.4	102.2	2.05	2.01

$$\% \text{ recovery} = \frac{\text{concentration at determined time} \times 100}{\text{concentration at zero time}}$$

2.5.2 Stability of in-processed analyte in the autosampler

For in-processed analyte that was transferred into the autosampler (4 °C) waiting for injection into HPLC, α-tocopherol could be withstood only within 6 hours. The percentage of average recovery was observed to be 101.9 (Table 19). Therefore, it was necessary to manage the number of processed analyte in autosampler to be injected within less than 6 hours to ensure no occurrence of decomposition.

2.5.3 Stability of α-tocopherol or IS stock solution and working solution

As shown in Table 20, after 28 days storage at –18±2 °C and protected from light, the percentage of average recovery of α-tocopherol and IS calculated from peak height were ranged between 90.93 to 93.87 for α-tocopherol and 93.09 to 100.1 for IS. The results indicated that methanolic stock solution of both α-tocopherol and IS were stable at least 28 days in these conditions.

The working solution of α-tocopherol was stable within 7 days, the percentage of average recovery calculated from peak height were ranged between 97.53 to 106.3 (Table 21). While the working solution of IS could be stable within 3 days, the percentage of average recovery were ranged between 93.39 to 102.0 (Table 22). Thus, it is recommended to prepare working solution of α-tocopherol every week and IS freshly prepared for IS solution.

Table 19. Stability of in-processed analyte in the autosampler (4 °C)

Standing time in the autosampler (hrs.)	N1		N2		N3		Mean of % recoveries	SD	%RSD
	α-tocopherol conc. (μg/ml)	% recovery	α-tocopherol conc. (μg/ml)	% recovery	α-tocopherol conc. (μg/ml)	% recovery			
0	6.20	100.0	6.12	100.0	6.56	100.0	100.0	0.00	0.00
6	6.01	96.94	6.54	106.9	6.68	101.8	101.9	4.96	4.87
12	6.77	109.2	6.46	105.6	6.71	102.3	105.7	3.45	3.27

$$\% \text{ recovery} = \frac{\text{concentration at determined time}}{\text{concentration at zero time}} \times 100$$

Table 20. Stability of stock standard methanolic α-tocopherol or IS solution stored at –18±2 °C

Storage time in freezer (days)	α-tocopherol (1.0 mg/ml) (n = 3)				IS (1.0 mg/ml) (n = 3)			
	Mean of PH	%recovery			Mean of PH	%recovery		
		Mean	SD	%RSD		Mean	SD	%RSD
0	7926	100.0	0.00	0.00	1767	100.0	0.00	0.00
7	7207	90.93	1.15	1.26	1768	100.1	2.52	2.52
14	7270	91.55	1.29	1.41	1644	93.09	1.67	1.79
21	7299	92.11	1.73	1.88	1671	94.64	5.16	5.45
28	7438	93.87	2.29	2.44	1678	94.98	1.86	1.96

$$\% \text{ recovery} = \frac{\text{PH at determined time}}{\text{PH at zero time}} \times 100$$

Table 21. Stability of working standard methanolic α-tocopherol solution stored at –18±2 °C

Storage time in freezer (days)	α-tocopherol (0.30 mg/ml) (n = 3)				α-tocopherol (0.10 mg/ml) (n = 3)			
	Mean of PH	%recovery			Mean of PH	%recovery		
		Mean	SD	%RSD		Mean	SD	%RSD
0	8134	100.0	0.00	0.00	7525	100.0	0.00	0.00
1	8631	106.3	2.35	2.21	7494	99.61	1.20	1.90
2	8134	100.6	4.95	4.92	7339	97.53	2.45	2.51
3	8268	101.8	2.46	2.42	7638	101.5	2.11	2.08
7	8421	103.7	4.72	4.56	7933	105.5	3.95	3.75

$$\% \text{ recovery} = \frac{\text{PH at determined time}}{\text{PH at zero time}} \times 100$$

Table 22. Stability of working standard methanolic IS solution stored at –18±2 °C

Storage time in freezer (days)	IS (0.20 mg/ml) (n = 3)			
	Mean of PH	%recovery		
		Mean	SD	%RSD
0	1655	100.0	0.00	0.00
1	1545	93.39	2.24	2.40
2	1555	93.90	3.10	3.31
3	1689	102.0	2.45	2.41
7	1854	112.0	4.90	4.37

$$\% \text{ recovery} = \frac{\text{PH at determined time}}{\text{PH at zero time}} \times 100$$

3. To determine the appropriate calibration method for detection of endogenous α-tocopherol

3.1 The standard addition method for determining endogenous α-tocopherol in plasma

The mean values of endogenous α-tocopherol (n=9) obtained from single-point standard addition of α-tocopherol 10.0 μg/ml or 30.0 μg /ml were 6.68 and 6.36 μg/ml, respectively (Table 23). More fluctuation of endogenous α-tocopherol concentration was observed with the addition of 10.0 μg/ml standard α-tocopherol as shown in the percentage of relative standard deviation (%RSD) value. The %RSD value of 10.0 μg/ml α-tocopherol standard addition was 12.27 % while that for 30.0 μg/ml addition was 9.36%. This result confirmed Renman's report (1997) that the increase in standard addition magnitude could decrease in the %RSD value.

For multiple-point standard addition method, the mean values of endogenous α-tocopherol (n=6) obtained from either extrapolation or interpolation were 6.22 and 6.71 μg/ml, respectively (Table 24). The interpolation method seemed to achieve the better precision with the %RSD of 10.25% comparing to 19.19% for extrapolation. Meier (2000) also reported that the interpolation could yield the more precise of the results from the GC signal.

To consider the appropriate calibration method for practical endogenous α-tocopherol determination, only single-point standard addition of 30.0 μg/ml of α-tocopherol were compared to the multiple-point interpolation standard addition method due to their similarity in %RSD values.

The single-point standard addition method was not practical for routine analysis because each sample requires its own addition. For multiple-point standard addition, interpolation type, the plasma pooled could be used for constructing the represent calibration curve for the same day analysis. This would be more convenient and practical used. Therefore, the multiple-point standard addition was further used for volunteers study. For preparing a calibration curve, the series of α-tocopherol concentration of 0, 0.80, 10.0, 20.0 and 30.0 μg/ml were analyzed. The calibration

curve was plotted between the absolute PHR value at any concentration in Y-axis and α-tocopherol concentration in X-axis. The absolute PHR value could be determined by subtracting the PHR value from standard addition with the PHR value of zero concentration.

3.2 Matrix effect from endogenous α-tocopherol on application of the standard addition method

To determine the effect of matrix complication in plasma on the standard addition method, the calibration curve prepared from spiked α-tocopherol into blank plasma and into deproteinizing solution were compared as displayed in Figure 13. The calibration curve from blank plasma exhibited the slope value of 0.4602 that was very similar to that from solution (0.4559). The difference in the slope value was only 0.0043. Therefore, it may be concluded that no matrix effect was observed in the standard addition used in this study.

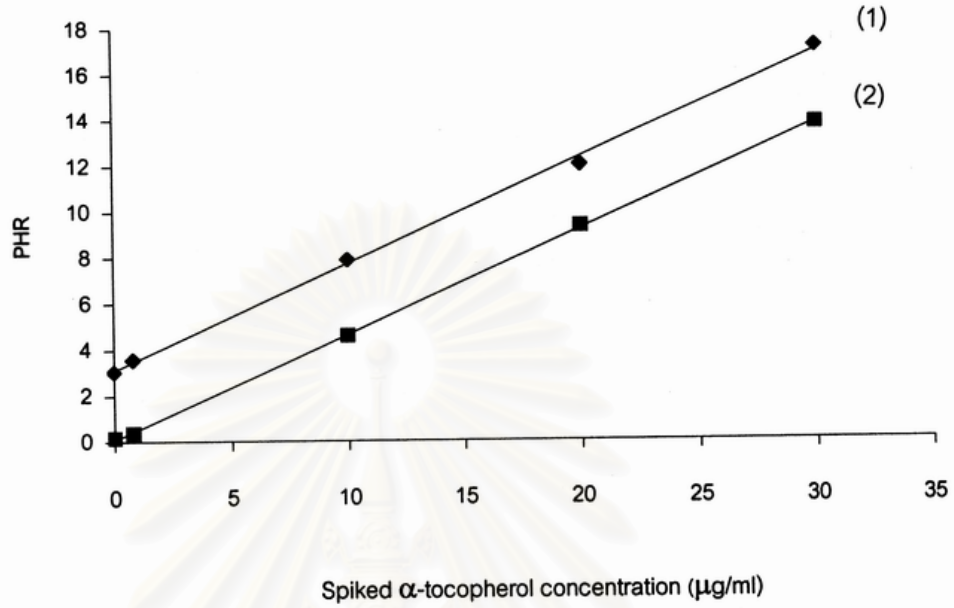
Table 23. Endogenous α-tocopherol concentration calculated from single-point standard addition

N	Endogenous α-tocopherol (μg/ml)	
	Spiked α-tocopherol 10.0 μg/ml	Spiked α-tocopherol 30.0 μg/ml
1	6.57	6.54
2	6.58	7.25
3	7.25	5.47
4	6.34	6.99
5	6.77	6.60
6	5.81	5.96
7	8.05	6.70
8	7.41	5.88
9	5.36	5.85
Mean	6.68	6.36
SD	0.82	0.60
%RSD	12.27	9.36

Table 24. Endogenous α-tocopherol concentration calculated from multiple-point standard addition

N	Endogenous α-tocopherol (μg/ml)	
	Extrapolation calculation	Interpolation calculation
1	6.28	6.81
2	6.96	7.54
3	4.02	6.91
4	7.52	6.45
5	6.13	7.06
6	6.40	5.51
Mean	6.22	6.71
SD	1.19	0.69
%RSD	19.19	10.25

Figure 13. Comparison of calibration curve prepared in spiked plasma (line 1) and deproteinizing agent (line 2)



Regression equation of line (1) : $Y = 0.4602X + 3.1251$, $R^2 = 0.9986$

Regression equation of line (2) : $Y = 0.4559X + 0.0901$, $R^2 = 0.9998$

4. To determine the concentration of endogenous α-tocopherol in Thai smokers and non-smokers

To confirm the similarity of characteristics between smokers and non-smokers

In this study, only Thai male smoker and non-smoker volunteers were included. Two of non-smoker volunteers were excluded from analysis because the concentrations of SGPT and SGOT were higher than three times of normal limit that implied unhealthy status. Therefore, total fifty-eight volunteers enrolled in this study including thirty smoker volunteers and twenty-eight non-smoker volunteers.

No statistically significant difference in the age and BMI between both groups was observed (p = 0.444 and 0.920, respectively) as tabulated in Table 25.

Table 25. Characteristics of smoker and non-smoker volunteers

Parameters	Age (years)		BMI (kg/m^2)	
	Smokers (n = 30)	Non-smokers (n = 28)	Smokers (n = 30)	Non-smokers (n = 28)
Mean	33.7	31.3	22.38	22.30
SD	9.4	8.3	2.70	3.36
Range	20.0-50.0	21.0-50.0	16.53-28.69	15.43-29.38
p-value	0.444		0.920	
Statistical significance Student t-test (2-tailed) at α = 0.05	NS		NS	

NS = non significance

To determine endogenous α-tocopherol in volunteers

Interpreting in term of α-tocopherol concentration

No statistically significant difference of endogenous α-tocopherol was observed between smokers and non-smokers (p = 0.931). As exhibited in Table 26, the mean values of endogenous α-tocopherol in smokers and non-smokers were determined to be 12.23 and 12.16 μg/ml, respectively. According to endogenous α-tocopherol guideline, this level of α-tocopherol in Thai volunteers could be indicated as non-deficient status. The grand total mean endogenous α-tocopherol among all fifty-eight volunteers was then determined to be 12.20 μg/ml (SD = 2.93, SE = 0.38). Ong-Ajyooth (1987) also reported the mean endogenous α-tocopherol in 173 Thai males with 18-55 years of age to be approximate 13.20 μg/ml (SD=4.60, SE=0.35). Since these both studies involve healthy Thai male volunteers, this would imply the normal α-tocopherol in Thai people.

There have been many reports concerned the influence of smoking habit on the level of endogenous α-tocopherol in plasma (Comstock, 1988; Bolton-smith, 1991; Faruque, 1995; Mezzetti, 1995; Driskell, 1996 and Dietrich, 2003). No absolute conclusion could be made. However, it is noticeable that in healthy subjects, smoking habit may or may not affect endogenous α-tocopherol level depending upon the number of subjects studied as well as the study design. Endogenous α-tocopherol level reported by Bolton-smith (1991) showed the significant difference among smokers and non-smokers when the number of subjects studied were 79 and 117, respectively. While no statistically difference was observed in Comstock (1988), Faruque (1995), Mezzetti (1995), and Dietrich (2003) studies.

Even though endogenous α-tocopherol in plasma of smokers and non-smokers were not significantly different (8.8 $\pm$ 0.7 and 10.5 $\pm$ 1.6 μg/ml, respectively), the α-tocopherol in the alveolar fluid of smokers (3.1 $\pm$ 0.7 ng/ml) were significantly lower than those in non-smokers (20.7 $\pm$ 2.4 ng/ml) (Pacht, 1986). Mezzetti (1995) determined endogenous α-tocopherol level in patient that undergoing

aortocoronary bypass surgery. The very low endogenous α-tocopherol in the arterial tissue of smokers (0.23 ± 0.03 μg/mg protein) comparing to non-smokers (0.41 ± 0.04 μg/mg protein) was observed. In the meantime endogenous α-tocopherol in plasma of smokers and non-smokers were not significantly difference.

It implied that concentration of endogenous α-tocopherol in plasma did not change until the regulatory system of α-tocopherol in the body was damage. However, the other ways for interpreting endogenous α-tocopherol in term of ratio of α-tocopherol to cholesterol and triglyceride might indicate the significant difference of endogenous α-tocopherol between smokers and non-smokers.

Interpreting in term of α-tocopherol/cholesterol ratio

No statistically significant difference of α-tocopherol/cholesterol ratio was observed between smokers and non-smokers ($p = 0.495$). As exhibited in Table 27, the mean values of α-tocopherol/cholesterol ratio in smokers and non-smokers were determined to be 6.16 and 6.00 μg/mg, respectively. This confirmed the reports of Bolton-smith, 1991; Faruque, 1995 and Mezzetti, 1995.

Interpreting in term of α-tocopherol/triglyceride ratio

Statistically significant difference of α-tocopherol/triglyceride ratio was observed between smokers and non-smokers ($p = 0.010$). As exhibited in Table 28, the mean values of α-tocopherol/triglyceride ratio in smokers and non-smokers were determined to be 11.63 and 15.65 μg/mg, respectively. This result agreed with Lui's report (Lui, 2000) that indicated the significant difference of α-tocopherol/triglyceride ratio between both groups.

Interpreting in term of α-tocopherol/(cholesterol plus triglceride) ratio

Statistically significant difference of α-tocopherol/(cholesterol plus triglyceride) ratio was observed between smokers and non-smokers ($p = 0.034$). As exhibited in Table 29, the mean values of α-tocopherol/(cholesterol plus triglyceride) ratio in smokers and non-smokers were determined to be 3.79 and 4.20 μg/mg, respectively.

For these four parameters in indicating endogenous α-tocopherol status in this study, it couldn't be concluded which one was the most appropriate parameter. According to the function of α-tocopherol in the body, α-tocopherol was a major antioxidant of unsaturated fatty acids that were the composition of cell membranes and plasma lipoproteins. The minimum requirement of α-tocopherol in plasma depended on the amount and type of unsaturated fatty acids in plasma but analysis of unsaturated fatty acids in plasma was not simple for routine work. Then, Nagaya (1998) studied the relationship of these four parameters with the minimum requirement of α-tocopherol in plasma. It was reported that α-tocopherol/(cholesterol plus triglyceride) ratio was the best correlated with the minimum requirement of vitamin E in plasma. Therefore, the ratio of α-tocopherol/(cholesterol plus triglyceride) might be the most appropriate parameter for endogenous serum α-tocopherol status in healthy volunteers.

In this study, significantly difference of endogenous α-tocopherol concentration between smokers and non-smokers couldn't be observed until the values were interpreted in term of the ratios of α-tocopherol/triglyceride or α-tocopherol/ (cholesterol plus triglyceride). It was observed that average plasma triglyceride concentration between smokers and non-smokers was quite different (133.93 and 89.43 mg/dl, respectively) comparing to the concentration of cholesterol (199.57 and 201.96 mg/dl, respectively). These needed further provement whether the triglyceride in smokers should usually be higher than non-smokers? If it is, the interpreting of α-tocopherol by normalizing with triglyceride would probably be the appropriate value.

Table 26. Endogenous α-tocopherol concentration of smokers and non-smokers

Volunteer number	Smokers (n = 30)			Non-smokers (n = 28)
	Average cigarettes/day	Time of smoking (years)	α-tocopherol conc. (μg/ml)	α-tocopherol conc. (μg/ml)
1	20	10	11.80	10.15
2	20	7	9.62	6.97
3	20	10	11.75	12.86
4	20	7	7.98	10.75
5	20	6	15.06	12.16
6	20	5	18.46	13.38
7	20	16	11.05	10.97
8	35	25	9.59	12.73
9	35	31	14.18	14.71
10	20	10	17.94	12.39
11	20	20	10.77	13.70
12	20	26	11.42	9.26
13	20	7	13.07	12.51
14	20	30	15.80	12.44
15	20	15	9.23	14.33
16	20	20	11.08	11.66
17	20	20	16.95	15.21
18	20	7	10.41	16.57
19	20	14	13.22	7.60
20	20	6	17.39	12.04
21	20	10	12.06	7.70
22	25	5	11.31	11.16
23	20	13	9.42	16.82
24	20	20	11.77	10.24
25	30	15	8.73	18.13
26	30	13	11.53	18.09
27	25	6	12.25	7.42
28	20	2	10.56	8.66
29	20	6	13.11	-
30	20	5	9.46	-
Mean			12.23	12.16
SD			2.81	3.11
p-value			0.931	
Statistical significance Student t-test (2-tailed) at α = 0.05			NS	
Grand total mean (μg/ml)			12.20	
SD			2.93	
SE			0.38	

NS = non significance

Table 27. Endogenous α-tocopherol in term of α-tocopherol/cholesterol ratio

Volunteer number	Smokers (n = 30)		Non-smokers (n = 28)	
	α-tocopherol conc. (μg/ml)	α-tocopherol /TC ratio (μg/mg)	α-tocopherol conc. (μg/ml)	α-tocopherol /TC ratio (μg/mg)
1	11.80	5.06	10.15	5.61
2	9.62	5.98	6.97	4.98
3	11.75	6.87	12.86	6.43
4	7.98	5.58	10.75	5.72
5	15.06	5.86	12.16	6.95
6	18.46	7.10	13.38	5.79
7	11.05	4.91	10.97	5.71
8	9.59	6.57	12.73	5.11
9	14.18	7.54	14.71	5.79
10	17.94	8.08	12.39	6.63
11	10.77	5.01	13.70	6.43
12	11.42	5.60	9.26	5.51
13	13.07	6.28	12.51	5.51
14	15.80	7.15	12.44	7.40
15	9.23	4.94	14.33	5.95
16	11.08	7.29	11.66	5.61
17	16.95	6.75	15.21	5.41
18	10.41	5.54	16.57	7.85
19	13.22	6.89	7.60	5.59
20	17.39	5.13	12.04	6.40
21	12.06	6.15	7.70	4.53
22	11.31	5.86	11.16	5.37
23	9.42	5.57	16.82	8.37
24	11.77	6.65	10.24	4.88
25	8.73	5.32	18.13	7.00
26	11.53	5.52	18.09	7.09
27	12.25	5.47	7.42	4.70
28	10.56	6.18	8.66	5.55
29	13.11	7.85	-	-
30	9.46	6.14	-	-
Mean		6.16		6.00
SD		0.90		0.94
p-value	0.495			
Statistical significance Student t-test (2-tailed) at α = 0.05	NS			

NS = non significance

TC = cholesterol

Table 28. Endogenous α-tocopherol in term of α-tocopherol /triglyceride ratio

Volunteer number	Smokers (n = 30)		Non-smokers (n = 28)	
	α-tocopherol conc. (μg/ml)	α-tocopherol /TG ratio (μg/mg)	α-tocopherol conc. (μg/ml)	α-tocopherol /TG ratio (μg/mg)
1	11.80	3.82	10.15	19.52
2	9.62	13.94	6.97	18.34
3	11.75	12.50	12.86	17.62
4	7.98	8.06	10.75	13.61
5	15.06	5.98	12.16	21.33
6	18.46	11.91	13.38	16.94
7	11.05	25.11	10.97	15.03
8	9.59	3.95	12.73	16.32
9	14.18	7.05	14.71	31.30
10	17.94	8.01	12.39	19.67
11	10.77	11.22	13.70	15.05
12	11.42	5.86	9.26	8.74
13	13.07	6.02	12.51	22.75
14	15.80	10.90	12.44	10.54
15	9.23	9.61	14.33	16.28
16	11.08	6.44	11.66	6.66
17	16.95	18.83	15.21	18.55
18	10.41	9.13	16.57	10.17
19	13.22	17.39	7.60	12.46
20	17.39	11.59	12.04	18.52
21	12.06	10.86	7.70	15.71
22	11.31	5.60	11.16	5.29
23	9.42	18.12	16.82	16.49
24	11.77	23.08	10.24	7.53
25	8.73	9.59	18.13	10.30
26	11.53	10.39	18.09	20.33
27	12.25	12.01	7.42	14.00
28	10.56	19.56	8.66	19.24
29	13.11	7.99	-	-
30	9.46	24.26	-	-
Mean		11.63		15.65
SD		5.95		5.54
p-value	0.010			
Statistical significance Student t-test (2-tailed) at α = 0.05	S*			

S* = significance

TG = triglyceride

Table 29. Endogenous α-tocopherol in term of α-tocopherol /(cholesterol plus triglyceride) ratio

Volunteer number	Smokers (n = 30)		Non-smokers (n = 28)	
	α-tocopherol conc. (μg/ml)	α-tocopherol /(TC+TG) (μg/mg)	α-tocopherol conc. (μg/ml)	α-tocopherol /(TC+TG) (μg/mg)
1	11.80	2.18	10.15	4.36
2	9.62	4.18	6.97	3.92
3	11.75	4.43	12.86	4.71
4	7.98	3.30	10.75	4.03
5	15.06	2.96	12.16	5.24
6	18.46	4.45	13.38	4.32
7	11.05	4.11	10.97	4.14
8	9.59	2.47	12.73	3.89
9	14.18	3.65	14.71	4.89
10	17.94	4.02	12.39	4.96
11	10.77	3.46	13.70	4.51
12	11.42	2.86	9.26	3.38
13	13.07	3.08	12.51	4.44
14	15.80	4.32	12.44	4.35
15	9.23	3.26	14.33	4.36
16	11.08	3.42	11.66	3.04
17	16.95	4.97	15.21	4.19
18	10.41	3.45	16.57	4.43
19	13.22	4.93	7.60	3.86
20	17.39	3.56	12.04	4.76
21	12.06	3.93	7.70	3.52
22	11.31	2.86	11.16	2.66
23	9.42	4.26	16.82	5.55
24	11.77	5.16	10.24	2.96
25	8.73	3.42	18.13	4.17
26	11.53	3.60	18.09	5.26
27	12.25	3.76	7.42	3.52
28	10.56	4.69	8.66	4.31
29	13.11	3.96	-	-
30	9.46	4.90	-	-
Mean		3.79		4.20
SD		0.76		0.70
p-value	0.034			
Statistical significance Student t-test (2-tailed) at α = 0.05	S*			

S* = significance

TC = cholesterol, TG = triglyceride

In this study, the developed method was used to determine endogenous α-tocopherol only in healthy volunteers including smokers and non-smokers; therefore, it was challenged to determine endogenous α-tocopherol in unhealthy volunteers to approve the efficiency of the method for more applications. The extra experiment was performed in two lung cancer patients because some previous studies reported that plasma α-tocopherol in lung cancer patients was significantly lower than that of control (Menkes, 1986 and Miyamoto, 1987). Moreover, a case control studies (Tominaga, 1992) and a cohort study (Woodson, 1999) reported inversely association between plasma α-tocopherol level and risk of lung cancer.

The results showed that endogenous α-tocopherol in plasma of the two lung cancer patients were determined to be 6.98 and 5.13 μg/ml that were lower than the grand total mean of endogenous α-tocopherol concentration in fifty-eight healthy subjects enrolled in this study (12.20 μg/ml). According to Machlin (1991) that categorized the α-tocopherol status, both patients were in the low level status (5-7 μg/ml). Therefore, by the mean of developed α-tocopherol analytical method, the method was also confirmed in its reliable in endogenous α-tocopherol analysis.

CHAPTER IV

CONCLUSION

1. The rapid, simple and efficient method for determining endogenous α-tocopherol in plasma was developed based on deproteinization by using acetonitrile with isopropanol (7:3) and then analyzed by HPLC technique. The advantages of this method were simple composition of deproteinizing agent and less time of analysis and used only 100 μl of plasma comparing with the previous reports (Teissier, 1996; Cooper, 1997 and Julianto, 1999). Therefore, it was practical for determining endogenous α-tocopherol in a large group of volunteers.

2. This proposed method has been validated to ensure the linearity, accuracy, precision, sensitivity, specificity and stability of the method according to the criteria of bioanalytical method validation. The method was also successfully proven for determining endogenous α-tocopherol in smokers and non-smokers based on multiple-point standard addition method by using interpolation calculation.

3. The mean values of endogenous α-tocopherol in smokers and non-smokers were determined via the developed analytical method to be 12.23 and 12.16 μg/ml, respectively. Additionally, very low concentration of endogenous α-tocopherol was also detected in two lung cancer patients (6.98 and 5.13 μg/ml).

4. Although no statistically significant difference of endogenous α-tocopherol was observed between smokers and non-smokers ($p = 0.931$), in interpreting concentration ratio of α-tocopherol to triglyceride or to cholesterol plus triglyceride, the statistically significant difference was observed with the p-value of 0.010 and 0.034, respectively.

5. This developed method could be recommended for any further study on α-tocopherol in human.

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APPENDIX

Appendix i

Table A1. Age, weight, height, BMI of 30 smokers

Number (code)	age (years)	weight (kg)	height (m)	BMI (kg/m^2)
1 (05)	39	55	1.63	20.70
2 (06)	29	65	1.70	22.49
3 (26)	33	72	1.76	23.24
4 (27)	22	62	1.78	19.57
5 (32)	27	65	1.60	25.39
6 (33)	36	70	1.69	24.51
7 (34)	36	52	1.65	19.10
8 (35)	42	57	1.63	21.45
9 (36)	48	65	1.65	23.88
10 (40)	28	70	1.65	25.71
11 (42)	43	56	1.63	21.08
12 (43)	46	63	1.60	24.61
13 (44)	26	75	1.72	25.35
14 (45)	49	78	1.80	24.07
15 (46)	41	80	1.67	28.69
16 (47)	35	60	1.65	22.04
17 (48)	50	56	1.65	20.57
18 (49)	24	75	1.71	25.65
19 (51)	30	60	1.78	18.94
20 (52)	50	58	1.67	20.80
21 (53)	35	65	1.64	24.17
22 (56)	25	63	1.76	20.34
23 (58)	31	58	1.70	20.07
24 (59)	43	52	1.72	17.58
25 (60)	25	68	1.70	23.53
26 (61)	27	45	1.65	16.53
27 (62)	21	75	1.82	22.64
28 (63)	22	73	1.75	23.84
29 (64)	28	61	1.67	21.87
30 (65)	20	65	1.68	23.03

Appendix ii

Table A2. Age, weight, height, BMI of 28 non-smokers

Number (code)	age (years)	weight (kg)	height (m)	BMI (kg/m^2)
1 (01)	21	60	1.57	24.34
2 (02)	21	52	1.70	17.99
3 (03)	22	62	1.74	20.48
4 (04)	22	70	1.78	22.09
5 (07)	25	67	1.65	24.61
6 (08)	50	74	1.63	27.85
7 (09)	29	85	1.75	27.76
8 (11)	26	65	1.78	20.52
9 (12)	47	65	1.69	22.76
10 (13)	32	62	1.63	23.34
11 (14)	25	47	1.60	18.36
12 (15)	32	65	1.67	23.31
13 (16)	36	52	1.64	19.33
14 (19)	41	60	1.70	20.76
15 (21)	32	48	1.71	16.42
16 (22)	43	70	1.69	24.51
17 (23)	40	65	1.70	22.49
18 (24)	28	54	1.60	21.09
19 (25)	30	73	1.81	22.28
20 (28)	31	64	1.65	23.51
21 (30)	30	65	1.65	23.88
22 (31)	45	60	1.55	24.97
23 (37)	29	70	1.69	24.51
24 (38)	29	60	1.64	22.31
25 (41)	30	63	1.66	22.86
26 (50)	45	80	1.65	29.38
27 (55)	26	56	1.80	17.28
28 (57)	26	50	1.80	15.43

Appendix iii

Table A3. Concentration of cholesterol and triglyceride in plasma of smokers and non-smokers

Volunteer number	Smokers		Non-smokers	
	TC (mg/dl)	TG (mg/dl)	TC (mg/dl)	TG (mg/dl)
1	233	309	181	52
2	161	69	140	38
3	171	94	200	73
4	143	99	188	79
5	257	252	175	57
6	260	155	231	79
7	225	44	192	73
8	146	243	249	78
9	188	201	254	47
10	222	224	187	63
11	215	96	213	91
12	204	195	168	106
13	208	217	227	55
14	221	145	168	118
15	187	96	241	88
16	152	172	208	175
17	251	90	281	82
18	188	114	211	163
19	192	76	136	61
20	339	150	188	65
21	196	111	170	49
22	193	202	208	211
23	169	52	201	102
24	177	51	210	136
25	164	91	259	176
26	209	111	255	89
27	224	102	158	53
28	171	54	156	45
29	167	164	-	-
30	154	39	-	-

TC = cholesterol

TG = triglyceride

Appendix iv

แบบบันทึกอาสาสมัคร

สำหรับงานวิทยานิพนธ์ เรื่อง

การพัฒนาวิธีไฮเพอร์ฟอร์แมนซ์ลิควิดโครมาโทกราฟีสำหรับตรวจหาปริมาณแอลฟา-โทโคฟีรอลในพลาสมาของคนไทยในกลุ่มสูบบุหรี่และกลุ่มไม่ได้สูบบุหรี่

วันที่บันทึกข้อมูล วันที่……..เดือน……..พ.ศ…….

ผู้บันทึก ชื่อ……นามสกุล……

1 ข้อมูลพื้นฐาน

ชื่อ……นามสกุล……

อายุ……ปี อาชีพ…….

อาศัยอยู่บ้านเลขที่……หมู่ที่……ซอย…….. ……..ถนน……..…

แขวง……เขต……จังหวัด……....รหัสไปรษณีย์……..…

โทรศัพท์ที่ติดต่อได้สะดวก…….

2 ข้อมูลสุขภาพ

น้ำหนัก..... กิโลกรัม ส่วนสูง…...เซนติเมตร ความดันโลหิต……..…มิลลิเมตรปรอท

ระดับโคเลสเตอรอลในเลือด……..……..มิลลิกรัม/เดซิลิตร

ระดับไตรกลีเซอไรด์ในเลือด……..……..……..มิลลิกรัม/เดซิลิตร

ท่านมีปัญหาสุขภาพในเรื่องต่อไปนี้หรือไม่

	มีปัญหา	ไม่มีปัญหา
โรคหัวใจและหลอดเลือด	( )	( )
โรคเบาหวาน	( )	( )
โรคตับ/เกี่ยวกับตับ	( )	( )
โรคเกี่ยวกับถุงน้ำดี	( )	( )
โรคเกี่ยวกับตับอ่อน	( )	( )
ภาวะไขมันในเลือดสูง	( )	( )
ภาวะไขมันในเลือดต่ำ	( )	( )

ปัญหาสุขภาพอื่นๆ (โปรดระบุ) ……

2.2 ท่านมีการรับประทานวิตามินเสริม ภายในระยะเวลา 3 เดือนจนถึงปัจจุบันหรือไม่

( ) รับประทาน ( ) ไม่ได้รับประทาน

หากรับประทานโปรด (ระบุชนิดของวิตามินที่รับประทาน)……..

……

3 ข้อมูลเกี่ยวกับการสูบบุหรี่

สูบบุหรี่ หมายถึง สูบบุหรี่มาเป็นเวลาอย่างน้อย 6 เดือน และสูบวันละไม่น้อยกว่า 1 ซอง (20 มวน)

ไม่สูบบุหรี่ หมายถึง ไม่สูบบุหรี่, ไม่เคยมีประวัติสูบบุหรี่ และไม่มีสมาชิกในบ้านสูบบุหรี่

( ) สูบบุหรี่ (ระบุปริมาณการสูบ)

1 จำนวนมวนที่สูบเฉลี่ยต่อวัน……มวนต่อวัน

2 ระยะเวลาตั้งแต่เริ่มสูบบุหรี่จนถึงปัจจุบัน……ปี……เดือน

( ) ไม่สูบบุหรี่

4 ข้อมูลด้านโภชนาการ

ความถี่ในการรับประทานอาหารแต่ละประเภท ดังต่อไปนี้

4.1 อาหารประเภททอดหรือผัดที่ปรุงจากน้ำมันพืช

( ) มากกว่า 5 ครั้งต่อสัปดาห์

( ) 2-5 ครั้งต่อสัปดาห์

( ) น้อยกว่า 2 ครั้งต่อสัปดาห์

ระบุชนิดของน้ำมันพืชที่ใช้บ่อยที่สุด……

4.2 อาหารประเภทถั่ว หรือถั่วเมล็ดแห้ง

( ) มากกว่า 5 ครั้งต่อสัปดาห์

( ) 2-5 ครั้งต่อสัปดาห์

( ) น้อยกว่า 2 ครั้งต่อสัปดาห์

4.3 ผักใบเขียว

( ) มากกว่า 5 ครั้งต่อสัปดาห์

( ) 2-5 ครั้งต่อสัปดาห์

( ) น้อยกว่า 2 ครั้งต่อสัปดาห์

4.4 ผลไม้

( ) มากกว่า 5 ครั้งต่อสัปดาห์

( ) 2-5 ครั้งต่อสัปดาห์

( ) น้อยกว่า 2 ครั้งต่อสัปดาห์

ระบุชนิดของผลไม้ที่รับประทานบ่อยที่สุด 3 ชนิด……..……

4.5 การรับประทานอาหารนอกบ้าน

( ) มากกว่า 5 ครั้งต่อสัปดาห์

( ) 2-5 ครั้งต่อสัปดาห์

( ) น้อยกว่า 2 ครั้งต่อสัปดาห์

รับรองข้อมูลถูกต้อง

……อาสาสมัคร

วันที่……..เดือน……พ.ศ……

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