Chapter One

Introduction

&

Literature Review

1.1 Introduction:

Prostate cancer is the most prevalent cancer in men with a median age at diagnosis of 68 years, two-thirds of prostate cancer-related deaths occur in men aged > 75 years, older men tend to have larger tumors of a higher grade than younger patients.(Liu et al, 2010). Benign prostatic hyperplasia (BPH) is an enlarged prostate gland, located at the inner part of the prostate (around the urethra) often keeps growing as men get older. In BPH, the prostate may squeeze the urethra leading to make problems with urinating. BPH is not cancer but can develop into cancer.

The prostate is an exocrine gland of the male reproductive system and consisted of muscular tissue and glandular. It located just below the bladder and in front of the rectum. It is about the size of a walnut and it surrounds a part of the urethra. The prostate gland makes fluid that is part of the semen.

Prostate –Specific Antigen (PSA) is a glycoprotein produced primarily by the epithelial cells that line the acini and ducts of the prostate gland. PSA concentrates in prostatic tissue. The level of PSA in the serum are normally very low (Giai M et al.,1995). Disruption of the normal prostatic architecture, such as by prostatic disease, inflammation, or trauma, allows greater amounts of PSA to enter the general circulation. Elevated serum PSA level has become an important marker of many prostate diseases – including benign prostatic hyperplasia, prostatitis, and prostate cancer. Prostatic intraepithelial neoplasia (PIN) does not appear to raise serum PSA levels (Mohler, J., Lee,C.L,et al 2009). The disadvantages of PSA is related to detect a slow growing cancer that may never cause symptoms nor shorten life span; however, the ‘cancer’ diagnosis may cause significant anxiety

which can affect the quality of life, it may lead to undergo treatment(s) for early prostate cancer that may not help live longer. The main treatments for early prostate cancer do carry risks and can cause distressing side-effects, it can miss cancer in the prostate and give false reassurance, it may cause anxiety and lead to a biopsy when have no cancer. The decision should be taken along with Urologist who can give further advice and know particular medical history and circumstances. (Andriole GL. 2012).

Alternatively, some other marker can be employed among these are sphingolipid which are one of the major lipid families in eukaryotes, incorporating a diverse array of structural variants that exert a powerful influence over cell fat and physiology, increased expression of sphingosine kinase 1 (SPHK1), which catalysis the synthesis of the pro-survival, pro-antigenic metabolite sphingosine 1-phosphate (S1P), is well established as a hallmark of multiple cancer (Webb, T.J.et al 2012).

Metabolic alterations that reduce levels of the pro-apoptotic lipid ceramide, particularly its glycosylation by glucosylceramide synthase (GCS), have frequently been associated with cancer drug resistance. However, the simple notion that the balance between ceramide and S1P often referred to as the sphingolipid rheostat, dictates cell survival contrasts with recent studies showing that highly potent and selective SPHK1 inhibitors do not affect cancer cell proliferation or survival, and studies demonstrating higher ceramide levels in some metastatic cancers. Recent reports have implicated other sphingolipid metabolic enzymes such as acid ceramidase (AC) sphingosinen-1-phosphate lyase (SPL) acid sphingomyelinases (ASM) more strongly in cancer pathogenesis, and highlight lysosomal sphingolipid

metabolism as a possible weak point for therapeutic targeting in cancer. (Xin Y. Lim and Timothy A. Couttas, 2014).

There are no previous investigations about the role of these markers in detection of PC and BPH in Iraq.

Aim of Study :

The main aim of this study was to find new sensitive and specific bio markers for diagnosis of prostate cancer Other goals of conducting this study are:

1- For measurement activity of acid ceramidase and sphingosine-1-phospate Lyase in sera and prostate tissue of patients with benign prostatic hyperplasia and prostate cancer and compare these activities of enzymes with normal controls.
2- For determination the concentration of bioactive sphingolipids ceramide and sphingosine-1- phosphate in tissues and sera of the patients and control.
3- For correlation the level of bioactive lipids with serum PSA concentration to detect their use as additional biomarker for diagnosis and follow up of patient with prostate cancer.

1.2 Review of literatures

1.2.1 The Prostate:

The prostate is the largest accessory gland in the male reproductive system. A healthy human prostate is classically said slightly larger than walnut. The mean weight of the normal prostate is about 11 grams, usually ranging between 7 and 16 grams (saladin, 2010). It is a compound tubule-alveolar exocrine gland which is located below the bladder and in front of the rectum and pierced by urethra and the ejaculatory ducts.it is responsible for producing a slightly alkaline white fluid (pH=7.29) that makes up 17% of the seminal plasma. This fluid, which protects, carries and nourishes sperm cells, is rich in lipids, polyamines, proteolytic enzymes, acid phosphatase, fibrinolysis and citric acids (Berman et al., 2011).

1.2.2 Prostate Cancer (PC):

Cancer is a disease of cell, the building block of tissue in the body. Inside of cells are coded instructions for building new cells and controlling how cells behave. These instructions are called genes. PC occurs when normal cells begin to grow faster or die slower. Some PC occurs from changes, called mutations, in genes. Cancer cells do not behave like normal cells in three key ways.

First, the changes in genes cause normal prostate cells to grow more quickly and live longer. Normal cells grow and then divide to form new cells when needed. They also die when aged or damaged. In contrast, cancer cells make new cells that are not needed and do not die quickly when aged or damaged. Over time cancer cells form a mass called the primary tumor (Nelson WG.et al., 2014).

The second way, cancer cells differ from normal cells in way they can grow into (invade) other tissues. If not treated, the primary tumor can grow large and take

over most of the prostate. It can also grow beyond the prostatic capsule and invade nearby tissues. This growth is called extra capsular extension.

Third, unlike normal cells, cancer cells can leave the prostate. This process is called metastasis. PC can then grow and form tumors in other parts of the body. PC can spread through blood or lymph vessels that are in the prostate. Most men with prostate cancer will not die of this disease. However, PC is the second most common cause of death from cancer in men. Most PCs grow slowly but some are aggressive and grow quickly. Why some PCs grow fast is unknown and is being studied by researchers (Robert R. Bahnson, MD, 2015). BPH anatomy is shown in Figure **(1.1)** (Albertsen PC et al 2011).

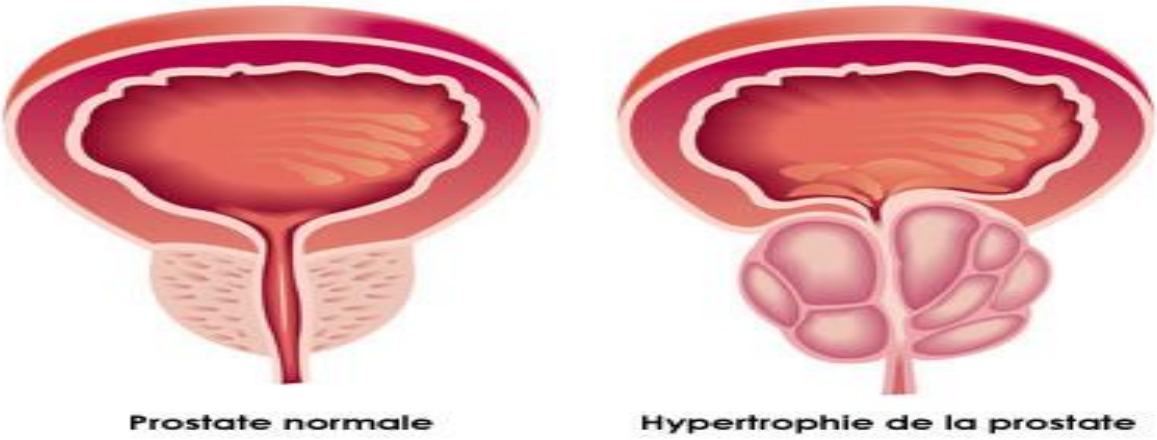

Figure (1.1) Prostate Anatomy (BPH)(Geo Espinosa, 2016).

1.2.3 Benign Prostatic Hyperplasia (BPH):

Benign prostatic hyperplasia (BPH) was one of the ten most prominent and costly diseases in men older than 50 years of age in a study in the United States

(Edwards, 2008). BPH is the most common cause of lower urinary tract symptoms (LUTS), Bladder outlet obstruction (BOO) can be caused by BPH.

BPH is a benign (noncancerous) increase in size of the prostate, BPH involves hyperplasia of prostatic stromal and epithelial cells resulting in the formation of large, fairly discrete nodules in the transition zone of the prostate. When sufficiently large, the nodules impinge on the urethra and increase resistance to flow urine from the bladder (Ziada et al.,1999). This is commonly referred to "obstruction," although the urethral lumen is no less patent, only compressed. Resistance to urine flow requires the bladder to work harder during voiding, possibly leading to progressive hypertrophy, instability, or weakness (atony) of the bladder muscle. BPH involves hyperplasia (an increase in the number of cells) rather than hypertrophy (a growth in the size of individual cells), but the two terms are often used interchangeably, even among urologists(Wilt TJ et al 2012). Although prostate specific antigen levels may be elevated in these patients because of increased organ volume and inflammation due to urinary tract infections, BPH does not lead to cancer or increase the risk of cancer. Adenomatous prostatic growth is believed to begin at approximately age 30. An estimated 50% of men have histologic evidence of BPH by age 50 and 75% by age 80; in 40–50% of these men, BPH becomes clinically significant. Symptoms are abdominal pain, a continuous feeling of a full bladder, frequent urination, acute urinary retention (inability to urinate), pain during urination (dysuria), problems starting urination (urinary hesitancy), slow urine flow, starting and stopping (urinary intermittence), and nocturia. BPH can be a progressive disease, especially if left untreated. Incomplete voiding results in residual urine or urinary stasis, which can lead to an increased risk of urinary tract infection.

1.2.4 Malignant Prostatic Cancer:

Several types of cells are found in the prostate, but almost all PCs develop from the gland cells, which are the cells that make the prostate fluid added to the semen. The medical term of cancer that starts in gland cells is adenocarcinoma. Other types of cancer can also start in the prostate gland, including sarcomas, small cell carcinomas, and transitional cell carcinomas. But these other types of PCs are so rare that if you have PC it is almost certain to be an adenocarcinoma. Some PC can grow and spread quickly, but most grow slowly. (cathepsin B et al 2012).

1.2.5 Risk Factor of Prostatic Cancer:

Risk factor is anything that affects chance of getting a disease such as cancer. Different cancers have different risk factors. The risk factors are smoking, age, diet, race/ethnicity, family history, geography, obesity, gene changes, smoking, workplace exposures, sexually transmitted infections, inflammation of the prostate, vasectomy (Algotar AM, Thompson PA,et al, 2010).

1.2.6 Prostate Cancer Grading:

Prostate Cancer Grade describes how much cancer cells look like normal cells. The grade of the cancer can help the physician to predict how quickly the cancer will spread. The Gleason system is the most common grading system and describes the cell patterns seen under the microscope (Droz JP et al 2012).

1.2.7 Prostatic Cancer Staging:

Staging is a way of describing a cancer, such as the depth of the tumor and where it has spread Staging is the most important tool doctors have to determine a patient's prognosis. Staging is described by the TNM system: the size of the

tumor, whether cancer has spread to nearby lymph **nodes**, and whether the cancer has **metastasized** (spread to organs such as the liver or lungs). Another staging system assigns letters (A, B, and C, D). The type of treatment a person receives depends on the stage of the cancer (Gevaert T et al, 2014).

1.2.8 The malignant Prostatic Cancer Stage and Anatomy :

The malignant Prostatic Cancer Stage and Anatomy **Stage 1** cancer is found only in the prostate and usually grows slowly as , **Stage 2** cancer has not spread beyond the prostate gland, but involves more than one part of the prostate, and may tend to grow more quickly, **Stage 3** cancer has spread beyond the outer layer of the prostate into nearby tissues or to the seminal vesicles, the glands that help produce semen , and finally **Stage 4** cancer has spread to other areas of the body such as the bladder, rectum, bone, liver, lungs, or lymph nodes as **Figure (1.2)** (Morgan et al., 2011).

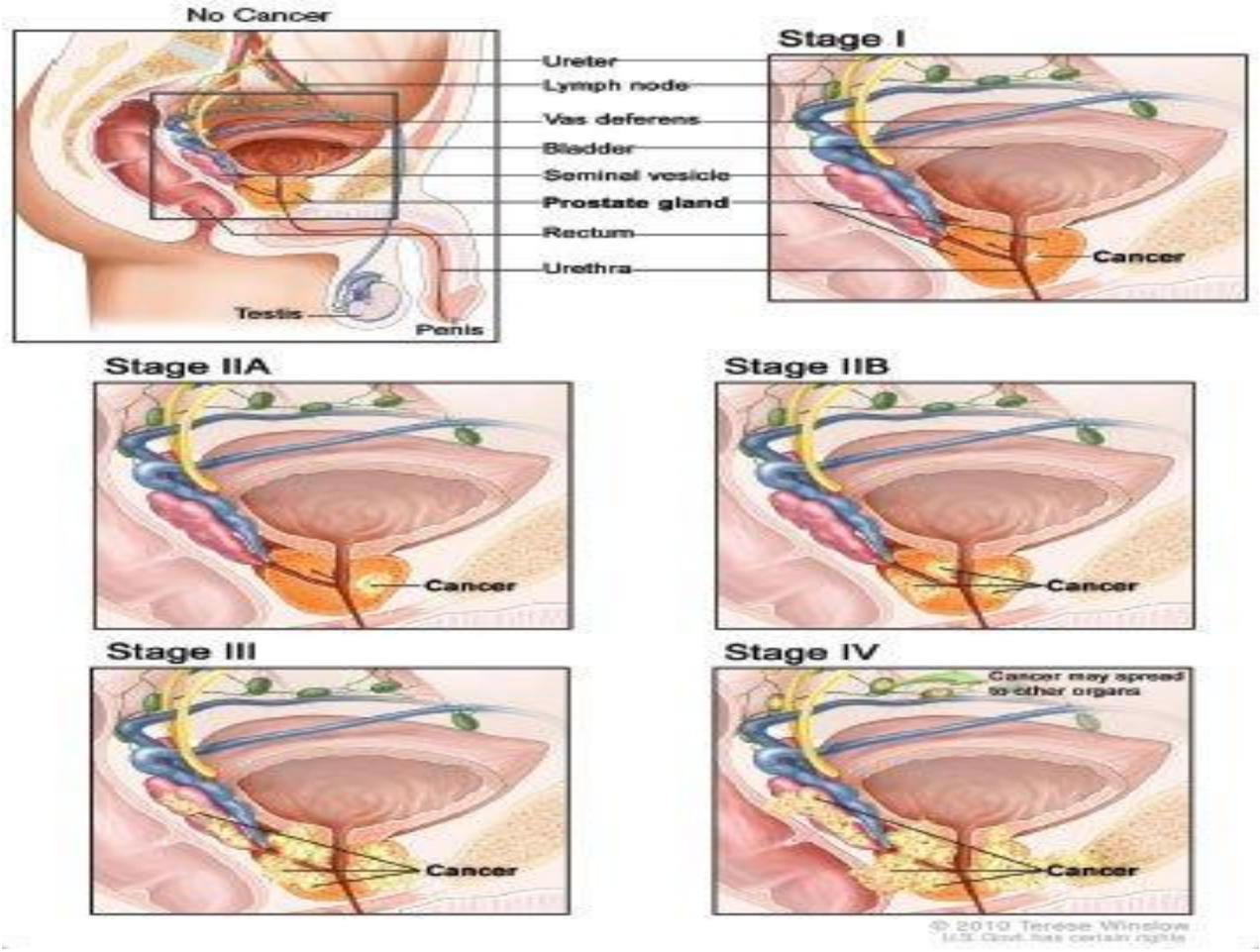

Figure (1.2) Stages for Prostatic Cancer (Fowke J H. et al., 2015)

1.2.9 Symptoms of Prostatic Cancer:

PC usually early causes no symptoms. But more advanced PC can sometimes cause symptoms, such as: problems urinating, including a slow or weak urinary stream or the need to urinate more often, especially at night, blood in the urine, trouble getting an erection (erectile dysfunction**)**, pain in the hips, back (spine), chest (ribs), or other areas from cancer spread to bones, weakness or numbness in the legs or feet, or even loss of bladder or bowel control from cancer pressing on

the spinal cord (Harper, 2013). Other conditions can also cause many of these same symptoms. For example, trouble urinating is much more often caused by (BPH) than cancer (Kristal AR, Darke AK, et al, 2014)

1.2.10 Diagnosis of Prostatic Cancer:

PC can often be found early by testing the amount of prostate-specific antigen (PSA) in a man's blood. Another way to find PC early is the digital rectal exam (DRE). If the results of either one of these tests are abnormal, further testing is needed to see if there is a cancer. If PC is found as a result of screening with the PSA test or DRE, it will probably be at an earlier, more treatable stage than if no screening were done (Komisaruk et al., 2011).

1.2.11 Prostate Specific Antigen (PSA) Detect Prostate Cancer:

PSA tests are used to look for warning signs of PC but these early detection tests cannot tell for sure if a man has cancer. If the result of one of these tests is abnormal, it will probably need a prostate biopsy. (Anderson RU et al., 2006).

PSA is a substance made by cells in the prostate gland (both normal cells and cancer cells). PSA is mostly found in semen, but a small amount is also found in the blood. Most healthy men have levels under 4 (ng/mL) of blood. The chance of having PC goes up as the PSA level goes up. When PC develops, the PSA level usually goes above 4. Still, a level below 4 does not guarantee that a man doesn't have cancer – about 15% of men with a PSA below 4 will have prostate cancer on a biopsy. Men with a PSA level between 4 and 10 have about a 1 in 4 chance of having prostate cancer. If the PSA is more than 10, the chance of having prostate cancer is over 50%. If PSA level is high, the doctor may advise either waiting a

while or repeating the test, or getting a prostate biopsy to find out if have cancer. Not all doctors use the same PSA cutoff point when advising whether to do a biopsy. Some may advise it if the PSA is 4 or higher, while others might recommend it at 2.5 or higher. Other factors, such as age, race, and family history, may also come into play (Wolf A. Wender RC, 2014).

1.3 Bioactive Sphingolipids and Enzymes:

Sphingolipids (SLs) represent a major class of lipids that are ubiquitous constituents of membranes in eukaryotes. First discovered by (J. L. W. Thudichum in 1876), for a long time SLs were considered to play primarily structural roles in membrane formation. However, intensive research on SL metabolism and function has revealed members of the SL family, including Ceramide (Cer), sphingosine (Sph), Sph-1-phosphate (S1P), and Cer-1-phosphate (C1P), as bioactive molecules playing roles from regulation of signal transduction pathways, through direction of protein sorting to the mediation of cell-to-cell interactions and recognition. SLs have also been reported to dynamically cluster with sterols to form lipid micro domains or rafts, which function as hubs for effective signal transduction and protein sorting. Various stimuli such as tumor necrosis factor-a (TNF-a), interleukin (IL)-1, Fas ligand, ionizing radiation , phorbol esters, heat stress, oxidative stress, and chemotherapeutics induce the formation of Cer (Fyrst H, et al 2010).

In **Figure (1.3)** Scheme of SL metabolism. Pathways are shown as described Bioactive SL metabolites are highlighted in red. Sphingolipids have emerged as bio effector molecules, controlling various aspects of cell growth and proliferation in cancer, these lipid molecules have also been implicated in the mechanism of

action of cancer chemotherapeutics. Ceramide, the central molecule of Sphingolipids metabolism, generally mediates anti proliferative responses, such as cell growth inhibition, apoptosis induction, senescence modulation, endoplasmic reticulum (ER) stress responses and/or autophagy (oskouian ,B & Sabe ,et al 2010). In addition, Ceramide metabolism to generate (S1P) by sphingosine kinases 1 and 2 mediates, with or without the involvement of G protein coupled S1P receptor signaling, pro survival, angiogenesis, metastasis and/or resistance to drug induced apoptosis. Importantly, recent findings regarding the mechanisms by which Sphingolipids metabolism and signaling regulate tumor growth and progression, such as identifying direct intracellular protein targets of SLs, they have been key for the development of new chemotherapeutic strategies. It present conclusions of recent studies that describe opposing roles of de novo generated Ceramide by Ceramide synthases and/or S1P in the regulation of cancer pathogenesis, as well as the development of Sphingolipids based cancer therapeutics and drug resistance (park, J.H., et al 2012).

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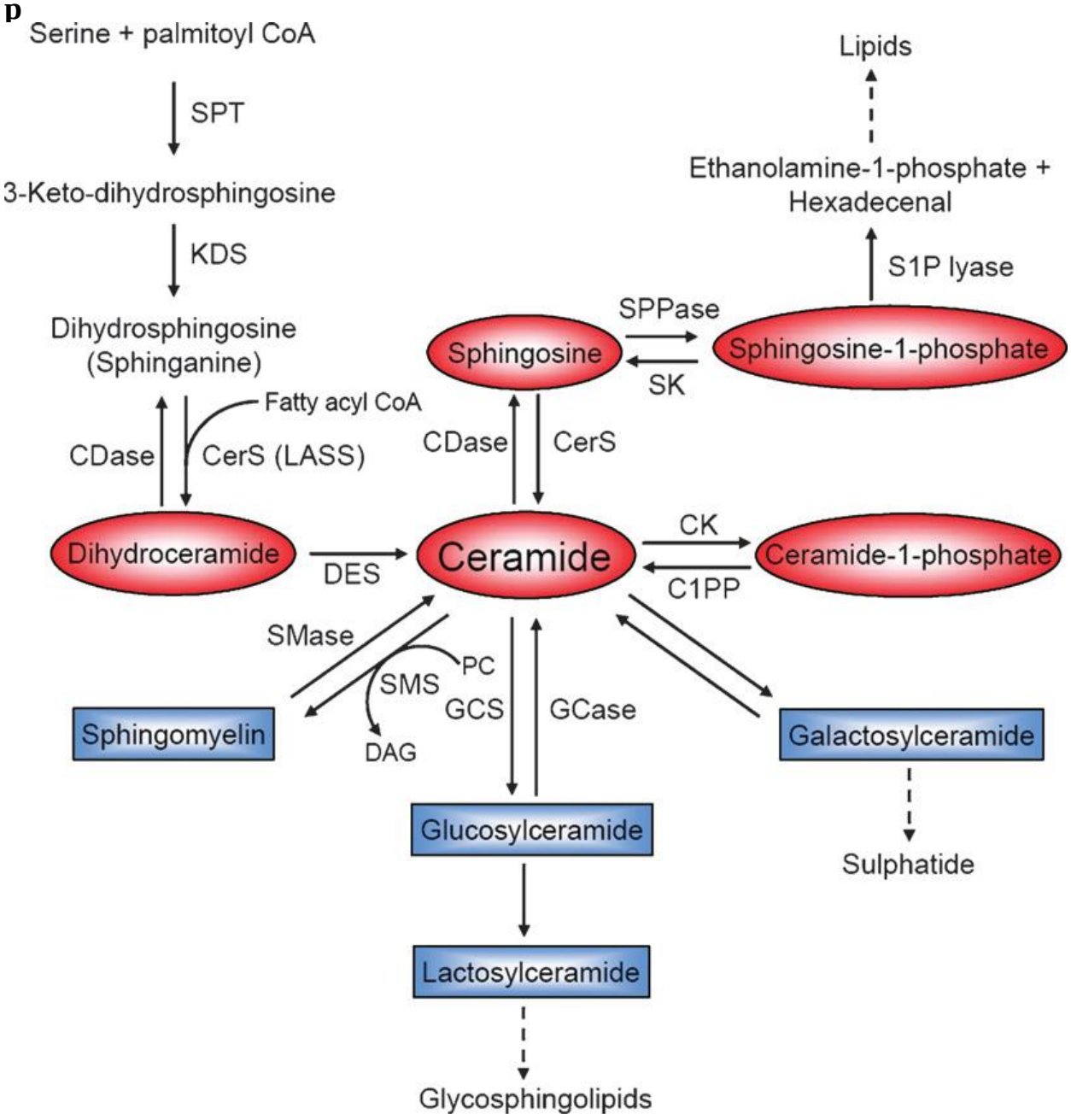

Figure (1.3) Scheme of SL metabolism Pathways are shown as described in the text. Bioactive SL metabolites (Hannun, Y. A., and L. M. Obeid. 2008).

1.3.1 Compartmentalization and Regulation of Sphingolipids:

Enzymatic reactions in SL metabolism are distributed throughout different cellular compartments. The functions of SLs require insight into the specific subcellular localization of the enzymes involved in the SL pathway. The initial steps of SL de novo synthesis leading to Cer formation take place on the cytosolic surface of the endoplasmic reticulum (ER) and potentially in ER-associated membranes, such as the perinuclear membrane and mitochondria-associated membranes. Synthesis of more complex SL metabolites like SM and GluCer occurs in the Golgi apparatus. Two specific pathways are known for the transport of Cer from the ER to the Golgi. First, SM is formed of Cer provided by the Cer transfer protein, CERT. Second, the synthesis of GluCer is based on Cer deriving from vesicular transport. Subsequently, the transport protein FAPP2 delivers GluCer as precursor for glycosphingolipids (GSL) synthesis (Yamaji, T., Kumagai,K., 2008).

The synthesis of complex GSLs (e.g., gangliosides) occurs in the luminal side of the Golgi. Therefore, GluCer needs to flip from the cytosolic surface to the inside of the Golgi. This mechanisms supported by the ABC transporter, P-glycoprotein (also known as MDR1) (Cowart, L. A.,2007).

Subsequently, SM and complex GSLs are transported to the plasma membrane via vesicular trafficking. There, SMcan be metabolized to Cer, and accordingly other bioactive lipids, either by a SMases on the outer leaflet of the membrane or by nSMase, which resides on the inner leaflet of the bilayer. From the plasma membrane, SLs may be recirculated through the endosomal pathway. Thereby, SM and GluCer are metabolized to Cer in the lysosomal compartment by SMases and

glycosidase and Cer is then degraded by acid CDase to form Sph. Due to its ionizable positive charge, the salvaged Sph is able to leave the lysosome and show

Solubility in the cytosol to move between membranes, including the ER, where it would be available for recycling. It is also important to realize that the subcellular localization of enzymes of SL metabolism is a key determinant of site of action of bioactive SLs. SLs are either hydrophobic or amphipathic molecules that have hydrophobic as well as hydrophilic attributes. Hence, it is not surprising that these molecules are mostly integral components of bio logical membranes and show little movement between membranes, unless acted upon by specific transport mechanisms (such as Cer transfer protein or FAPP2) (Tafesse, F. G.,Ternes,P.,2006). Figure (1.4) shows the compartmentalization of metabolites and enzymes of the SL pathway.(Yamaji, T. et al., 2008).

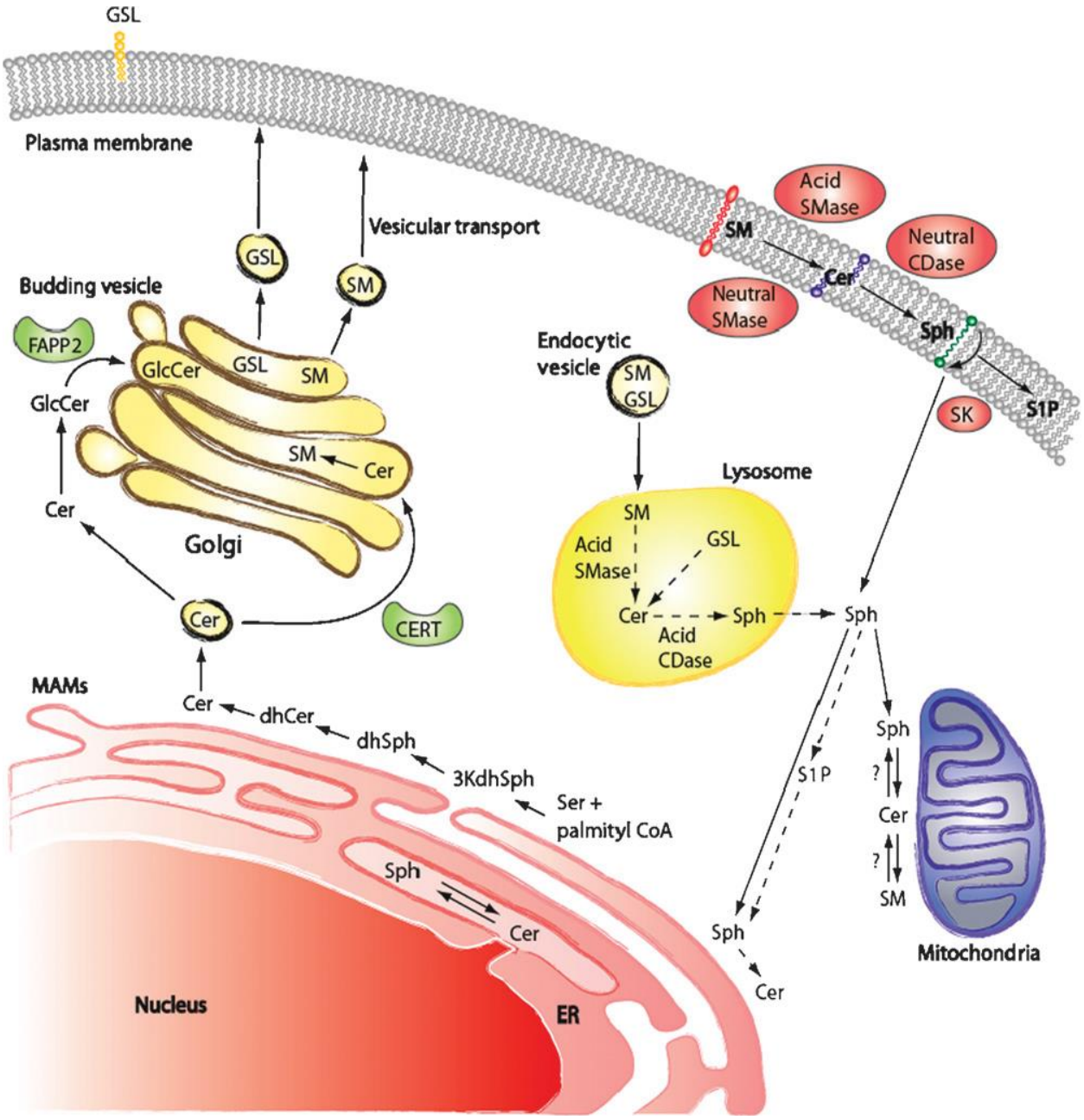

Figure (1.4) Compartmentalization of metabolites and enzymes of the SL pathway (Yamaji, T. et al., 2008).

1.3.2 Ceramide

1.3.2.1 Pathways for Ceramide Synthesis:

There are three major pathways of ceramide generation. The sphingomyelinases pathway uses an enzyme to break down sphingomyelin in the cell membrane and release ceramide de novo pathway creates ceramide from less complex molecules, Ceramide generation can also occur through breakdown of complex sphingolipids that are ultimately broken down into sphingosine, which is then reused by reacylation to form ceramide, This latter pathway is termed the Salvage pathway (Gomez-Munoz, A, et al, 2004).

1.3.2.2 Physiological Roles of Ceramide:

Role of ceramide as a bioactive lipid, ceramide has been implicated in a variety of physiological functions including apoptosis, cell growth arrest, differentiation, cell senescence, cell migration, adhesion and its downstream metabolites have also been suggested in a number of pathological states including cancer, neuro degeneration, diabetes, microbial pathogenesis, obesity, and inflammation.(Fyrst H, Saba JD.2010).

1.3.2.3 Apoptosis:

Apoptosis or type I programmed cell death, is essential for the maintenance of normal cellular homeostasis and is an important physiological response to many forms of cellular stress. One of the most studied roles of ceramide pertains to its function as a proapoptotic molecule. Ceramide accumulation has been found following treatment of cells with a number of apoptotic agents including ionizing radiation, UV light, TNF-alpha, and chemotherapeutic agents. (Saddoughi et al.,2008).This suggests a role for ceramide in the biological responses of all these

agents. Because of its apoptosis-inducing effects in cancer cells, ceramide has been termed the tumor suppressor lipid. Several studies have attempted to define further the specific role of ceramide in the events of cell death and some evidence suggests ceramide functions upstream of the mitochondria in inducing apoptosis. It is owing to the conflicting and variable nature of studies into the role of ceramide in apoptosis, the mechanism by which this lipid regulates apoptosis remains elusive as shown in Figure (1.5) (Spiegel, S, et al , 2002).

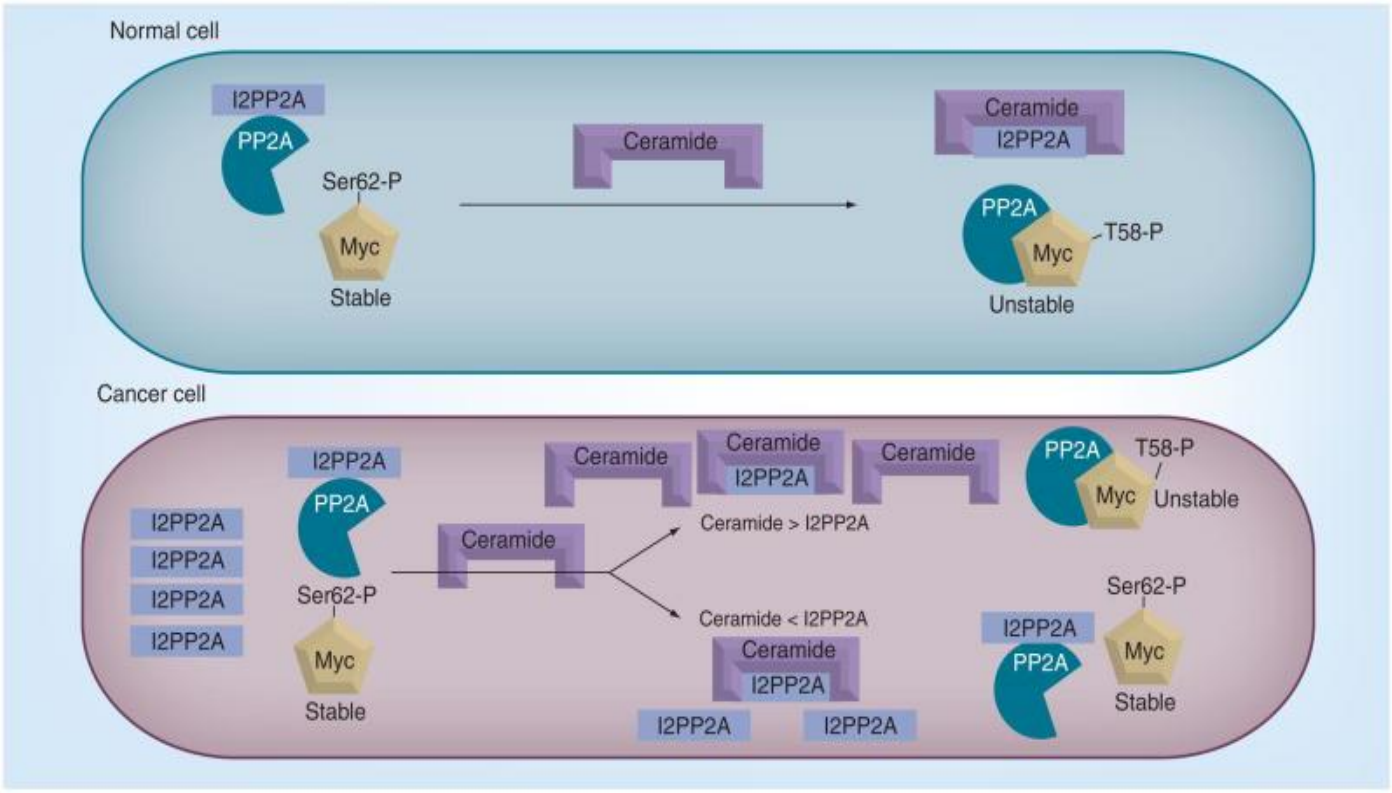

Figure (1.5) Comparison Between Normal Cell and Cancer Cell (Levade T., Sandhoff K., Schulze H., Medin J. A,2009).

1.3.3 Sphingosine -1-Phosphate :

Sphingosine-1-phosphate (S1P) is a signaling sphingolipid, also known as Lysosphingolipid and also referred to as a bioactive lipid mediator. Sphingosine can be released from ceramides, a process catalyzed by the enzyme ceramidase. Phosphorylation of sphingosine is catalyzed by sphingosine kinase, an enzyme ubiquitously found in the cytosol and endoplasmic reticulum (ER) of various types of cells. S1P can be dephosphorylated to sphingosine by sphingosine phosphatases and can be reversibly degraded by an enzyme Sphingosine phosphate lyase (ito, K., et al ,2007).

1.3.3.1 Function of S1P:

Sphingosine 1 phosphate (S1P) is a blood borne lipid mediator found in particular in association with lipoproteins such as high density lipoprotein (HDL). It is less abundant in tissue fluids. This is referred to as the S1P gradient, which seems to have biological significance in immune cell trafficking. Originally, thought as an intracellular second messenger, it was discovered to be an extracellular ligand for G protein-coupled receptor S1PR1 in 1998. It is now known that S1P receptors are members of the lysophospholipid receptor family. There are five described to date. Most of the biological effects of S1P are mediated by signaling through the cell surface receptors (Alvarez, S.E.; Harikumar, K.B.;et al 2010). S1P is of importance in the entire human body, it is a major regulator of vascular and immune systems. In addition, it might be relevant in the skin and in the vascular system, S1P regulates angiogenesis, vascular stability, and permeability. The immune system, it is now recognized as a major regulator of trafficking of T- and B-cells. S1P interaction with its receptor S1PR1 is needed for

the egress of immune cells from the lymphoid organs (such as thymus and lymph nodes) into the lymphatic vessels (Antoon, J.W.; White, M.D.; Burow, M.E.; et al 2012). Inhibition of S1P receptors was shown to be critical for immunomodulation. S1P has also been shown to directly suppress TLR mediated immune response from T cells (Sharma, N; et al. (2013).

1.3.4 Studies on Individual Enzymes and Sphingolipid Metabolites in Cancer:

Study on the role of individual sphingolipid metabolic enzymes in oncogenic transformation and cancer phenotype have focused primarily on SPHK1 and GCS, with a number of studies focusing on acid ceramidase and other enzymes. The literature on these enzymes in cancer is summarized below.

1.3.4.1 Sphingosine 1-Phosphate Lyase (SGPL):

Sphingosine 1phosphate lyase (SPL) is responsible for the irreversible catabolism of sphingosine 1phosphate, which signals through five membrane receptors to mediate cell stress responses, angiogenesis, and lymphocyte trafficking. The standard assay for SPL activity utilizes a radioactive dihydrosphingosine 1phosphate substrate and is expensive and cumbersome. (Roness, Kalich-Philosoph,H.; L.et al (2014).

The major phosphorylated sphingoid base in humans is S1P, which is synthesized in most cell types and circulates in blood and lymph at high concentrations. S1P signals through a family of five known G protein coupled membrane receptors that regulate cell survival, migration, and complex processes such as vascular maturation and lymphocyte trafficking. SPL activity in tissues and endothelium are required to regulate circulating S1P levels and maintain the

chemical gradient that facilitates S1Pmediated lymphocyte egress from peripheral lymphoid organs and thymus (Baran, Y.; Salas, A.; Senkal, C.E.; et al 2007). Inhibition of SPL through genetic or pharmacological approaches has been shown to block lymphocyte trafficking, indicating that SPL may be a useful target for immune modulation. The SPL gene and gene product are highly conserved throughout evolution and are essential in vertebrates and invertebrates, although its deletion in plants did not lead to deleterious effects. Studies in model organisms have demonstrated that SPL expression is necessary for normal development, survival, and tissue homeostasis. The requirement for SPL in mammals is primarily attributed to its regulation of S1P (Bieberich, E.; MacKinnon, S.; Silva, J.;et al 2010). Null mutation of SPL blocks infectivity of the parasite due to ethanolamine depletion, demonstrating that product regulation may be critical for some physiological processes. Loss of SPL expression has been described as a feature in colon and prostate cancers and in melanoma cell lines compared to normal melanocytes. SPL is a major sump for cellular S1P, so analogous to up-regulation of SPHK1, down-regulation of SPL promotes higher S1P levels. Accordingly, SPL expression enhances chemo sensitivity and pro-apoptotic responses, whilst its down regulation blocks these phenotypes. SPL loss inhibits sphingolipid degradation, thereby increasing sphingosine and ceramide levels. Therefore, the cancer supportive effects of SPL loss are presumably attributed to higher S1P rather than changes to ceramide levels (Sattler K, et al (2009).

1.3.4.2 Acid Ceramidase Enzyme:

Human acid ceramidase is catalyzes the hydrolysis of ceramide to sphingosine and fatty acid. Ceramide serves as the precursor for most sphingolipids and is a

signaling molecule that induces apoptosis in a number of different cell types. In contrast, sphingosine is converted into sphingosine 1-phosphate, another important cell signaling lipid that is anti-apoptotic and can counteract the effects of ceramide. The only source of sphingosine in vertebrate cells is through ceramide hydrolysis, whereas Ceramide can be generated by synthetic pathways or through the degradation of sphingomyelin or glycosphingolipids. Several ceramidase have been described in human that function at varying pH values and presumably reside in distinct intracellular locations. An acidic ceramidase (*i.e.* AC) was first described and partially purified by Gatt in 1963, 1972, (Hanker, L.C.; Karn, T.; Holtrich, U.;et al 2013). Patients with the lipid storage disorder, Farber disease. Ceramide, SPH, and S1P are bioactive lipids that mediate cell proliferation, differentiation, apoptosis, adhesion, and migration acid ceramidase (ASAH1) – cell survival(Mao C,et al 2008).

1.3.4.3 Ceramid Transfer Protein or (CERT) and (FAPP2):

Sphingolipid transfer proteins, CERT and FAPP2, have brought the field of sphingolipid metabolism to a more dynamic stage. CERT transfers ceramide from the endoplasmic reticulum (ER) to the Golgi apparatus, a step crucial for sphingomyelin (SM) synthesis. The pleckstrin homology (PH) domain and the FFAT motif of CERT restrict the direction of transfer and destination of ceramide through binding to phosphatidylinositol 4-monophosphate (PI4P) at the Golgi and the ER resident proteins, VAPs, respectively (Kumagai K1, Kawano-Kawada M, Hanada K, 2014). CERT is regulated by the phosphorylation and dephosphorylation of serine/threonine, in which protein kinase D, possibly casein

kinase I, and PP2Cepsilon are involved. On the other hand, FAPP2 transfers glucosylceramide (GlcCer) to appropriate sites for the synthesis of complex glycosphingolipids. Like CERT, FAPP2 contains a PH domain, the binding of which to PI4P is required for its localization to the Golgi. These observations indicate that lipid transfer proteins, CERT and FAPP2, spatially regulate lipid metabolism on the cytosolic side. proteins (Hanada K (2010).

1.3.4.4 Glycated Hemoglobin:

Glycated hemoglobin (hemoglobin A1c, HbA1c, A1C, or Hb1c; sometimes also referred to as being Hb1c or HGBA1C) is a form of hemoglobin that is measured primarily to identify the three-month average plasma glucose concentration. The test is limited to a three-month average because the lifespan of a red blood cell is four months (120 days). However, since RBCs do not all undergo lysis at the same time, HbA1C is taken as a limited measure of 3 months. It is formed in a non-enzymatic glycation pathway by hemoglobin's exposure to plasma glucose. HbA1c is a measure of the beta-N-1-deoxy fructosyl component of hemoglobin (Miedema K (2005).The origin of the naming derives from Hemoglobin type A being separated on cation exchange chromatography. The first fraction to separate, probably considered to be pure Hemoglobin A, was designated HbA0 , the following fractions were designated HbA1a, HbA1b, and HbA1c, respective of their order of elution. There have subsequently been many more sub fractions as separation techniques have improved (Denig P (2011). Normal levels of glucose produce a normal amount of glycated hemoglobin. As the average amount of plasma glucose increases, the fraction of glycated hemoglobin increases in a

predictable way. This serves as a marker for average blood glucose levels over the previous three months before the measurement as this is the lifespan of red blood cells.In diabetes mellitus, higher amounts of glycated hemoglobin, indicating poorer control of blood glucose levels, have been associated with cardiovascular disease, nephropathy, neuropathy, and retinopathy. A trial on a group of patients with Type 1 diabetes found that monitoring by caregivers of HbA1c led to changes in diabetes treatment and improvement of metabolic control compared to monitoring only of blood or urine glucose (Geistanger A, Arends S 2011). A trial designed specifically to determine whether reducing HbA1c below the normal 6% would reduce the rate of cardiovascular events in Type 2 Diabetes found higher mortality—the trial was terminated early Marcus RL, Smith S:2008).

1.3.4.5 Creatinine in serum and urine:

Creatinine derives from creatine and creatine phosphate in muscle tissue and is defined as a nitrogenous waste product. Creatinine dose not reutilize but is excrete from the body in the urine via the kidney. It produces and excretes at a constant rate which is proportional to the body muscle mass. As a consequence of the way in which creatinine is excreted by the kidney, creatinine measurement is used almost exclusively in the assessment of kidney function. Creatinine is regarded as the most useful endogenous marker in the diagnosis and treatment of kidney disease. Creatinine is measured primarily to assess kidney function and has certain advantages over the measurement of urea. The plasma level of creatinine is relatively independent of protein ingestion, water intake, rate of urine production and exercise. Since its rate of production is constant, elevation of plasma creatinine

is indicative of under-excretion, suggesting kidney impairment. Depressed levels of plasma creatinine are rare and not clinically significant. Creatinine in alkaline solution reacts with picric acid to form a colored complex. The amount of the complex formed is directly proportional to the creatinine concentration (Bartels, H., Bohmer, M., (1972).

1.4 Study Kinetic of Enzymes:

Studying enzyme activity is measured in vitro under many conditions that often do not closely resemble those in vivo. Measuring enzyme activity is normally to determine the amount of enzyme present under defined conditions, in way conditions chosen are usually at the optimum pH, saturating substrate concentrations, and at a temperature that is convenient to control. In many, cases the activity is measured in the irreversible reaction to that of the enzyme's natural function, with a complete study of the parameters that affect enzyme activity it should be possible to extrapolate to the activity expected to beoccurring in vivo (Chen, W.W.; Neipel, M.; Sorger, P.K., 2010).

1.4.1 Michaelis–Menten kinetics:

Michaelis–Menten kinetics is one of the best-known models of enzyme kinetics. This model was take form of an equation describing the rate of enzymatic reactions, by relating reaction rate velocity (V) to concentration of substrate [S]. This equation is called Michaelis–Menten equation represents the maximum rate achieved by the system, at maximum saturation of the substrate concentration Km the Michaelis constant—is characteristic of an enzyme and its particular substrate,

and reflects the affinity of the enzyme for that substrate. Km is numerically equal to the substrate concentration at which the reaction velocity is equal to 1⁄2Vmax. Km does not vary with the concentration of enzyme, and (V max) maximal velocity the rate of an enzyme-catalyzed reaction increases with substrate concentration until a maximal velocity (V max). rate of a reaction (v) is the number of substrate molecules converted to product per unit time; velocity is usually expressed as μmol of product formed per minute as shown in figure (1.6)(Walsh, R.et al (2012).

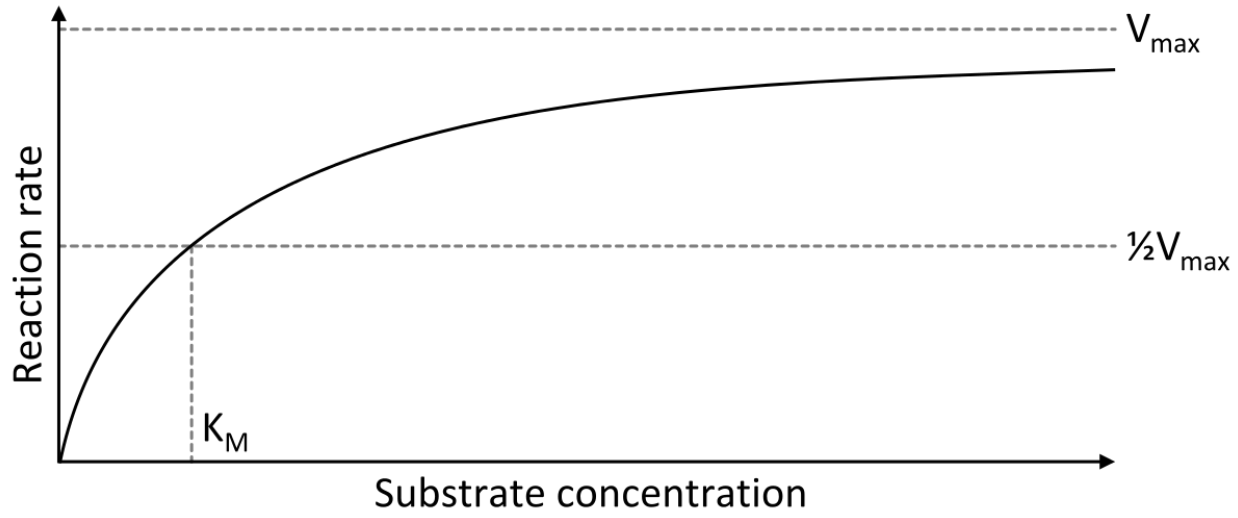

Figure (1.6) Michaelis–Menten saturation curve for an enzyme reaction showing the relation between the concentration substrate [s] and reaction rate (V) (Reuveni 2014).

1.4.2 Line Weaver-Burk plot:

The initial rate of the reaction (v_o) is plotted against [S], it is not always possible to determine when V max has been achieved, because of the gradual upward slope of the hyperbolic curve at high substrate concentrations. However, if $1/v_o$ is plotted versus 1/[S], a straight line is obtained, this plot, the Lineweaver-Burk plot (also called a double-reciprocal plot) can be used to calculate Km and Vmax, as well as to determine the mechanism of action of enzyme inhibitors. **Figure (1.7)**(Nagamine, T.; et al. (2006)

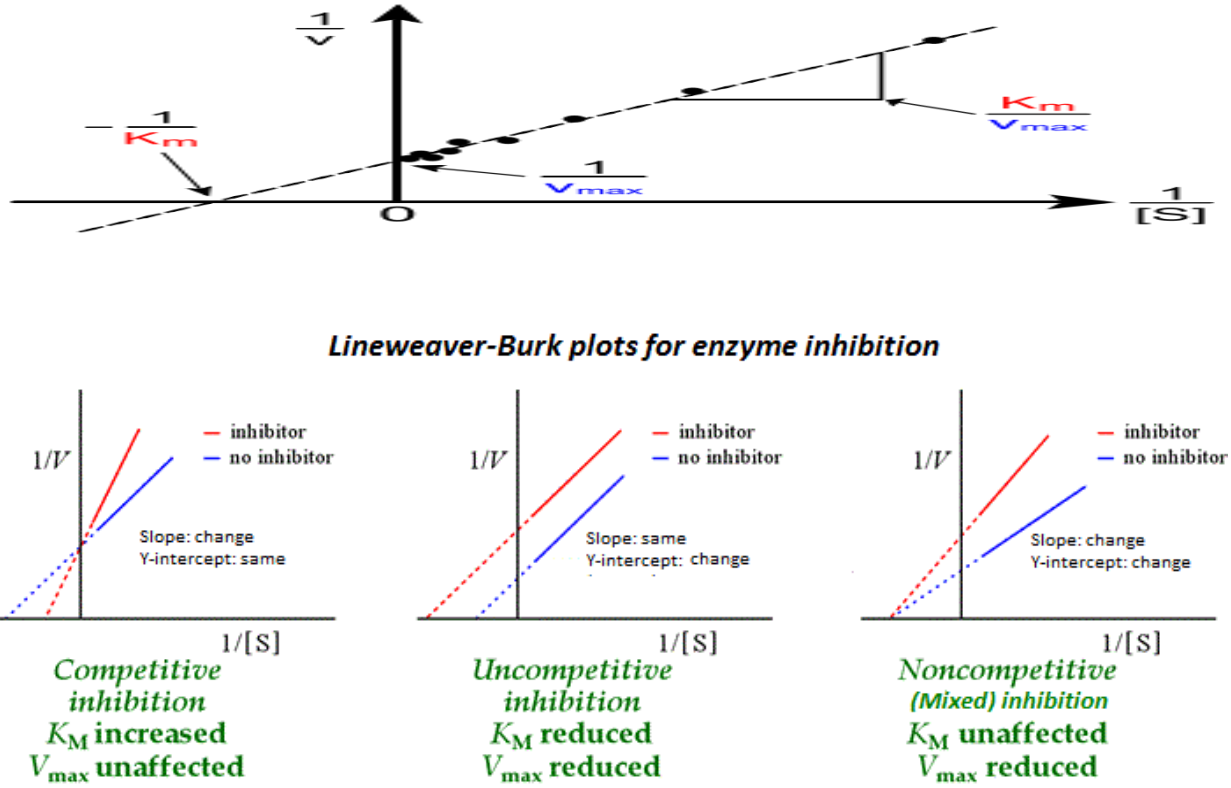

Figure (1.7) Line weaver-Burk plots for enzyme and enzyme inhibitions (Greco, W. R.; Hakala, M. T.,1979).

Useful to actively predict what the enzyme through a series of concentrations of the base material faced by the enzyme in the body and therefore can study the physiological role of the enzyme used for this purpose which describe the adoption of the reaction rate on the concentration of the base [S] if was assumed that reaction that stimulated enzyme together to form a complex compound of the enzyme and the article basically [ES] which it decays later to the enzyme and material basis or enzyme, and the resulting article. In this study, we have been tested SPL and AC and measured Km and V max in HPLC fluorescence detector analytical technique for the separation of mixture in tissue and serum and these were listed in chapter two.

Aim of study

The main aim of this study is found a new sensitive and specific bio markers for diagnosis prostatic cancer And other goals of conducting this study are:

1- To measure activity of Acid ceramidase and Sphingosine-1-Phospate Lyase in sera and prostate tissue of patients with benign prostatic hyperplasia and prostate cancer and compare these activities of enzymes with normal controls.

2- To determine the concentration of bioactive Sphingolipids Ceramide and Sphingosine-1- phosphate in tissues and sera of the patients and control.

3- To correlate the level of bioactive lipids with serum PSA concentration to detect their use as additional biomarker for diagnosis and follow up of patient with prostate cancer.

Chapter Two

Materials & Methods

2.1 Methodology, and Subjects

The experimental work was done at The Department of Chemistry and Biochemistry, College of Medicine AL-Nahrain University, and The Ministry of Science and Technology, during the period from (January to Jun) 2016.

2.1.1 Materials:

2.1.1.1 Chemical Materials

Chemical used in this study were mentioned in table (2-1)

Table (2-1): Chemical Compound

Chemicals	Purity %	Supplied company
Acetic acid (CH_3COOH)	99.9%	Sigma chemicals company U.S.A
Bovin albumin serum (BAS)	99.9%	Sigma chemicals company U.S.A
Cholic acid ($C_{24}H_{40}O_5$)	99.5%	Santa cruse Germany
Chloroform ($CHCl_3$)	99.9%	Scientific Solutions company Australia
Coomassie G 250 dye $C_{45}H_{44}N_3NaO_7S_2$ (Sodium salt)	97.5%	Sigma chemicals company U.S.A
Di ammonium hydrogen phosphate $(NH_4)_2HPO_4$	95.5%	Sigma chemicals company U.S.A
Dithiothreitol (DTT)	99%	Sigma chemicals company U.S.A
Ethanol C_2H_5OH	99.9%	Biosolve company, Australia
Ethylene di amine tetra acetate EDTA	99.5%	Fullka company

EverFluor Fl C_5-Ceramide [N-(4,4-Difluoro-5,7-Dimethyl-4-Bora-3a,4a-Diaza-s-Indacene-3-Pentanoyl)Sphingosine], BODIPY FL C_5-Ceramide, TM of Molecular Probes Acid Ceramidase substrate enzyme	99.9%	Setareh Biotech, LLC, U.S.A
Ever Fluor FL C_5-HPC [2-(4,4-Difluoro-5,7-Dimethyl-4-Bora-3a,4a-Diaza-s-Indacene-3-Pentanoyl)-1-Hexadecanoyl-sn-Glycero-3-Phosphocholine] [ß- BODIPY FL C_5-HPC TM of Molecular Probes] SPL substrate enzyme	99.9%	Setareh Biotech, LLC, U.S.A
Methanol	99.9%	Biosolve company Australia
Mono potassium phosphate (MONO basic) powder KH_2PO_4	95.5%	Sigma chemicals company U.S.A
Phosphric acid H_3PO_4	99.9%	Sigma chemicals company U.S.A
Potassium Phosphate ,di hydrogen Dibasic, Anhydrous (DI basic) powder K_2HPO_4	99.9%	Sigma chemicals company U.S.A
Pyriodoxal 5' -phosphate		Sigma chemicals company U.S.A
Sodium fluoride (NaF)	99.9%	Sigma chemicals company U.S.A
Sucrose ($C_{12}H_{22}O_{11}$)	99.9%	Sigma chemicals company U.S.A
Tetra butyl ammonium dihydrogen phosphate (TBAP)	99.9%	Sigma chemicals company U.S.A
Triton™ X-100 solution	99.9%	Sigma chemicals company U.S.A

2.1.1.2 Materials of Kits:

Table (2-2) shows the list of kits

Table (2-2): kits used for Analysis

Kits	Supplied company
Albumin (serum) BCG	Biolabo, France
Albumin (urine)	Nycocard Reader, Germany
Ceramide Kinase	Konobiotech, China
Creatinine (serum, urine)	Randox, U.K
Glucose serum	Randox, U.K
Prostate specific antigen (PSA)	Human, Spain
Sphingosine -1-phosphate	Konobiotech, China
Urea serum	Randox, U.K

2.1.1.3 Instruments:

All the instruments and tools which was used during this work are shown in table (2-3)

Table (2-3): The Instruments analysis and Tools

Instrument	Supplied company
Centrifuge	U.S.A.
Elisa Reader and Washer	U.S.A
High Performed Liquid Chromatography (HPLC) Fluorescence detector	JAPAN
Homogenizer	China
Incubator	U.S.A
Magnetic stirrer	Germany
PH meter	England
Sensitive balance	England
Sonicater	England
Spectrophotometer CECIL 1100	U.K.
Vortex mixer	Germany
Water bath	Germany

2.1.2 Subjects

All samples were collected from Al- Imameen Al-Kademen Medical City, AL-Jawad Oncology Centre, Medical City-Hospital Martyr Ghazi Al-Hariri for Specialized Surgery, AL-Yarmok Hospital, Educational Oncology Hospital, Special Nursing Hospital, and AL-Saadon Privet Hospital subject shown as figure (2.1) and table (2-4).

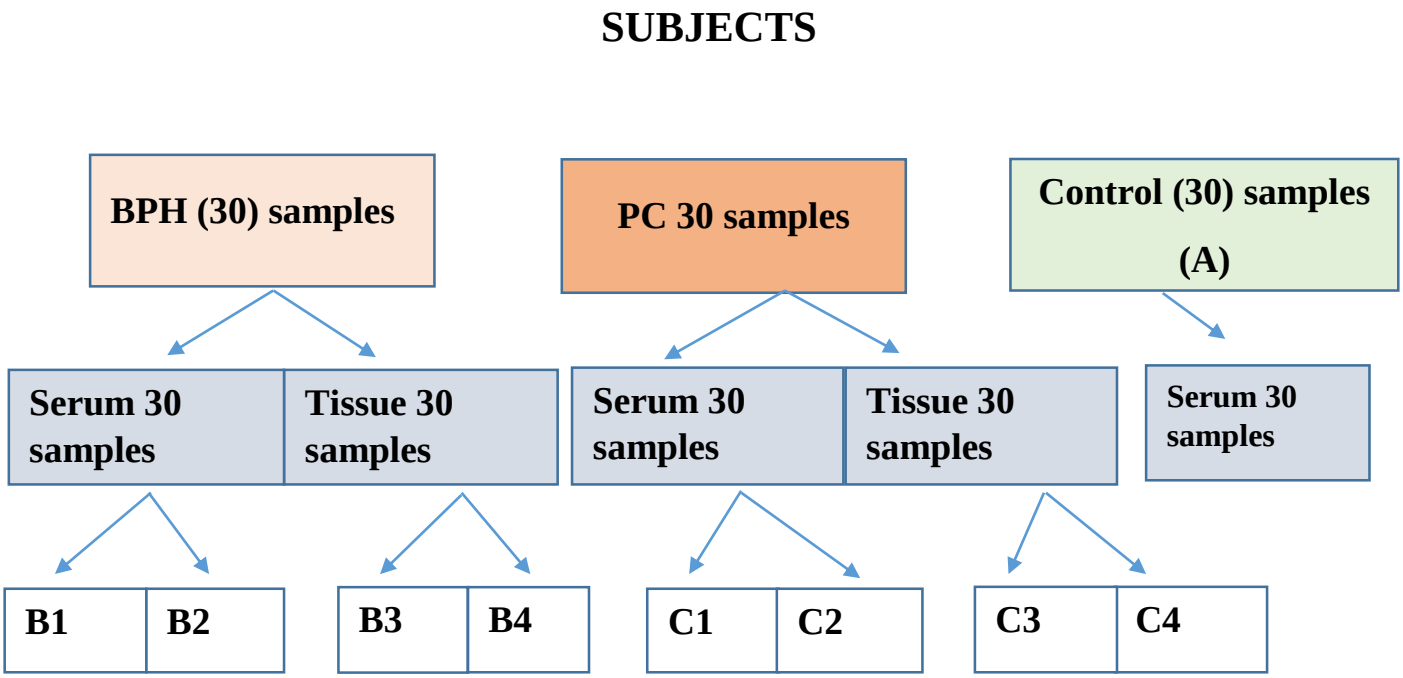

Figure (2.1) Scheme for All Subjects

Exclusion Criteria:

Men free D.M, hypertension, smokers, exposure to smokers, family history, and renal failure.

Table (2-4) shows the group for all subjects with doses of drugs and chemotherapy and matched with healthy group age ranged (50-70 yrs.).

Group	No. of sample	Described
A	**30**	Health men (control group) in (serum)
B1	**30**	Men with BPH (new cases) without drugs or chemotherapy in (serum)
B2	**30**	Men with BPH with drugs in (serum)
B3	**30**	Men with BPH low dose drugs in (tissue)
B4	**30**	Men with BPH high dose drugs in (tissue)
C1	**30**	Men with prostatic cancer low dose chemotherapy in (serum)
C2	**30**	Men with prostatic cancer high dose chemotherapy in (serum)
C3	**30**	Men with prostatic cancer low dose chemotherapy in (tissue)
C4	**30**	Men with prostatic cancer high dose chemotherapy in (tissue)

2.1.3 Sample Collection:

2.1.3.1 Serum collection

Samples were collected from each participant patients and control by taking 7 ml of venous blood the sample was divided to two aliquots. (4.5 ml) of sample was kept in plan tubes and left for coagulation to biochemistry analysis and subjected to centrifuge at 3000 rpm for 10 min at 4° C. after that serum was stored at (- 20 °C) immediately in multiple Eppendorf . The remaining aliquot (2.5) ml was kept in EDTA tubes and used immediately to HbA1c analysis (Oblinger, J.L.; 2006).

2.1.3.2 Tissue Collection and Preparation

Samples of tissue were collected and prepared within 3 days. In first day samples were washed by Phosphate buffer saline (pH 7.4) to remove that all wests and blood the tissue was cut into pieces weighted 500 mg by a sensitive electronic balance then. All pieces were washed off by adding 5 ml of PBS buffer. The sample were homogenized by Grinder Intermittent for 2 min to keep the enzymes from damage in heat of homogenizer was put sample tube in ice glass. A centrifuged was applied for 20 min at 2000-3000 rpm to collect the supernatant. The supernatant was freezed and kept the samples at (- 70° C) or (-20°C). At the Second day, thawing supernatant (tissue homogenates) was put in freeze. Finally at third day, the samples were thawing and after thawing Lyse the cells by ultra-sonication thaw (room temperature) 3 time, Centrifuged homogenate at 5000×g (United States Environmental Protection Agency, 1980).

Note, mince tissues and homogenize them in 5-10 mL of PBS and collect the supernatant for assaying freeze (- 70° C) for one year or under (-20°C) for sixth months.

2.1.3.3 Blood Collection

Samples were collected from patients and healthy people for control in coagulant tube the samples were immediately to analysis by HbA1C.

2.1.3.4 Urine Collection

Samples of urine were collected from patients and healthy people for control in test tube and were used for mastered creatinine and albumin in urine analysis.

2.1.3.5 Ethical Issues:

This study was performed with permission from Al-Imameen Al-Kademen Medical City, AL-Jawad Oncology Centre, Medical City - Hospital Martyr Ghazi Al-Hariri for Specialized Surgery, AL-Yarmok Hospital, Educational Oncology Hospital, and AL-Saadon Privet Hospital, and the Ministry Health in Iraq. All patients and their families' members were counseled. They signed a written informed or verbal consent from them before testing.

2.2 Methods:

2.2.1 Determination of Sphingosine-1- Phosphate Lyase Activity in Serum and Tissue Using Reversed- Phase High Performance Liquid Chromatography.

The HPLC method for the separation of SPL enzyme was applied by add (Ever Fluor FL C_5-HPC [2-(4,4-Difluoro-5,7-Dimethyl-4-Bora-3a,4a-Diaza-s-Indacene-3-Pentanoyl)- 1- Hexadecanoyl-sn-Glycero-3-Phosphocholine] [ß BODIPY FL C_5-HPC TM of Molecular Probes])(Allende, M.L, et al., 2011). Substrate enzyme described a useful system for this study of determination SPL in serum and tissue depending on special reagents that colored the solution, the method is concentration dependent that can be applied on serum and tissue. This method is low cost and high sensitivity (Ikeda M.2004).

2.2.1.1 HPLC Method:

Samples were analyzed by (HPLC) system, model Shimadzu S-1122 solvent equipped with binary delivery pump model S-1122, sample injected in sample injector S 5200, column thermo S-4011.The Eluted peaks were monitored by fluorescence RF-20A by prominence fluorescence detector. Standards of suspected compound were run at the same conditions applied on the samples for quantities and qualitative study the condition of separation was listed in Table (2-5).

2.2.1.2 Calculation:

SPL and AC enzymes concentrations were calculated by equation, the area under the peak is used for calculating the concentration of the sample as the following formula:-

Concentration of sample (ppb) = (The area of the sample / the area of the standard) × Standard Conc. × Dilution factor…..1

2.2.1.3 Preparation of Standard Solution

A total volume of 100 µL of (SPL) was dissolved in 350 µL of reaction buffer (0.6 mM EDTA, 0.4 mM Pyrodoixal 50- phosphate, 3 mM DTT, 70 mM sucrose, 36 mM potassium phosphate buffer, 36 mM NaF) containing 0.08% Triton X-100. This solution is stable for at least one month at (-20° C) (Bonifacino, E. et al., 2011).

2.2.1.4 Principle and Preparation of Samples:

A volume of 20 µL of BODIPY substrate with 100 µL of buffer reaction were Sonicated for 10 min then added to 200 µL of (serum or tissue) the mixture was Incubated for 30 min or more to enzymatic reaction. (100 µL) Triton X-100 was added to the mixture followed by 250 perchloric acid. One ml of chloroform/ methanol (1:2) was added. The mixture was centrifuged at 6000 rpm for 6 min. 2.5 ml of final was taken supernatant and added 50 µL of BODIPY to assure maximum reaction rate. The solution was suspended in methanol and injected into HPLC column (4.5×150 mm Luna C18-column). BODIPY-sphingosine was used as an internal standard and labeled compounds that separated by HPLC at a flow 1ml/min. The mobile phase consist of solvent A (water) /and B (methanol / 5 mm acetic acid

in water /1mm tetra butyl ammonium di hydrogen phosphate (TBAP), (95:4:1) (v:v:v)) (Osawa, Y.; Suetsugu, A.;2013).

Table (2-5): Conditions of High Performance Liquid Chromatography

Parameter	Characteristic for SPL identification	Characteristic for Acid ceramidase identification
Detector	Florescence Ex= 350 nm Em = 482 nm	Florescence Ex= 505 nm Em = 540 nm
Flow rate	1ml/min	1 ml/min
Volume injection sample	100 µL	100 µL
Type of Column	C18-ODS	C18-ODS
Mobile phase	A= (water) B= (methanol / 5 mM acetic acid in water /1Mm tetra butyl ammonium dihydrogen phosphate (TBAP) ,(95:4:1) (v:v:v)).	A= (95 ml methanol) B= (5 ml water)
Temperature	37ºC	37ºC

2.2.2 Determination of Acid Ceramidase Enzyme Activity in Serum and Tissue Using Reversed- Phase High Performance Liquid Chromatography.

HPLC method for the separation of Acid ceramidase enzyme by add EverFluor Fl C_5-Ceramide [N-(4,4-Difluoro-5,7-Dimethyl-4-Bora-3a,4a-Diaza-s-Indacene-3-Pentanoyl)Sphingosine], BODIPY FL C_5-Ceramide, tm of molecular probes acid ceramidase as substrate enzyme described a useful system for this study of determination acid ceramidase in serum and tissue. Substrate enzyme described a useful system for this study of determination AC in serum and tissue depending on special reagents that colored the solution, the method is concentration dependent that can be applied on serum and tissue. This method is low cost and high sensitivity (Hanker, L.C.et al., 2013).

2.2.2.1 Preparation of Standard Solution of Acid Ceramidase Enzyme Method

A standard solution of acid ceramidase (AC) was prepared by dissolved 250 µl of BODIPY-ceramide in 0.2 M buffer (citrate/phosphate) pH=4.5.

2.2.2.2 Principle and Preparation Samples:

A solution 200 µl of BODIPY substrate and 250 µl buffer reaction, and it was prepared then 250 µl of serum or tissue was added to the solution mixture was sitting for 10 min after that 250 µl of 0.5% each of (Triton X-100) cholic acid were added. The resulted mixture was Incubated at 37°C for 20 min to incubate the reaction, then 1.5 ml of ethanol was added to stop the reaction, then centrifuged at 6000 rpm for 6 min was applied . The supernatant was transferred to glass sampling vial. Eluted samples were carried out in mobile phase (methanol: water) (95:5) (v:v), original reaction mixture (100 µl) was injected into Reverse-phase column C18 (4.6 × 150 mm) (Breckenridge, D. G., Germain, M, 2005).

2.2.3 Determination of Concentration of Sphingosine -1-Phosphate by Using Enzyme Linked Immunosorbent Assay (ELIZA)

A double -antibody sandwich enzyme-linked immunosorbent one step process assay (ELISA) one stop process was used to measure sphingosine 1 phosphate (S1P) in serum and tissue samples.

2.2.3.1 Standard Preparation:

The standard was diluted with standard diluents in the method of Multiple proportion dilution and the concentrations were as follows: (0, 18.7, 37.5, 75, 150, 300) ng / ml .

2.2.3.2 Washing Method:

Manually washing method was used by upside the plate and shaking the content out. Then the absorbent papers were used to dry the plate At least 0.35ml of washing solution was added into each well, and the plate was soaked for1~2 minutes. This process was repeated for 5 times.

2.2.3.3 Operation Steps:

Fifty µl of standard were added to standard wells.40 µl of sample diluent were added to sample wells and ten µl of sample were added. 50 µl of horseradish peroxidase (HRP) were added into each well, except the blank well, the plate was shacked gently and was incubated 60 min at 37 ℃.

Excess liquid was discarded, the wells then dried ,washed with washing solution and dried again .Chromogen solution (A) followed by 50µl of Chromogen solution (B) were added to each well. The plate gently shacked and incubated for 10 minutes at 37℃ in dark place.

Fifty µl of stop solution were added into each well to stop the reaction (the blue changes into yellow immediately).

Measurement and standard were the blank wells were set at zero and the optical density (OD) was measured at 450 nm within 15 min after adding the stop solution.

2.2.3.4 Standard curve :

According to standards concentration and the corresponding OD values, the standard curve linear regression equation was calculated and then the OD values of the sample was applied on the regression equation to calculate the corresponding **Figure (2.1)** shows the calibration in curve concentration of samples with a rang > 0- <150 ng/ml.

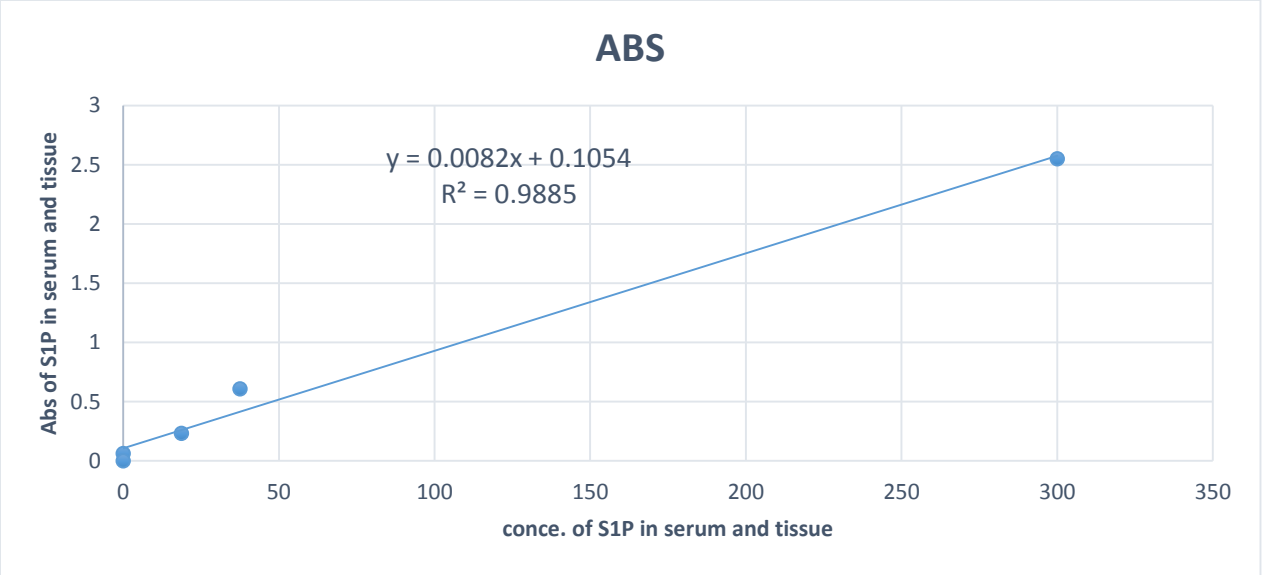

Figure (2.2) standard curve of sphingosine -1-phosphate concentration (ng/ml) in serum and tissue.

2.2.4 Determination Concentration of Ceramide kinase (CERK) in Enzyme Linked Immunosorbent Assay (ELIZA)

A double -antibody sandwich enzyme-linked immunosorbent one step process assay (ELISA) one stop process was used to measure Ceramide kinase (CERK) in serum and tissue samples.

2.2.4.1 Standard preparation :

The Standard was diluted with Standard diluents in the method of multiple proportion dilution and the concentrations were followed: (0, 75, 150, 300, 600, 1200 pg/ml).

2.2.4.2 Reagent Preparation :

Twenty times dilution of washing buffer: distilled water, diluted by 1:20, or 1 copy of the twenty times washing buffer plus 19 copies of the distilled water 5.

2.2.4.3 Washing Method:

Manually washing method was emptied the plate by inverting it and shaking the content out, and then it was taped on the absorbent papers to dry. At least 0.35ml washing solution was added into each well, and the plate was soaked for 1~2 minutes. This process was repeated for 5 times.

2.2.4.4 Operation Steps:

Fifty μl of standard were added to standard wells.40 μl of sample diluent were added to sample wells and ten μl of sample were added. 50 μl of horseradish peroxidase (HRP) were added into each well, except the blank well, the plate was shacked gently and was incubated 60 min at 37 ℃.

Excess liquid was discarded, the wells then dried ,washed with washing solution and dried again .Chromogen solution (A) followed by 50μl of Chromogen solution (B) were added to each well. The plate gently shacked and incubated for 10 minutes at 37℃ in dark place. Fifty μl of stop solution were added into each well to stop the reaction (the blue changes into yellow immediately).

Measurement and standard were the blank wells were set at zero and the optical density (OD) was measured at 450 nm within 15 min after adding the stop solution

2.2.4.5 Standard curve :

According to standards concentration and the corresponding OD values, the standard curve linear regression equation was calculated and then the OD values of the sample was applied on the regression equation to calculate the corresponding Figure (2.2) shows the calibration in curve concentration of samples with a rang > 0- <150 ng/ml.

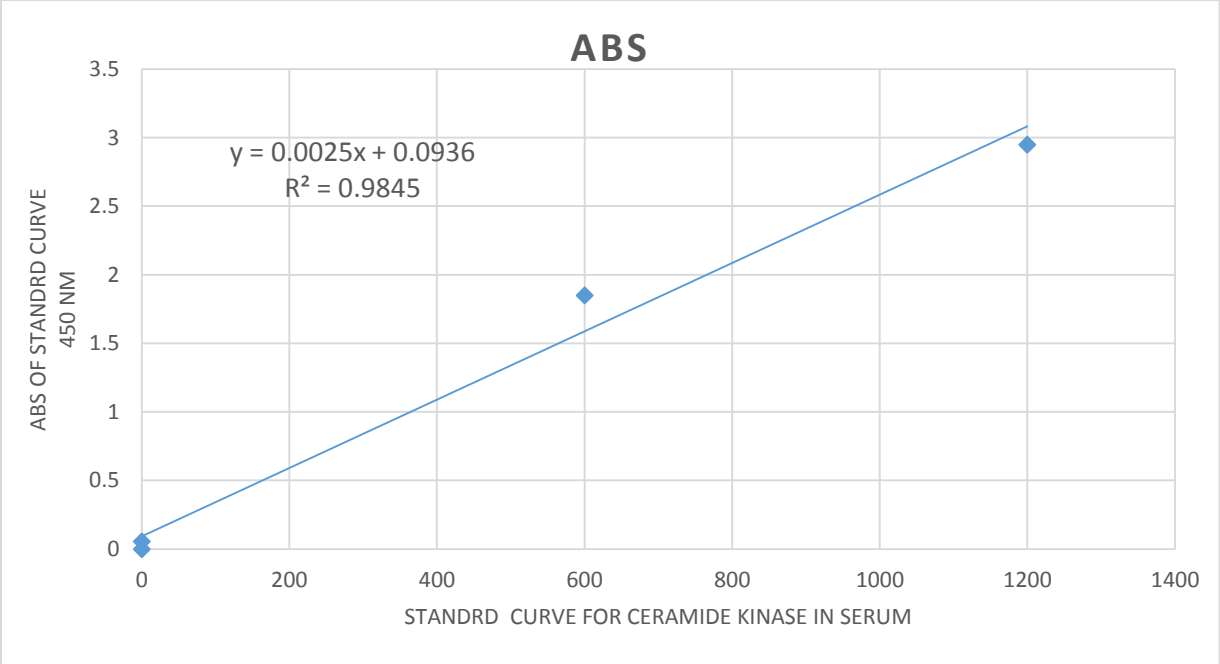

Figure (2.3) standard curve of (CERK) concentration (pg/ml) in serum and tissue

2.2.5 Brad Ford Method for Protein Quantitation in Tissue Using Spectrophotometer Method

2.2.5.1 Reagents:

The assay reagent was made by dissolving (100 mg) of Coomassie Blue (G 250) in (50 ml) of 95% ethanol. The solution was mixed with (100 ml) of 85 % phosphoric acid and made up to 1L with (D.W).The reagent was filtered through filter paper before storage in an amber bottle at room temperature (Albright, Brian ,2009).

2.2.5.2 Protein Standard:

Bovine Serum Albumin (BSA) at concentration (1mg/ml) or (100 µg /ml) for micro assay in (Di ionized. Water) prepared for micro assay.

2.2.5.3 Standard Method

A series concentration of BSA [10, 20, 40, 60, 80 and 100 µl] of (100 µg /ml) from (BAS) was prepared to set up the standard curve to 100 µl with DD.W.

This form of the assay is more sensitive to protein and interfering substance and is useful when the amount of the unknown protein is limited.

2- For the reagent blank : 100 µl of (DD.W) into a further tube then (1 ml) of protein reagent were pipetted to all tubes, mix well and the absorbance was measured at wave length (595-600) nm. The standard curve obtained by this procedure is shown in **Figure (2.3)**

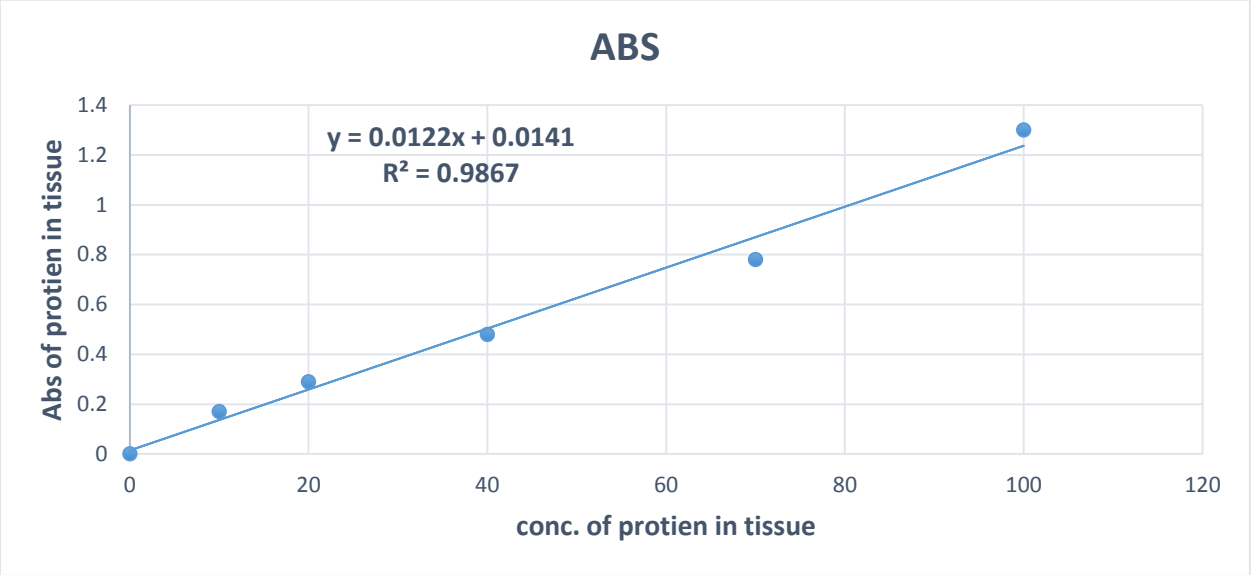

Figure (2.4) Standard Curve for concentration of protein (µg/l) in tissue

2.2.6 Determination of Glucose in Serum by Spectrophotometer

Glucose was determined after enzymatic oxidation in the presence of glucose oxidase. The hydrogen peroxide formed under catalysis of peroxidase, with phenol and 4-aminophenazone, a red - violet quinoneimine dye will form as indicator. According to the following reaction. (Kunst, A., Draeger, B. & Ziegenhorn, J. 1984)

Calculation:

Glucose concentration (mmol/l) = (Abs of sample / Abs of standard) × 5.55…..2

Glucose concentration (mg /l) = (Abs of sample / Abs of standard) × 100…...3

2.2.7 Determination of Urea in Serum by Spectrophotometer

The method is based on the following reaction:- Salicylate and hypochlorite in the reagent react with the ammonium ions to form a green complex (2.2 dicarboxylindophenol). (Patton, C.J., Crouch, S.R., Anal. Chem., 1977).

Calculation:

Urea concentration (m. mol/l) = (Abs of sample / Abs of standard) × 8.33….4

Urea concentration (mg /l) = (Abs of sample / Abs of standard) × 50……5

2.2.8 Determination of Creatinine in Serum by Spectrophotometer

Creatinine in alkaline solution reacts with picric acid to form a colored complex. The amount of the complex formed is directly proportional to the creatinine concentration (Bartels, H., Bohmer, M., (1972).

A2 - A1 = DA sample or DA standard

Concentration of creatinine in serum

Creatinine concentration = (Abs of sample / Abs of standard) × 177 = µmol/l….6

Creatinine concentration = (Abs of sample / Abs of standard) × 2 = mg/dl…..7

Concentration of creatinine in urine

Creatinine concentration = (Abs of sample / Abs of standard) × 8.85 = µmol/l….8

Creatinine concentration = (Abs of sample / Abs of standard) × 100 = mg/dl….9

2.2.9 Determination of Albumin in Serum by Spectrophotometer

In buffered solution at pH 4.2, bromocresol green binds albumin to form a colored compound which absorbance, measured at 630 nm. The absorbance is proportional to concentration of albumin in the specimen (Roche, M., Rondeau, P., Singh, N.R. 2008).

Calculation

Albumin concentration (Abs of sample / Abs of standard) × 5 =(g/dl)…….10

Albumin concentration (Abs of sample / Abs of standard) × 725 = (µmol /l)…11

2.2.10 Determination of Albumin in Urine by Solid phase, Sandwich-format, Immunometric Assay by a Gold-antibody Conjugate.

Micro albuminuria is defined a persistent elevation of the urinary albumin excretion to 20-200 μg /min or 20-200 mg/L when using for early morning urine. it has demonstrated that micro albuminuria independently predicts cardiovascular morbidity and all-cause mortality in essential hypertension. Monitoring of micro albuminuria is worth-while in order to monitor the effect of anti-hypertensive treatment on target-organ damage Low-protein diets, lowering blood pressure and the use of anti-hypertensive therapy have all been reported to have a positive effect on decreasing the urinary albumin excretion . The research group has shown the importance of strict glycemic control in preventing micro albumin urea Nycocard U-Albumin is simply quick and convenient within only 3 minutes a quantitative test result is obtained using the Nycocard READER (Barth, B.M.; Gustafson, S.J.;2012).

2.2.11 Determination of HbA1C by Using: Solid phase, Sandwich-Format, Immunometric Assay Using a Gold-antibody Conjugate.

Several studies have shown the importance of good metabolic control in preventing – and slowing - the progression of diabetic late complications for person with type I and type II diabetes. Measurement of glycated hemoglobin has proven to be an important tool in determining the quality of the metabolic control. Nycocard HbA1c is rapid and provides an accurate HbA1c result within 3 minutes during the patient consultation making a revisit to alter the treatment regime no longer necessary (Barth, B.M.; Gustafson, S.J.;2012).

Standardization

The International Federation of Clinical Chemistry (IFCC) has established a working group on glycol hemoglobin standardization. This will be based on HbA1c, as it is the easiest component to define. Methods that measure total hemoglobin, such as affinity methods, can also be standardized against HbA1c, as their respective values correlate well.

2.2.12 Determination the Concentration of Prostatic Specific Antigen (PSA) in Enzyme Linked Immunosorbent Assay (ELIZA)

The PSA (Human) ELISA Kit is based on the principle of a solid phase enzyme-linked immunosorbent assay. The assay system utilizes a goat anti-PSA antibody directed against PSA for solid phase immobilization (on the micro titer wells). A monoclonal anti PSA antibody conjugated to horseradish peroxidase (HRP) is in the antibody enzyme conjugate solution. The sample was reacted first with the immobilized goat antibody at room temperature for 60 min. The wells are washed to remove any unbound antigen, then monoclonal anti PSA-HRP conjugate is then added and allowed to react with the immobilized antigen for 60 min at room temperature resulting in the PSA molecules being sandwiched between the solid phase and enzyme-linked antibodies. The wells were washed with water to remove unbound-labeled antibodies. A solution of TMB reagent was added and incubated at room temperature for 20 min to enrich the blue color. The enrichment of blue color with the addition of stop solution. The color start to change to yellow. The concentration of PSA was directly proportional to the color intensity of the test sample. Absorbance is measure spectrophotometrically at 450 nm.

According the concentration of standards and the corresponding OD values, the standard curve linear regression equation was calculated and then the OD values of

the sample was applied on the regression equation to calculate the corresponding sample's concentration (Armstrong, A. J.et al., 2007). Figure (2.5) shows the standard curve of PSA in the serum.

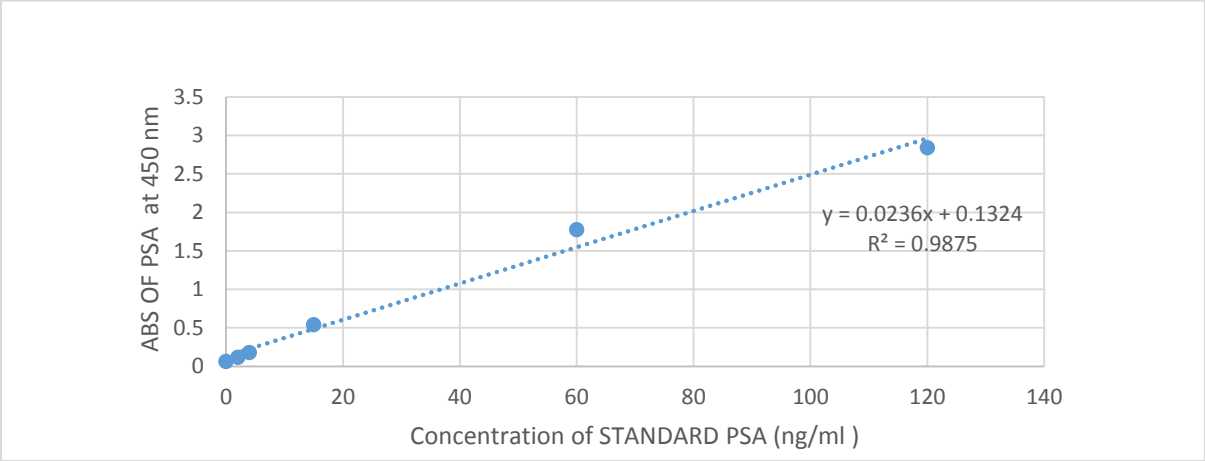

Figure (2.5) Standard Curve of (PSA) concentration (ng/ml) in serum

2.3 Calculation Body Mass Index (BMI) Formulas

Body mass index (BMI) was measured for body fat based on height and weight that applied to adult men. (Parr CL.et al., 2010).

The formula of BMI was calculated based on two of the most commonly used unit systems:

$$\text{BMI} = \text{weight (kg)} / \text{height}^2\ (\text{m}^2) \qquad \text{(Metric Units)}\ldots\ldots..12$$

2.4 Statistical Methods:

Data were encoded and entered into SPSS statistical package (v.20). Pearson correlation test was performed to examine the association between SPL, AC, S1P, Ceramide biomarkers and prostatic cancer. At the multivariate level of analysis, the Analysis of Variance (ANOVA) test was performed to examine the difference in the SPL, AC, S1P and ceramide markers between the control group and study groups

(BPH with and without drugs) (malignant PC with and without chemotherapy) in serum and tissue. Receiver Operating Characteristics (ROC) curve was calculated to assess the sensitivity and specificity of the used HPLC and kits in classifying the true positive as well. Finally, a series of Figures were produced and presented to show the concentration levels of SPL and AC in both tissue and serum among BPH and PC men. Groups respectively S1P, Ceramide different concentration and standard curve level in BPH and PC men in both tissue and serum and P values less than 0.05 was considered significant.

Chapter Three

Results

3.1 Serum and Tissue of Sphingosine Phosphate Lyase Enzyme (SPL) Concentration

In this study, HPLC was used for estimation of the concentration of SPL activity enzyme in serum and tissue. Figure (3.1) shows the chromatography of HPLC of standard of SPL and the retention time of suspected product peaks. Figure (3.2) healthy controls which were compared with peak of standard material BODIPY-sphingosine, figures (3.3), (3.4), (3.5), and (3.6) show peaks of serum SPL in BPH (with and without drugs) such as (5-alpha reductase inhibitors Finastreride and Dutasteride), and PC (with low and with high doses chemotherapy such as (zoladex and Taxotere). While figures (3.7), (3.8), (3.9), and (3.10) show the chromatography of HPLC of tissue SPL peaks in BPH (with and without drugs), and PC (with low doses chemotherapy and with high doses chemotherapy). Serum and tissue the concentration of SPL (PPb) in PC (with low and high doses of chemotherapy), BPH (with and without drugs), and healthy controls respectively were shown in appendix (5.2) and figure (3.11) show concentration of SPL (ppb) levels in men serum in control, serum and tissue in BPH (with and without doses of drugs) also PC (with low and high doses of chemotherapy).

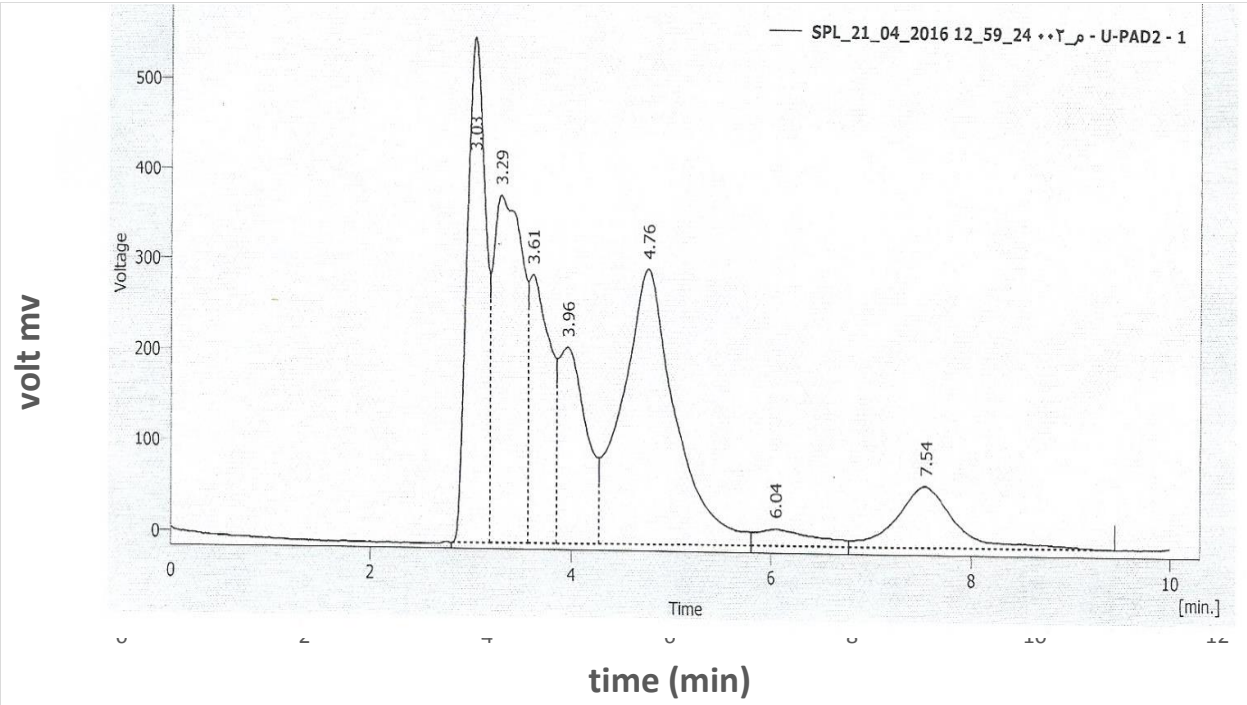

Figure (3.1) HPLC chromatogram analysis of BODIPY- labeled sphingosine SPL products as standard, suspected product peaks of the SPL-catalyzed reaction retention time suspected (RT= 7.54) min.

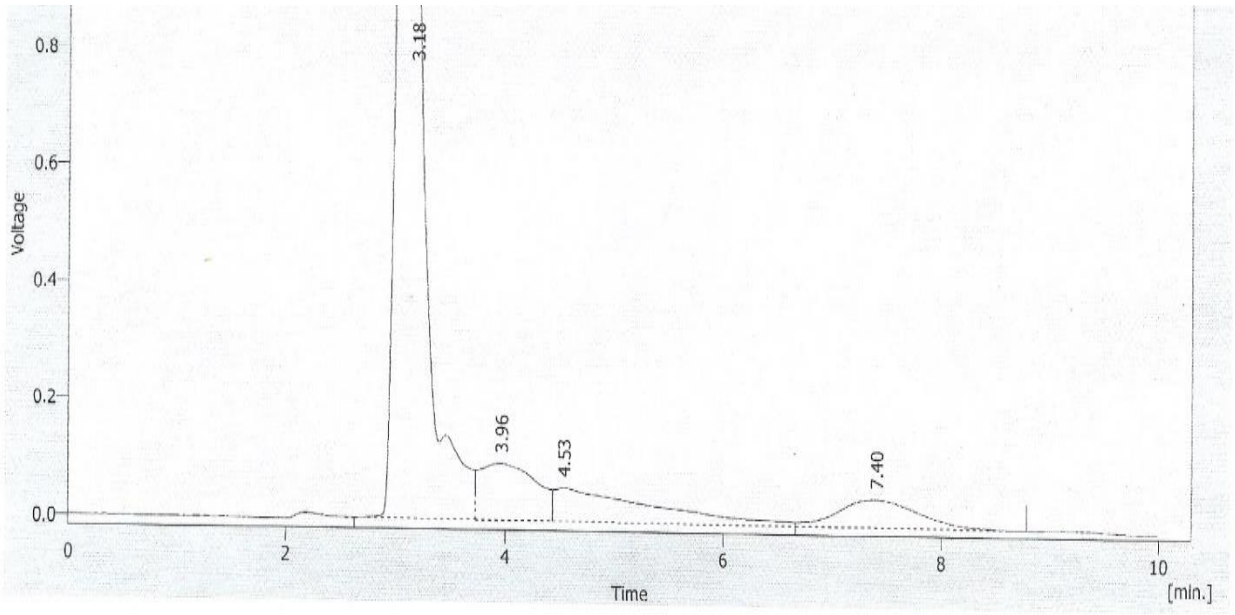

Figure (3.2) SPL in serum (control group) (RT= 7.40)min.

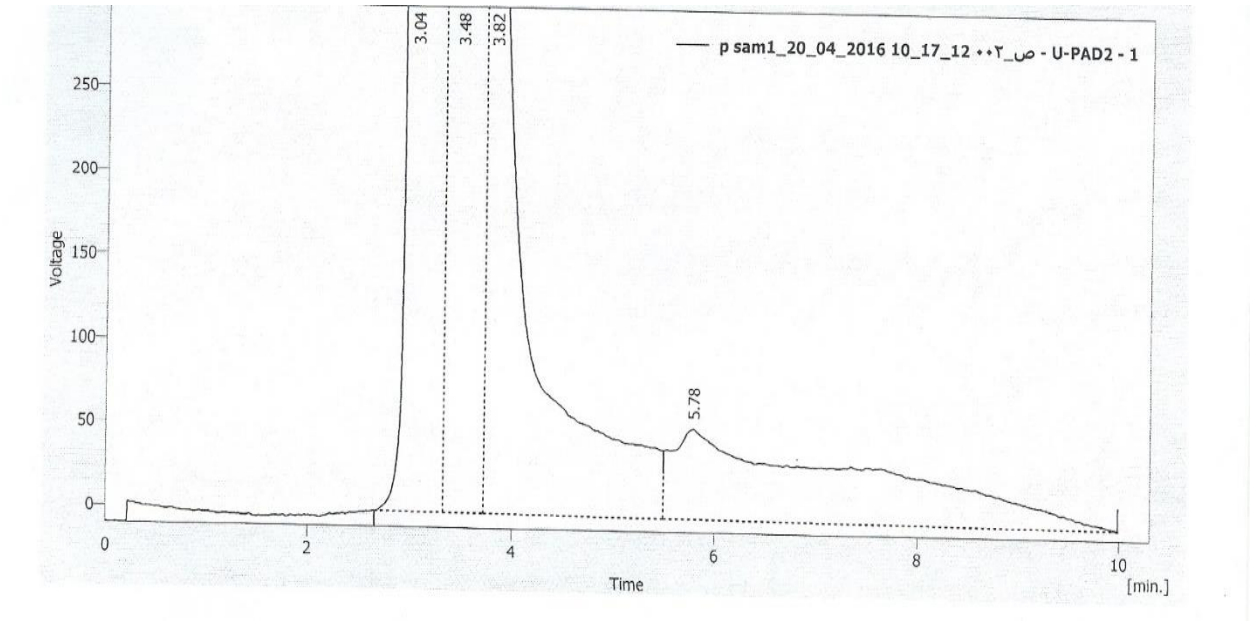

Figure (3.3) SPL in serum of BPH without drugs absence.

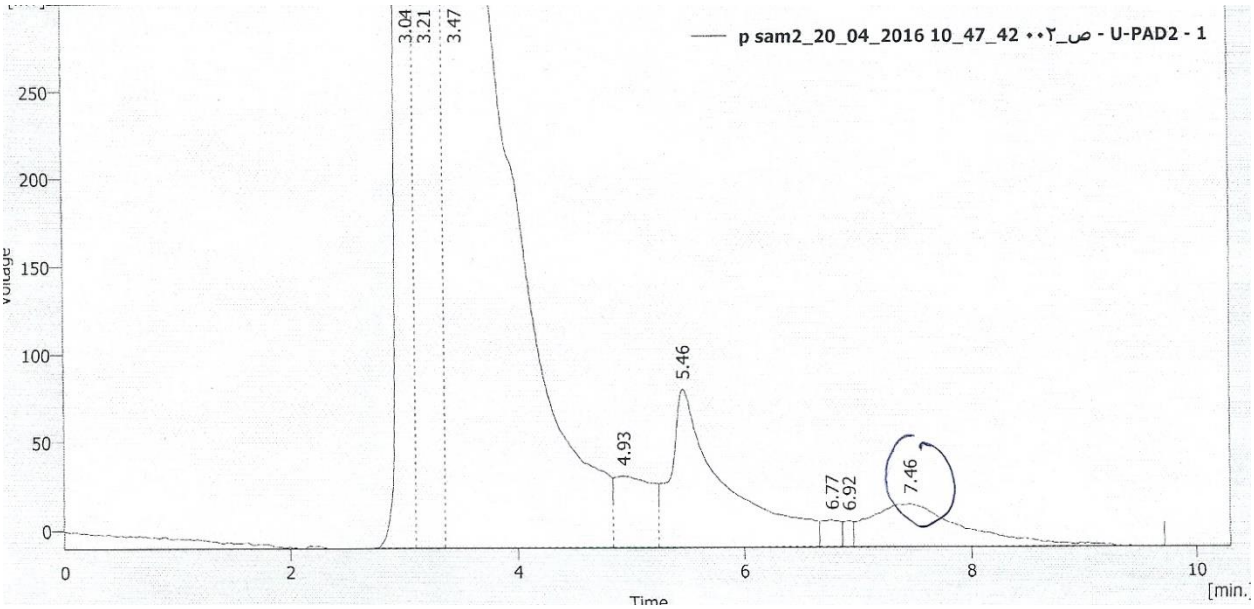

Figure (3.4) SPL in serum of BPH with drugs (RT= 7.46) min

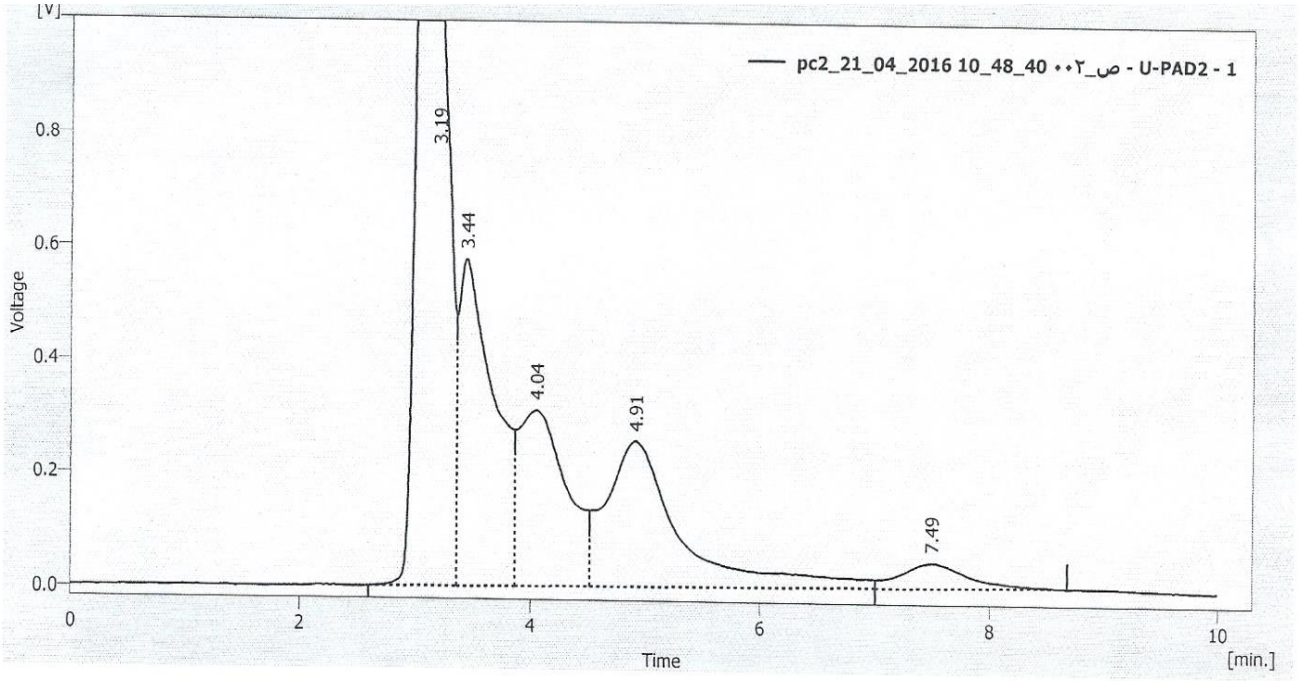

Figure (3.5) SPL in serum peaks in patients with PC with low doses of chemotherapy (RT= 7.49) min.

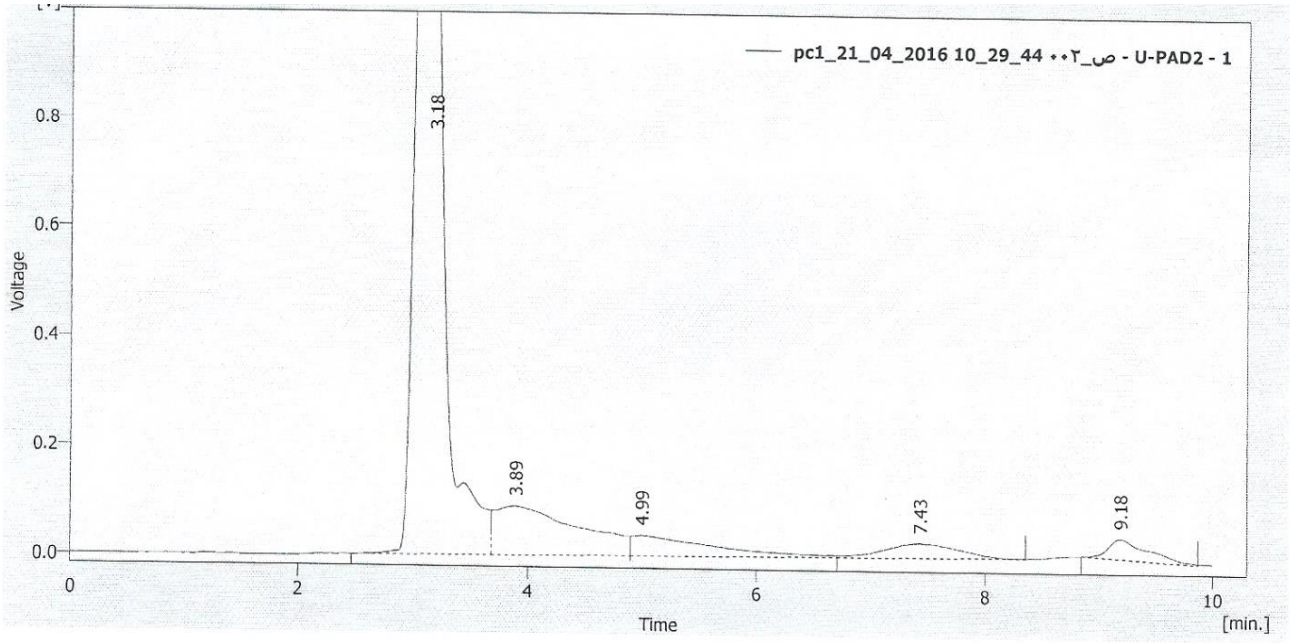

Figure (3.6) SPL in Serum peaks of PC with high doses chemotherapy (RT=7.43) min.

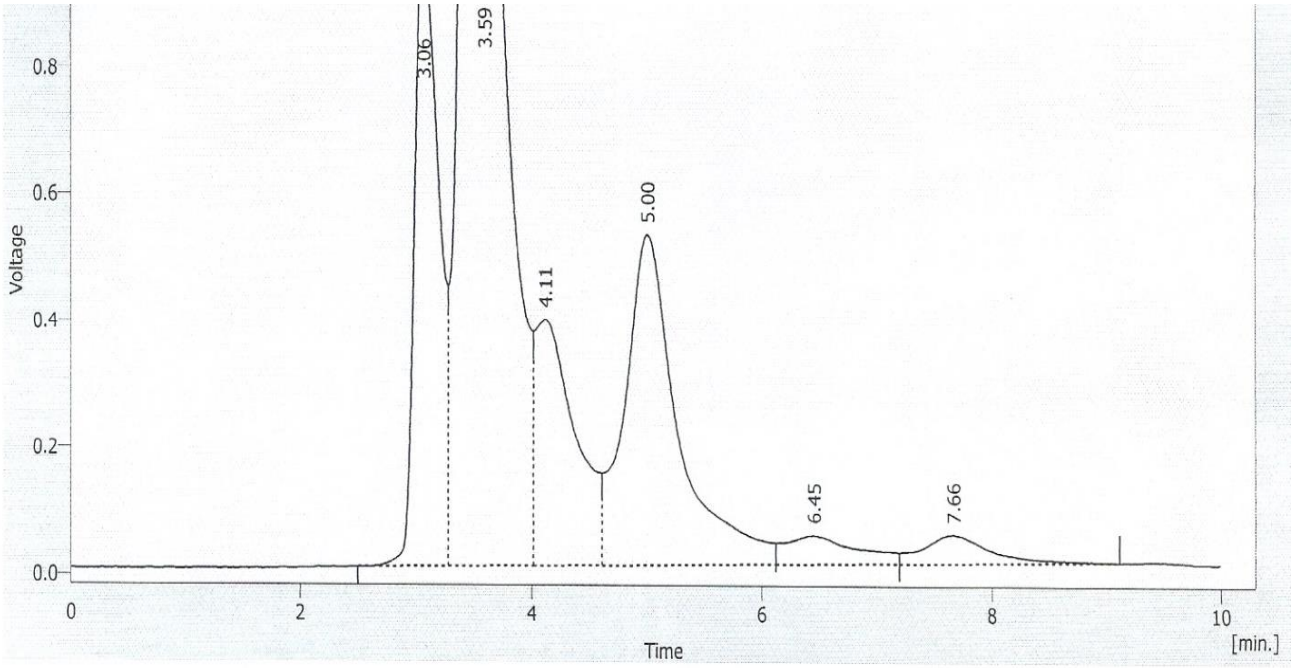

Figure (3.7) SPL in tissue of BPH without drugs (RT= 7.66) min.

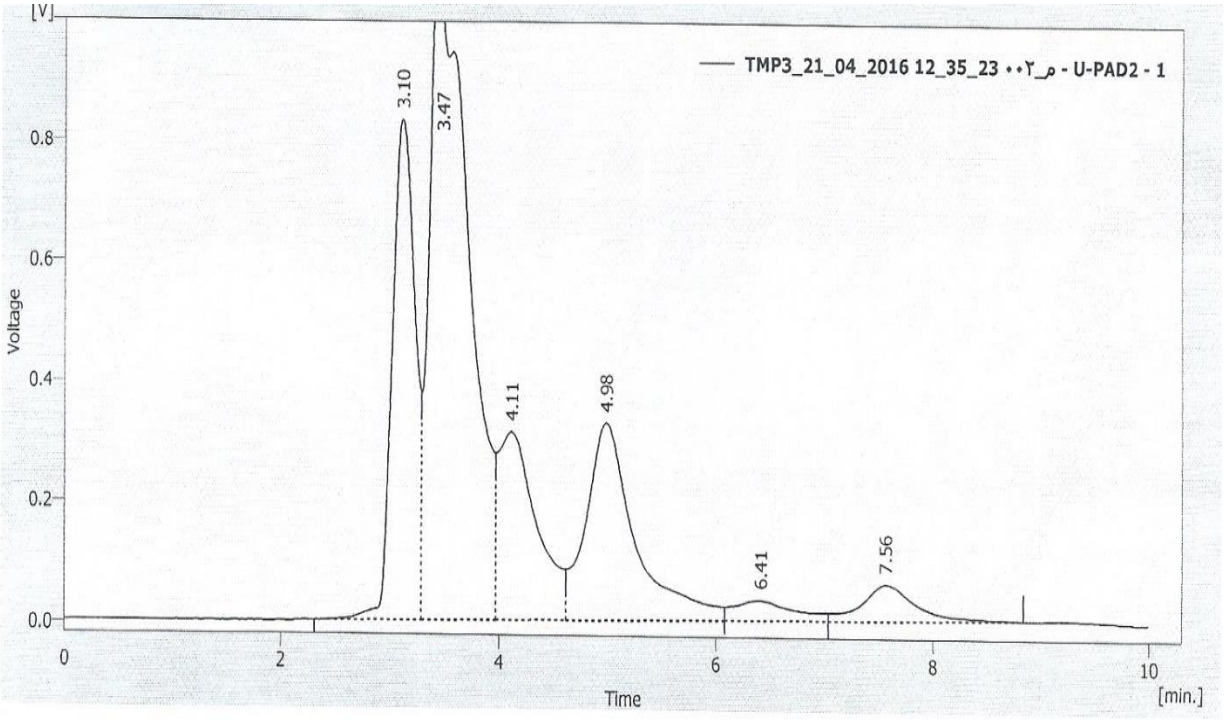

Figure (3.8) SPL in tissue of BPH with drugs (RT=7.56) min.

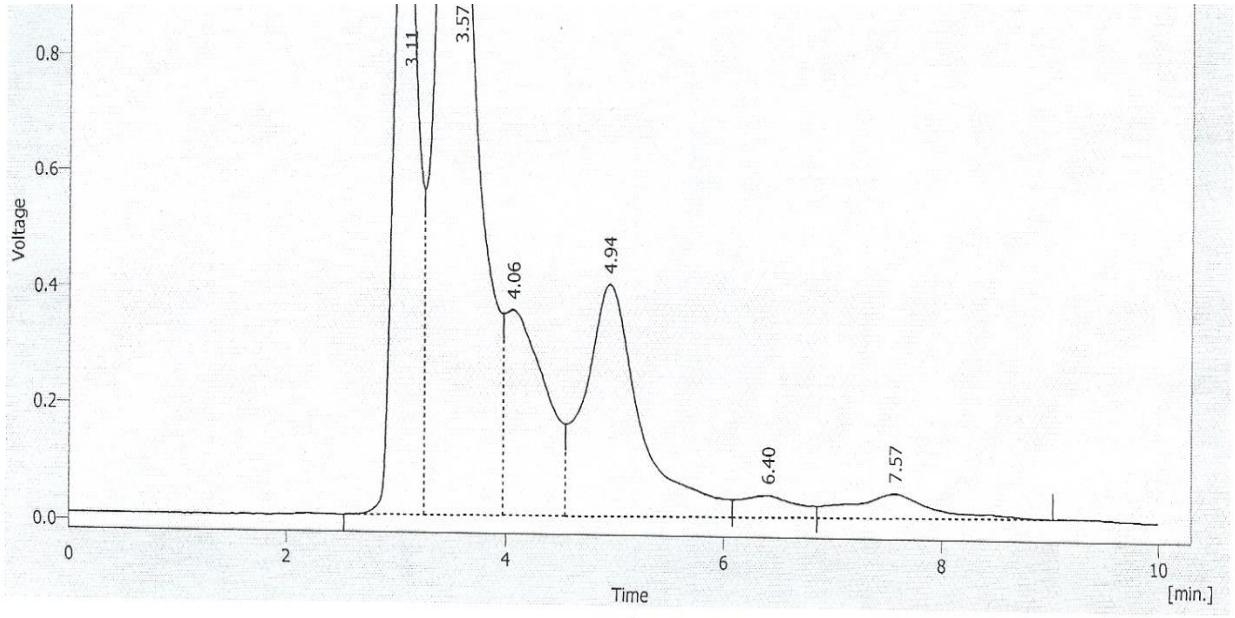

Figure (3.9) SPL in tissue peaks of patients with PC at low doses chemotherapy (RT=7.57) min.

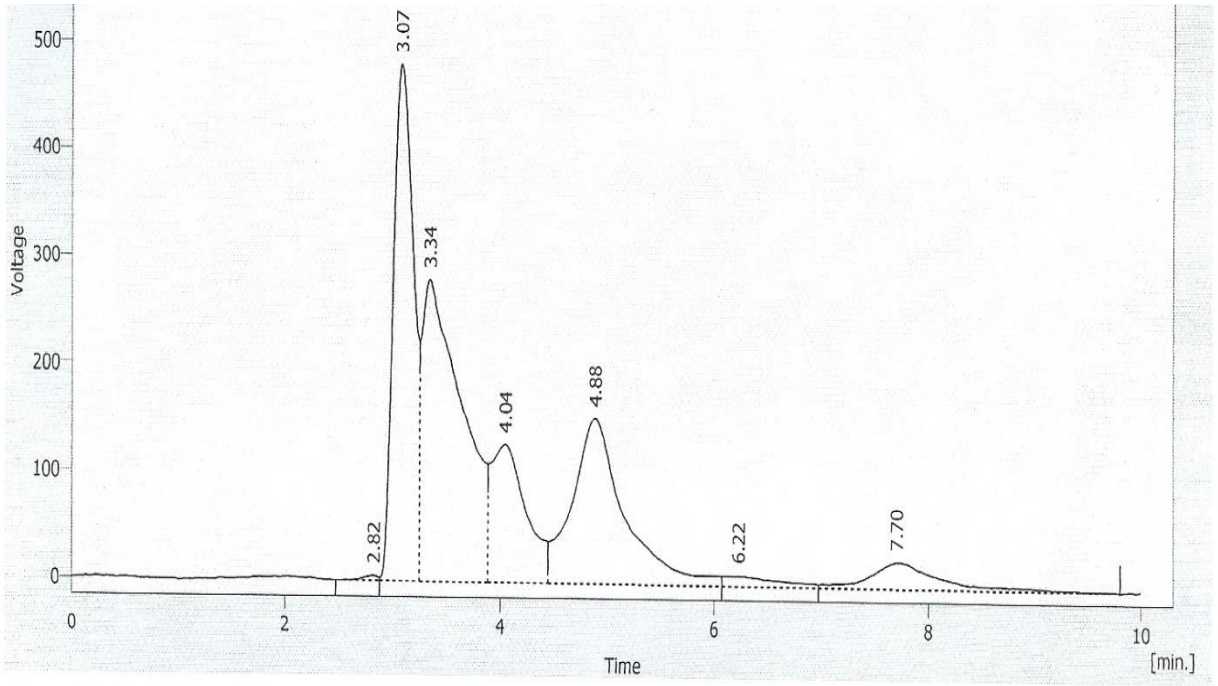

Figure (3.10) SPL in tissue peaks in patients with PC with high doses chemotherapy (RT=7.70) min.

The area under the peak for serum and tissue SPL at the same retention time and same conditions of standard for different groups shows that (Mean ± SD) of SPL in serum and tissue are absence in the patients with BPH without drugs comparing with BPH with drugs activity of SPL was decreased. The concentration of SPL is decreased with malignant prostatic cancer when low doses of chemotherapy is applied in comparison with patients applied to high doses of chemotherapy, so all two groups were inhibition activity of enzyme by pharmacological or genetic chemotherapy doses. However, the control group shows much more concentration comparing with the groups under the test. All the results are shown in in Table (3-1). Table (3-1) the peak of area for SPL (serum and tissue) in the groups under the test.

Groups	**Mean of peak area ± SD for serum groups ppb**	**P value between serum groups**	**Mean of peak area ± SD for tissue groups ppb**	**P value between tissue groups**
Control	59.20 ± 6.53			P<0.001
BPH without drugs	0.54 ± 0.39	P<0.001	16.87 ± 4.73	
BPH with drugs	14.71 ± 9.04		30.43 ± 3.91	
Prostatic cancer low doses chemotherapy	13.37 ± 4.46	P<0.001	17.49 ± 3.95	P<0.001
Prostatic cancer high doses chemotherapy	32.94 ± 5.19		30.75 ± 4.49	

According to (P value) (P < 0.001), there were significance difference between the mean of SPL in serum and tissue of patients and mean of SPL in serum of control. Also (P value < 0.001) were found significant between (BPH with drug and without drug) and (PC with high and low dose chemotherapy).

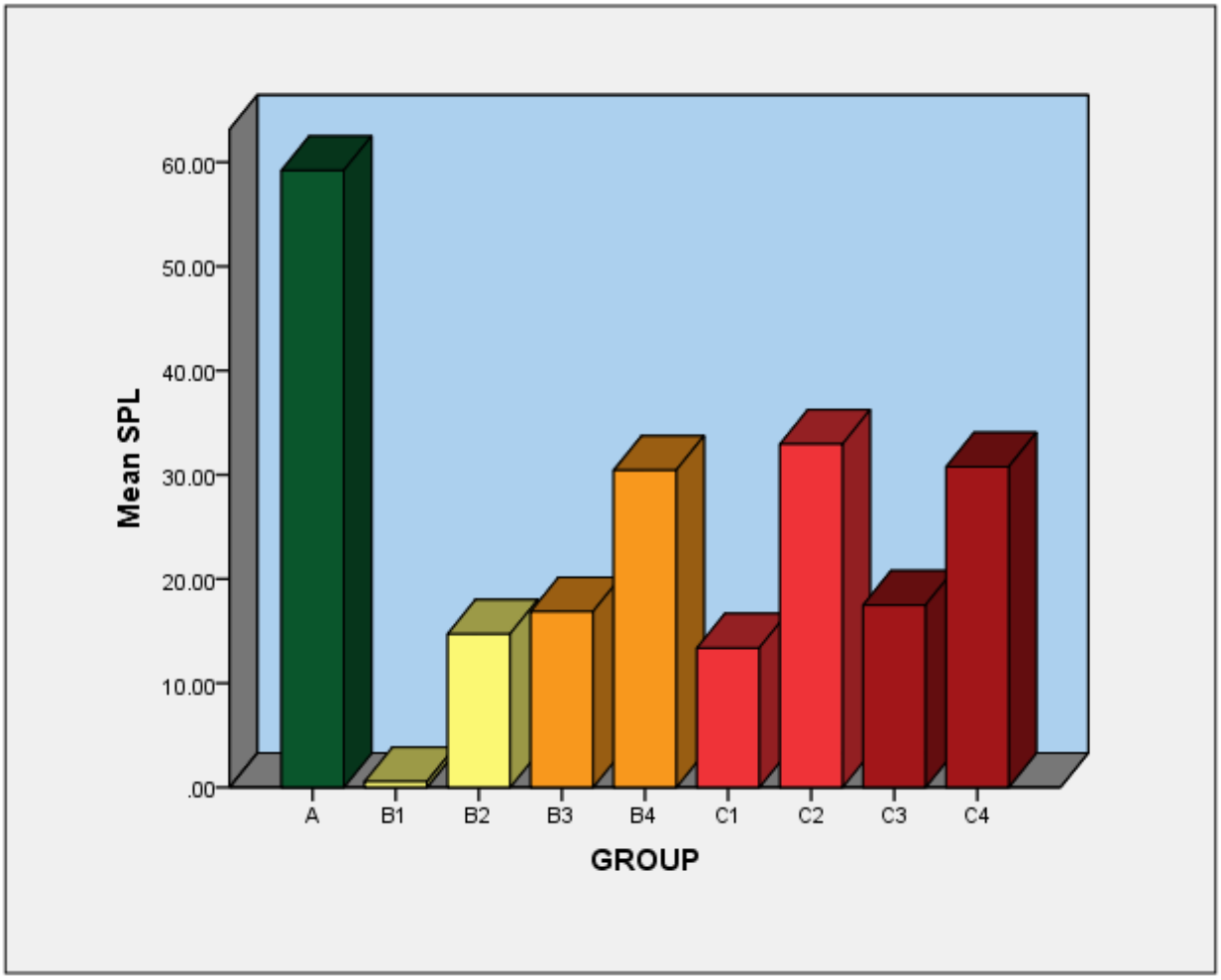

Figure (3.11) SPL concentration (ppb) levels in men serum and tissue control, Benign Prostatic Hyperplasia with low and high doses of drugs and Prostatic Cancer with low and high doses of chemotherapy. As statistical SPSS v. 20.

3.2 Serum and Tissue Acid Ceramidase (AC) Concentration

HPLC was used for estimation of the concentration of (AC) activity enzyme in serum and tissue. Figure (3.12) shows the chromatography of HPLC of standard of AC and the retention time of suspected product peaks. Figure (3.13) healthy controls which were compared with peak of standard material BODIPY- ceramide. Figures (3.14), (3.15), (3.16), and (3.17) show peaks of serum AC in BPH (with and without drugs) such as (5-alpha reductase inhibitors Finastreride and Dutasteride), and PC (with low and high doses of chemotherapy such as (zoladex and Taxotere). While figures (3.18), (3.19), (3.20), and (3.21) show the chromatography of HPLC of tissue AC peaks in BPH (with drugs and without drugs), and PC (with low doses chemotherapy and with high doses chemotherapy). Serum and tissue the concentration of AC (PPb) in PC (with low and high doses of chemotherapy), BPH (with and without drugs), and healthy controls respectively were shown in appendix (5.1) and Figure (3.22) show concentration of AC (ppb) levels in men serum in control, serum and tissue in BPH (with and without doses of drugs) and (PC with low and high doses of chemotherapy).

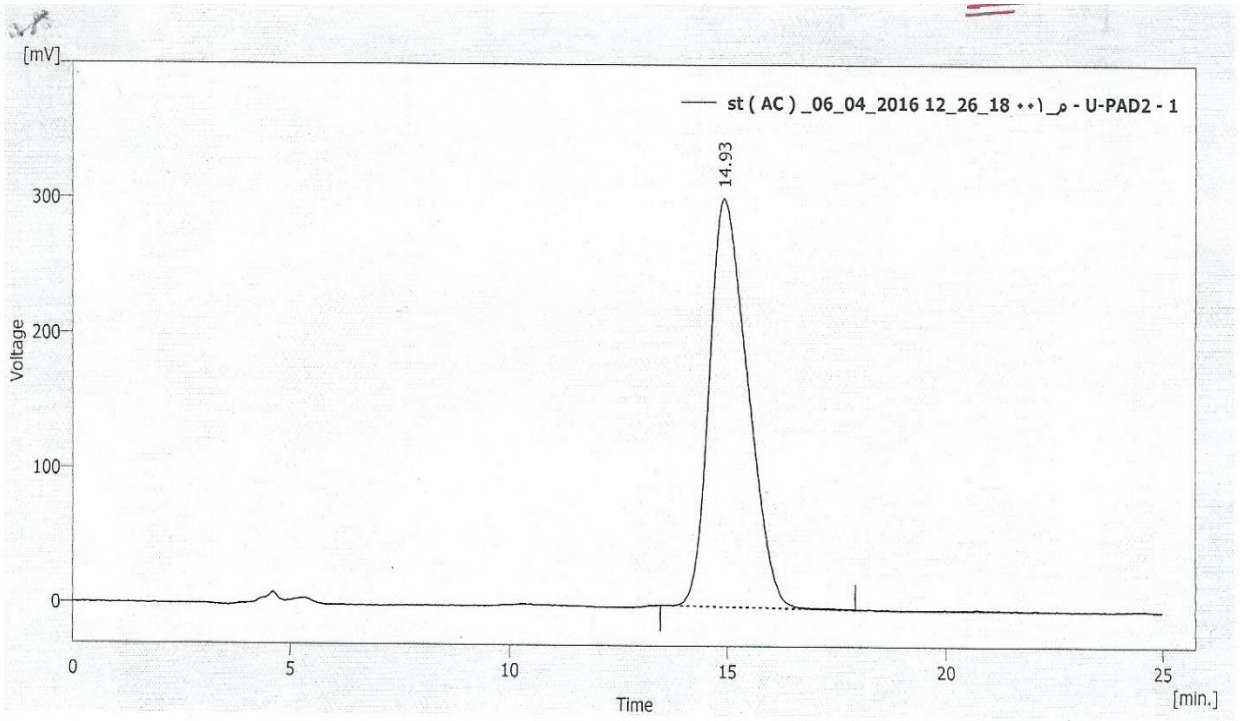

Figure (3.12) HPLC Chromatogram Peak of BODIPY-Ceramide as standard of AC the retention time (RT= 14.93) min.

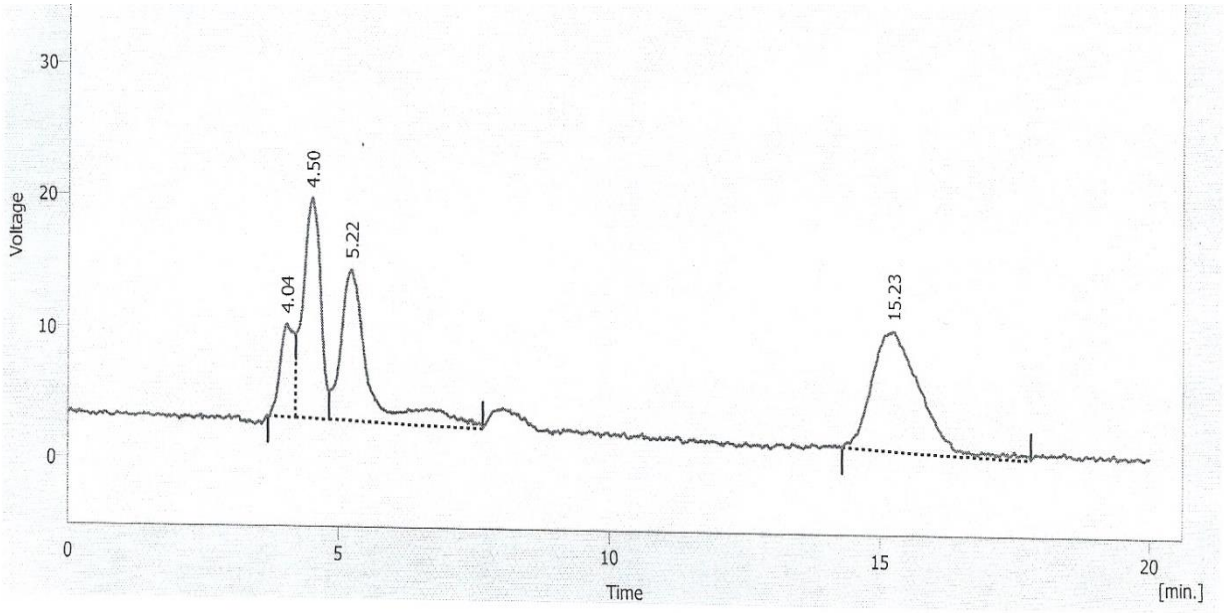

Figure (3.13) Peak of AC in serum of healthy men (RT= 15.23) min

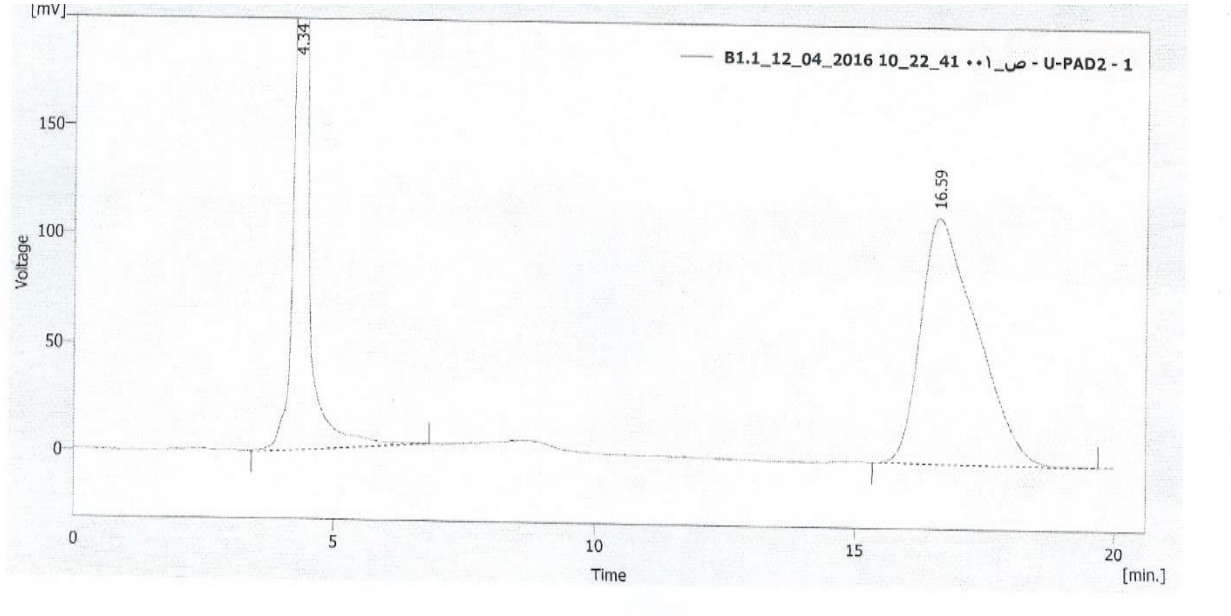

Figure (3.14) Peak of AC in serum of BPH without drugs (RT=16.59) min

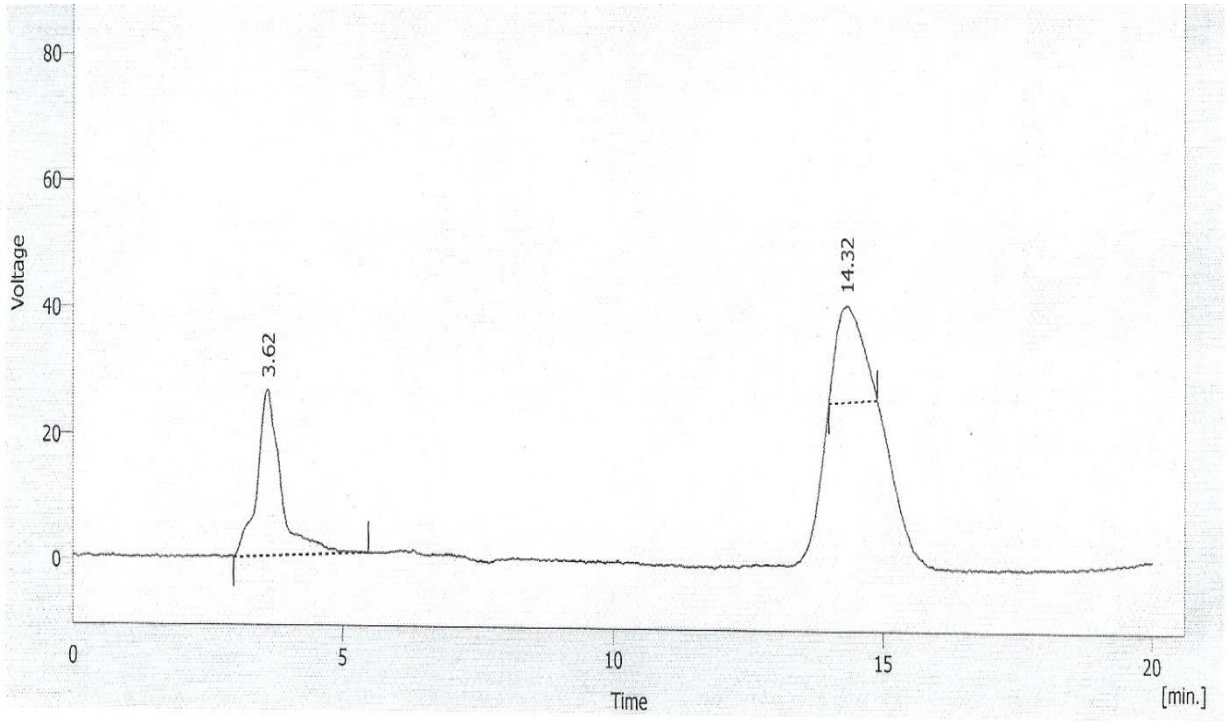

Figure (3.15) Peak of AC in serum of BPH with drugs (RT=14.32) min.

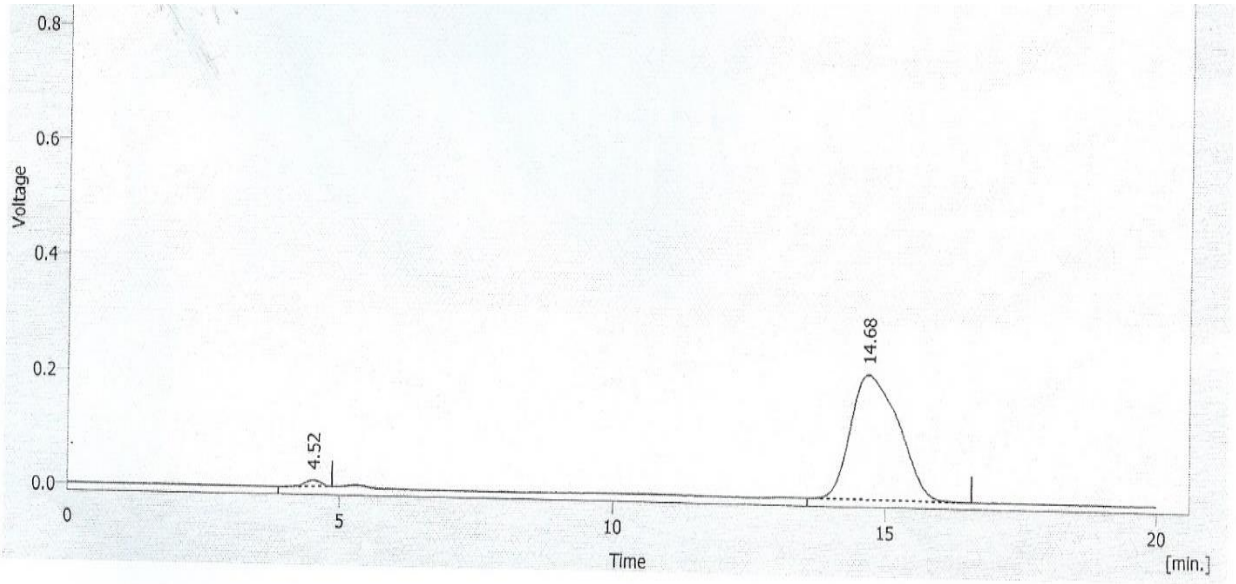

Figure (3.16) peak of AC in serum of PC with low doses of chemotherapy (RT=14.68) min.

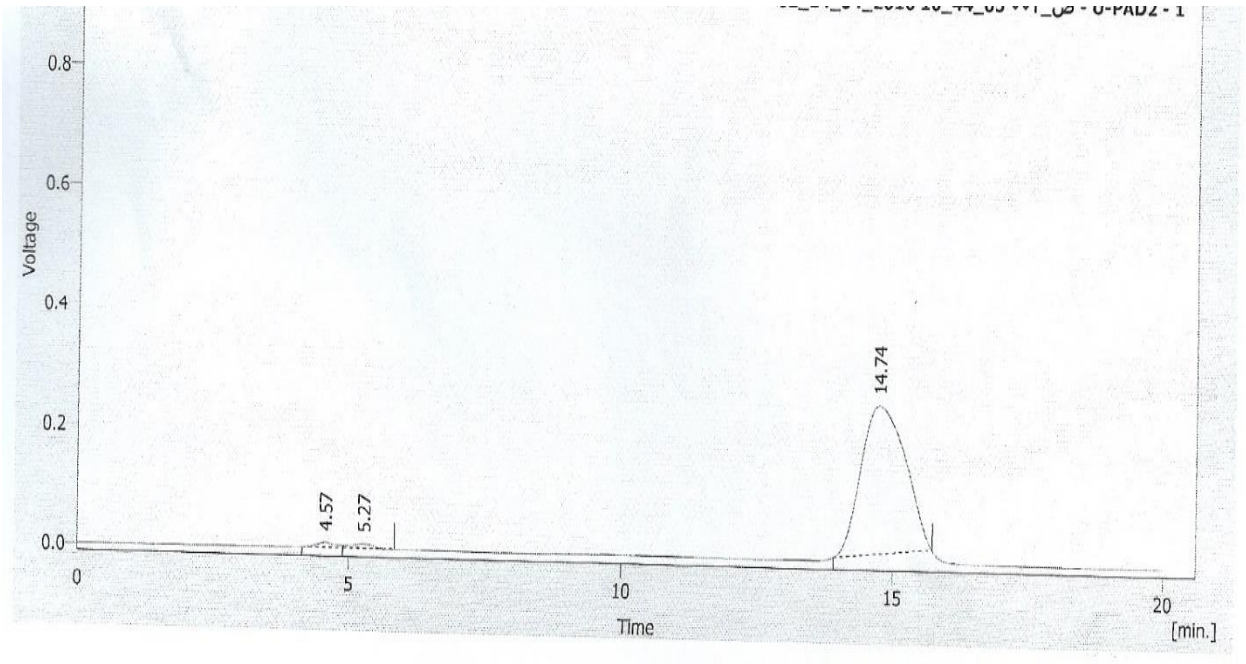

Figure (3.17) Peak of AC in serum of PC with high doses of chemotherapy (RT=14.74) min.

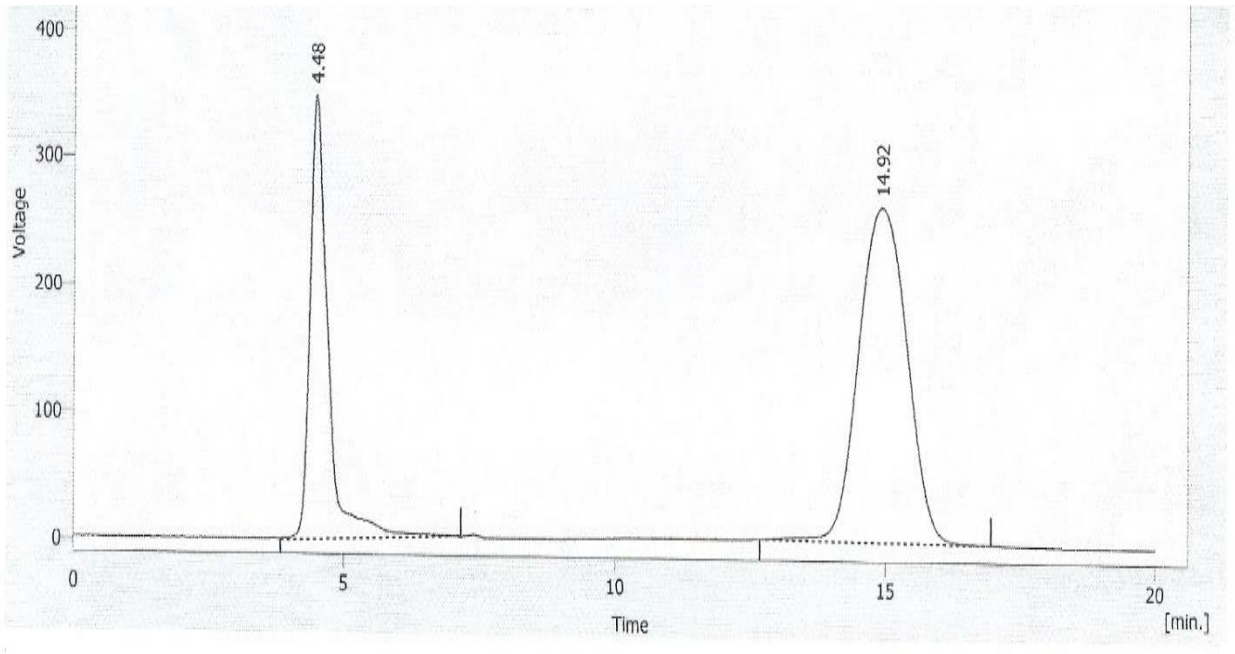

Figure (3.18) peak of AC in tissue of BPH with drugs (RT=14.92) min.

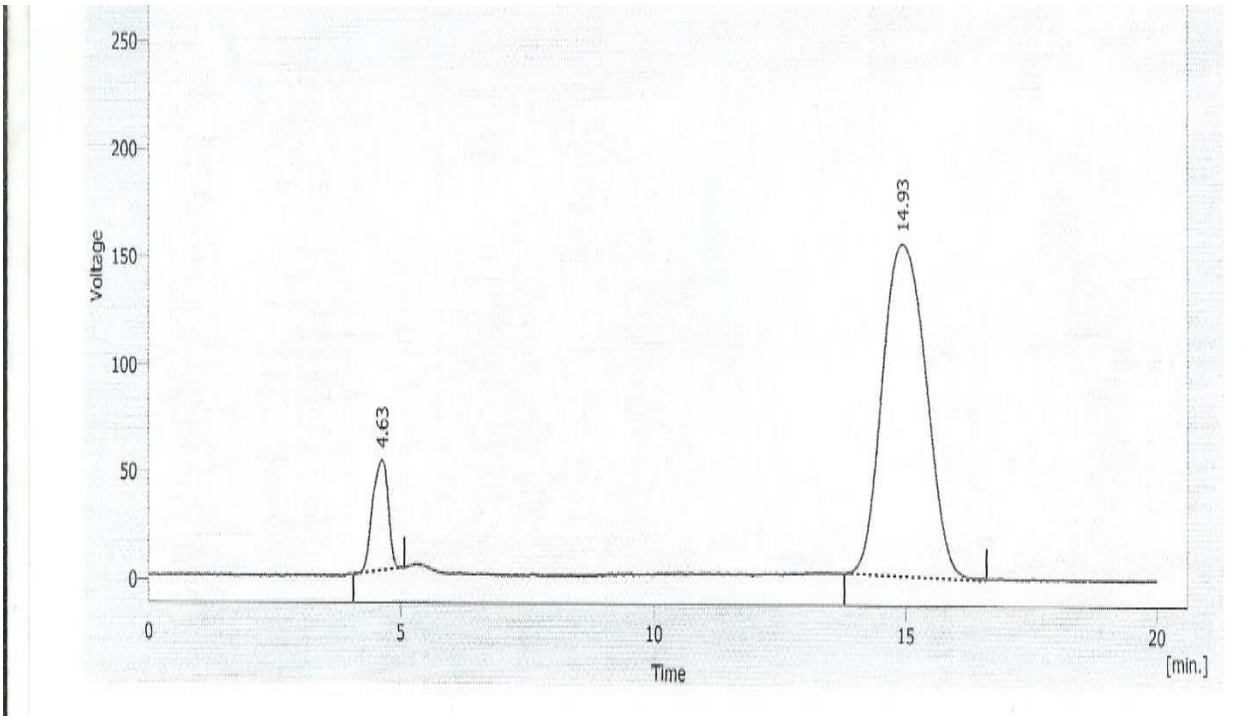

Figure (3.19) Peak of AC in tissue of BPH without drug (RT=14.93) min

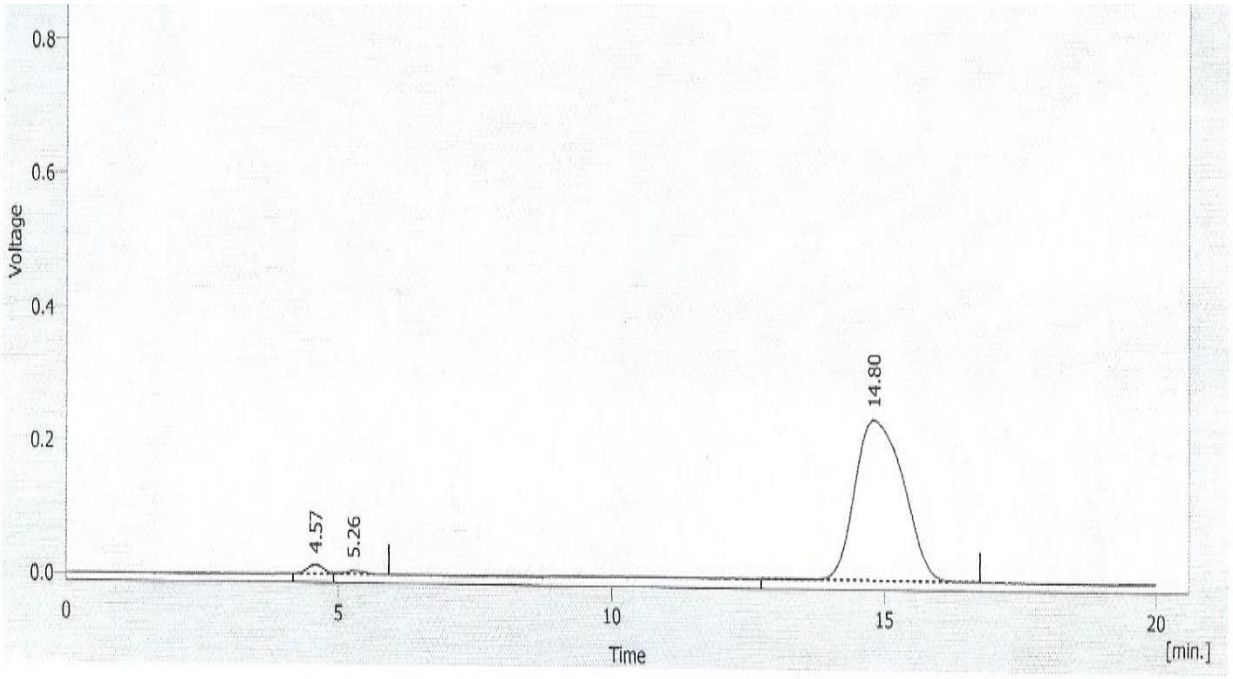

Figure (3.20) Peak of AC in Tissue of PC with low doses of chemotherapy (RT=14.80) min.

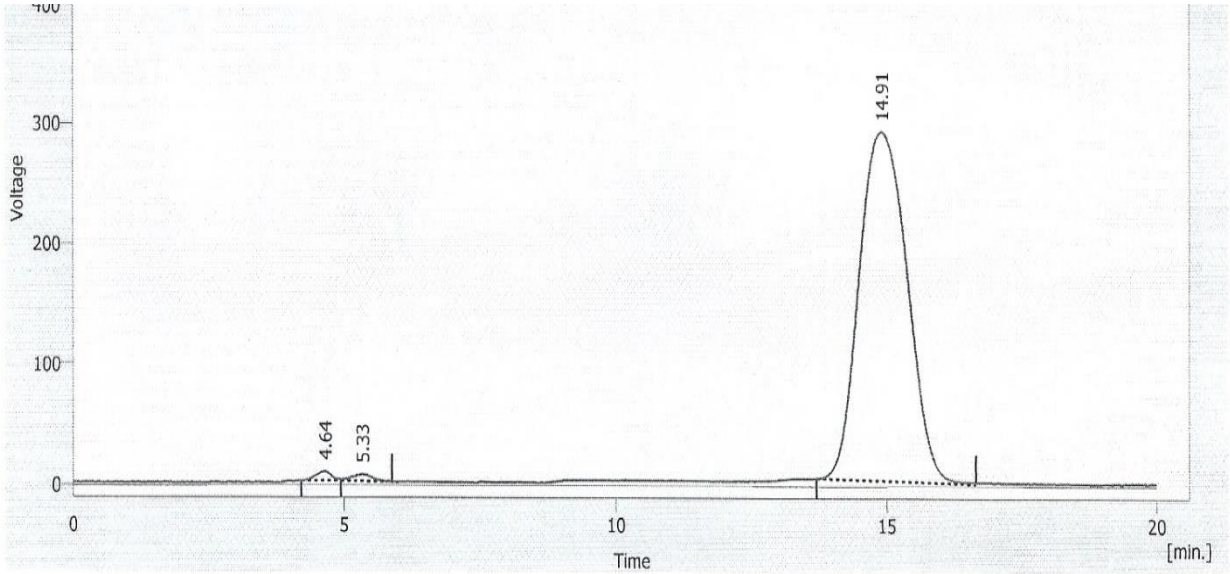

Figure (3.21) Peak of AC in tissue of PC with high doses of chemotherapy (RT=14.91) min.

The area under the peak for serum and tissue AC at the same retention time and same conditions of standard for different groups shows that (Mean ± SD) of AC in serum and tissue are increase in the patients with BPH without drugs comparing with BPH with drugs. The concentration of AC is increase with malignant prostatic cancer when low doses of chemotherapy is applied in comparison with patients applied to high doses of chemotherapy. However, the control group shows much less concentration comparing with the groups under the test. All the results are shown in in table (3-2). Table (3-2) the peak of area for AC (serum and tissue) in the groups under the test.

Groups	**Mean of peak area ± SD for serum groups ppb**	**P value between serum groups**	**Mean of peak area ± SD for tissue groups ppb**	**P value between tissue groups**
Control	1.658 ± 0.3			
BPH without drugs	37.85 ± 7.5	P<0.001	63.33 ± 8.8	P<0.001
BPH with drugs	17.53 ± 4.7		36.77 ± 9.3	
Prostatic cancer low doses chemotherapy	52.38 ± 9.6	P<0.001	93.07 ± 8.5	P<0.001
Prostatic cancer high doses chemotherapy	32.62 ± 10.2		71.76 ± 10.7	

According to (P value) (P < 0.001), there were significance difference between the mean of AC in serum and tissue of patients and mean of AC in serum of control. Also (P value < 0.001) were found significant between (BPH with drug and without drug) and (PC with high and low dose chemotherapy).

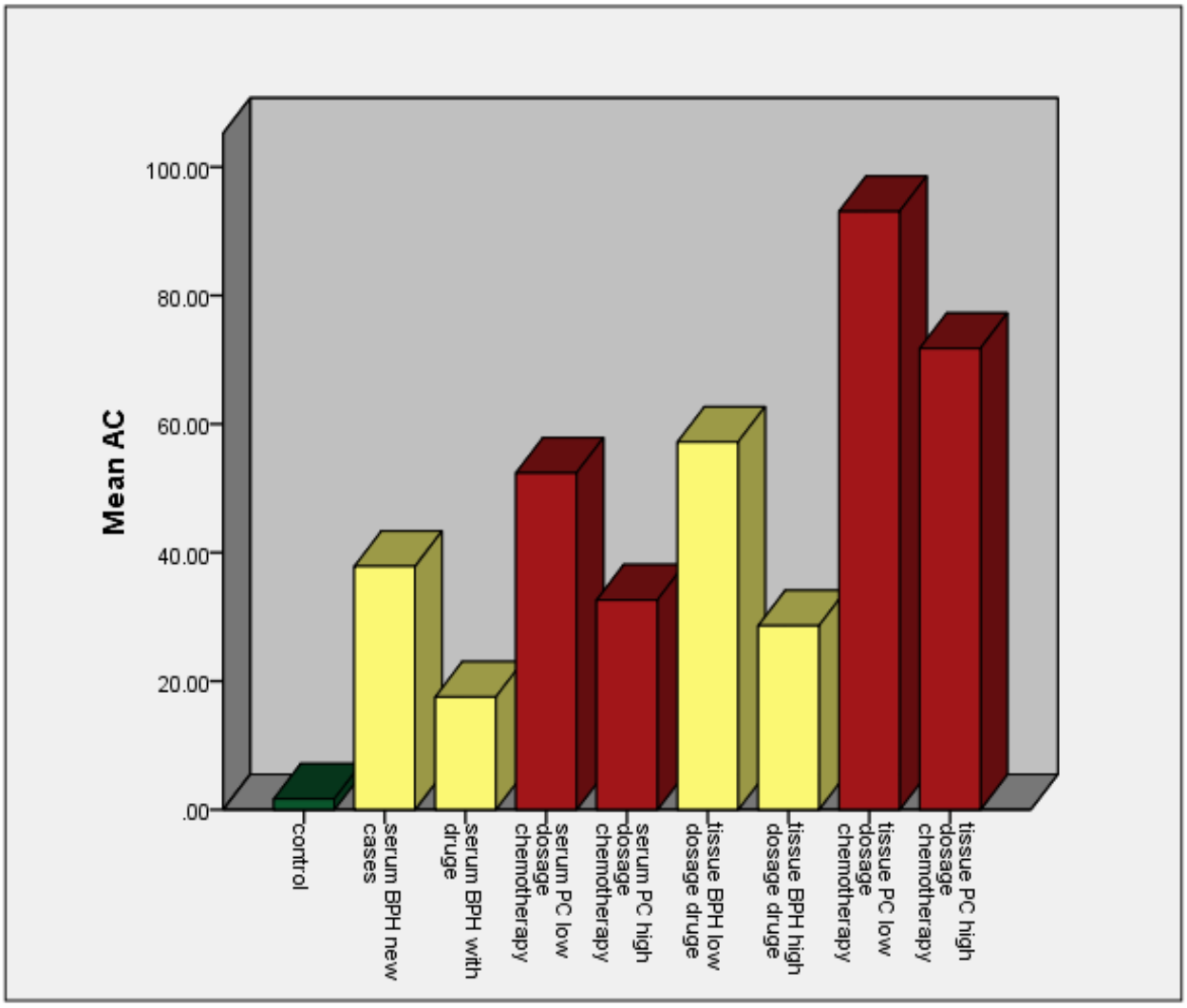

Figure (3.22) AC concentration (ppb) levels in men serum and tissue control, Benign Prostatic Hyperplasia with low and high doses of drugs and Prostatic Cancer with low and high doses of chemotherapy. As statistical SPSS v. 20.

3.3 Concentration of Sphingosine-1- Phosphate (S1P) in Serum and Tissue

Elisa technique was used to estimate concentrations of S1P serum and tissue Figure (3.23) shows the standard curve of S1P in serum and tissue.

The (Mean ± SD) of S1P in serum and tissue had shown an increase in the patients with BPH and malignant prostate cancer in comparing to the control group tables (3-3).

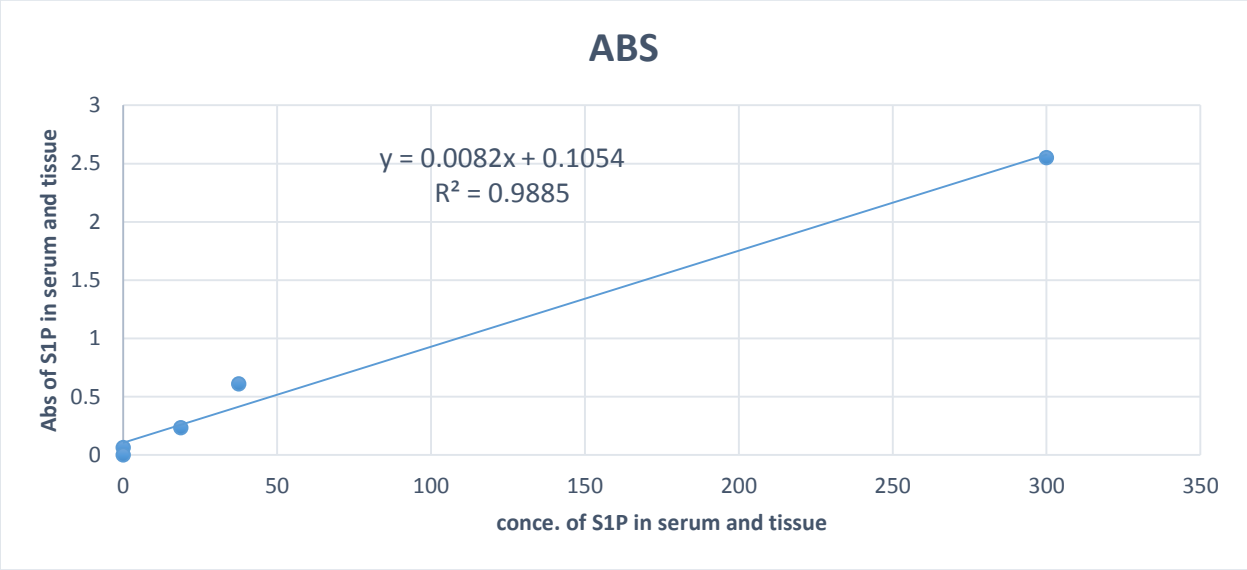

Figure (3.23) Standard Curve of S1P in Elisa Kit in Serum and Tissue.

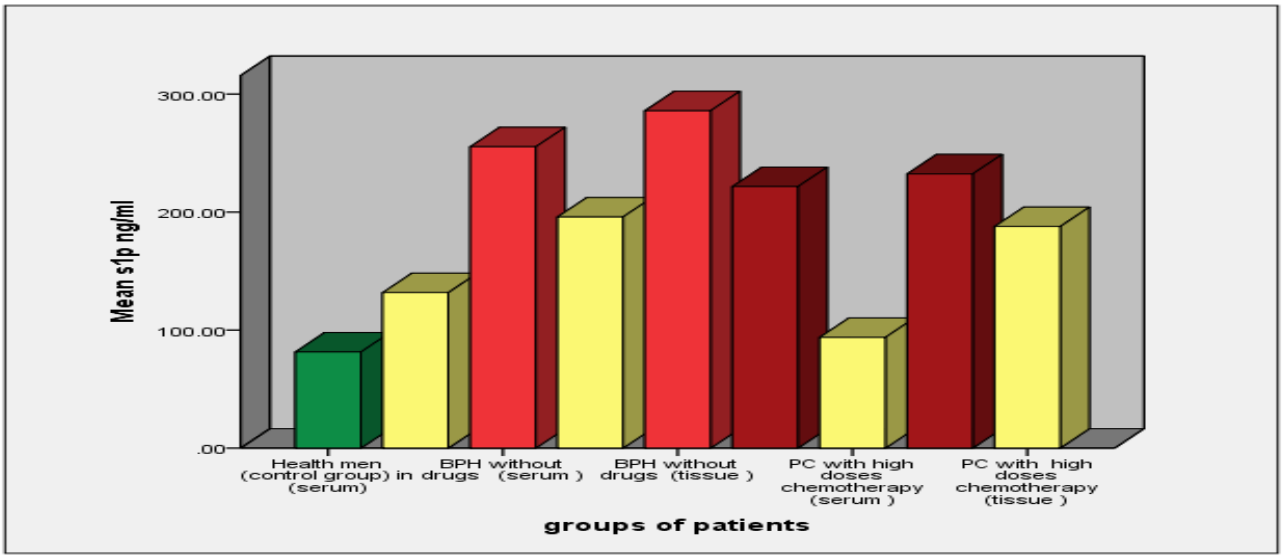

Figure (3.24) Concentration of S1P (ng/ml) in serum and Tissue in BPH, PC, and matched with the control.

Table (3-3) Mean ± SD of S1P levels in patient's serum and tissue with prostate Cancer, BPH and control.

Groups	Mean of peak area ± SD for serum groups ng/ml	P value between serum groups	Mean of peak area ± SD for tissue groups ng/ml	P value between tissue groups
Control	**81.46 ± 3.1**			
BPH without drugs	**131.78 ± 5.1**	**P<0.001**	**195.87 ± 1.1**	**P<0.001**
BPH with drugs	**255.36 ± 3.4**		**285.87 ± 3.2**	
Prostatic cancer low doses chemotherapy	**221.62 ± 7.5**	**P<0.001**	**232.37 ± 3.4**	**P<0.001**
Prostatic cancer high doses chemotherapy	**93.82 ± 0.10**		**187.75 ± 0.8**	

According to (P value) (P < 0.001), there were significance difference between the Mean of S1P in serum and tissue of patients and Mean of S1P in serum of control. Also (P value < 0.001) were found significant between (BPH with drug and without drug) and (PC with high and low dose chemotherapy).

3.4 Concentration of Ceramide (Cer) in Serum and Tissue

The result show that elisa technique was used to estimate serum and tissue concentrations of Cer this Figure **(3.25)** show standard curve of Cer in serum and tissue respectively

The (Mean ± SD) of cer. in serum and tissue had shown an increase in the patient with BPH and malignant prostatic cancer in comparison to that of control group as in tables (3-4). Figure (3.26) shows concentration of Ceramide (pg/ml) in serum and t/*issue in BPH, PC, and matched with healthy men.

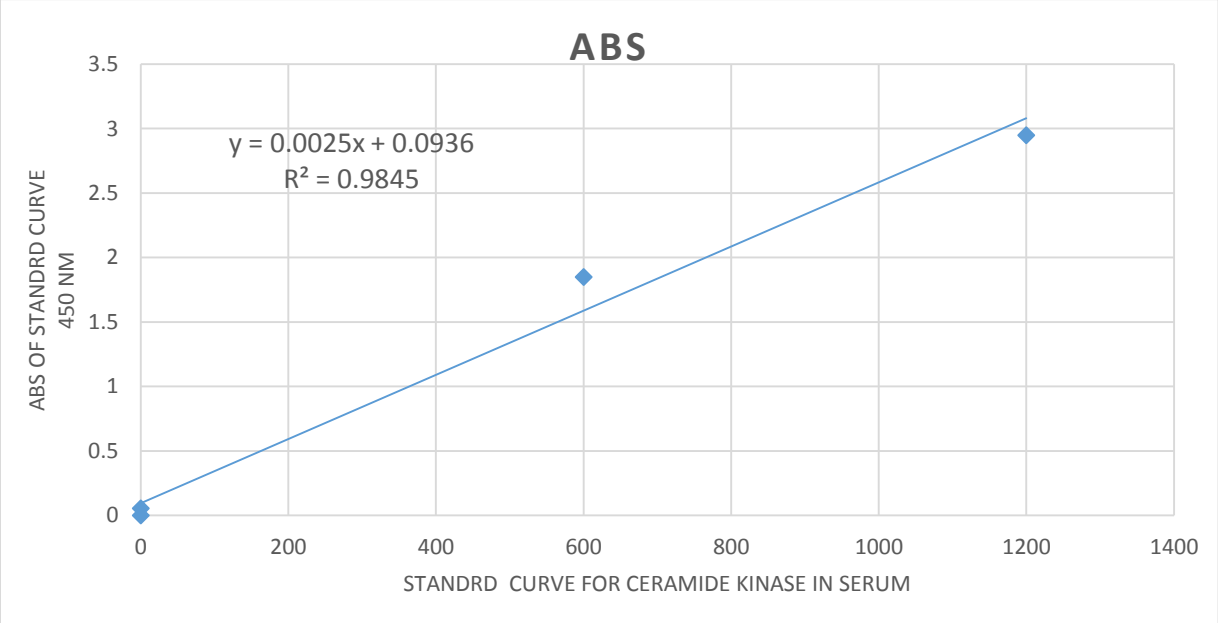

Figure (3.25) Standard Curve of Ceramide in Elisa Kit in Serum and Tissue

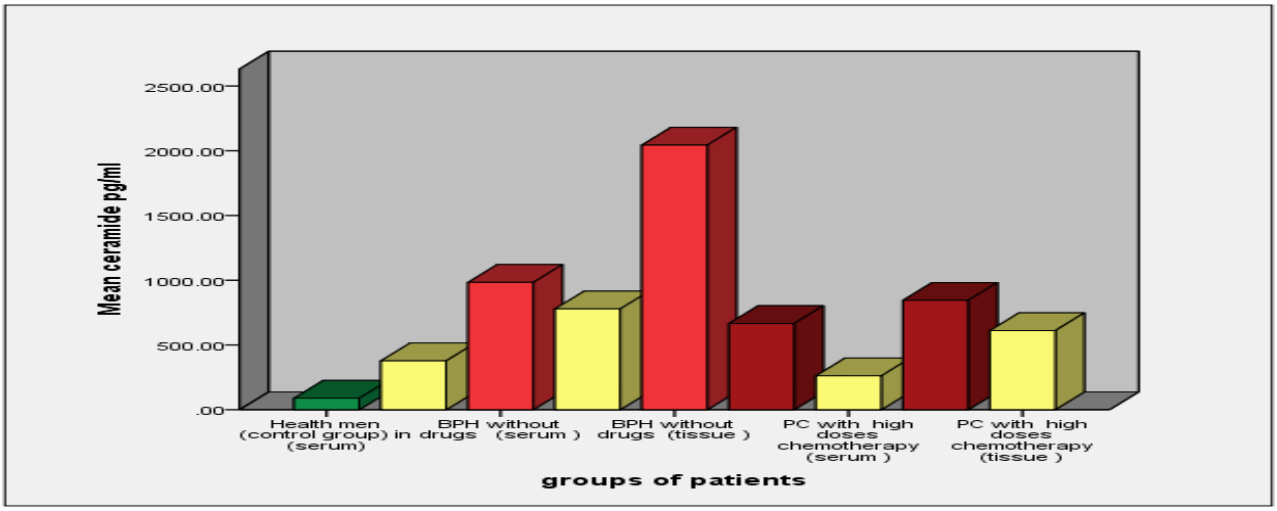

Figure (3.26) concentration of Ceramide (pg/ml) in serum and Tissue in BPH, PC, and matched with Healthy Men

Table (3-4) Mean ± SD of Cer levels in men Serum and Tissue with Prostatic Cancer, Benign Prostatic Hyperplasia and Healthy Men

Groups	Mean of peak area ± SD for serum groups pg/ml	P value between serum groups	Mean of peak area ± SD for tissue groups pg/ml	P value between tissue groups
Control	**88.73 ± 4.3**			**P<0.001**
BPH without drugs	**377.50 ± 2.0**	**P<0.001**	**778.25 ± 1.0**	
BPH with drugs	**983.50 ± 2.0**		**2043.62 ± 4.0**	
Prostatic cancer low doses chemotherapy	**665.62 ± 2.2**	**P<0.001**	**844.87 ± 1.0**	**P<0.001**
Prostatic cancer high doses chemotherapy	**261.50 ± 9.0**		**610.87 ± 8.0**	

According to (P value) (P < 0.001), there were significance difference between the Mean of Cer in serum and tissue of patients and Mean of Cer in serum of control. Also (P value < 0.001) were found significant between (BPH with drug and without drug) and (PC with high and low dose chemotherapy).

3.5 concentration of Prostatic Specific Antigen (PSA) in Serum and Correlation with SPL and AC

Figure (3.27) shows serum concentration of PSA in PC, BPH, and controls. Patients with PC had significantly high serum conce. of PSA than both of BPH and control as Table in appendix (5.5).

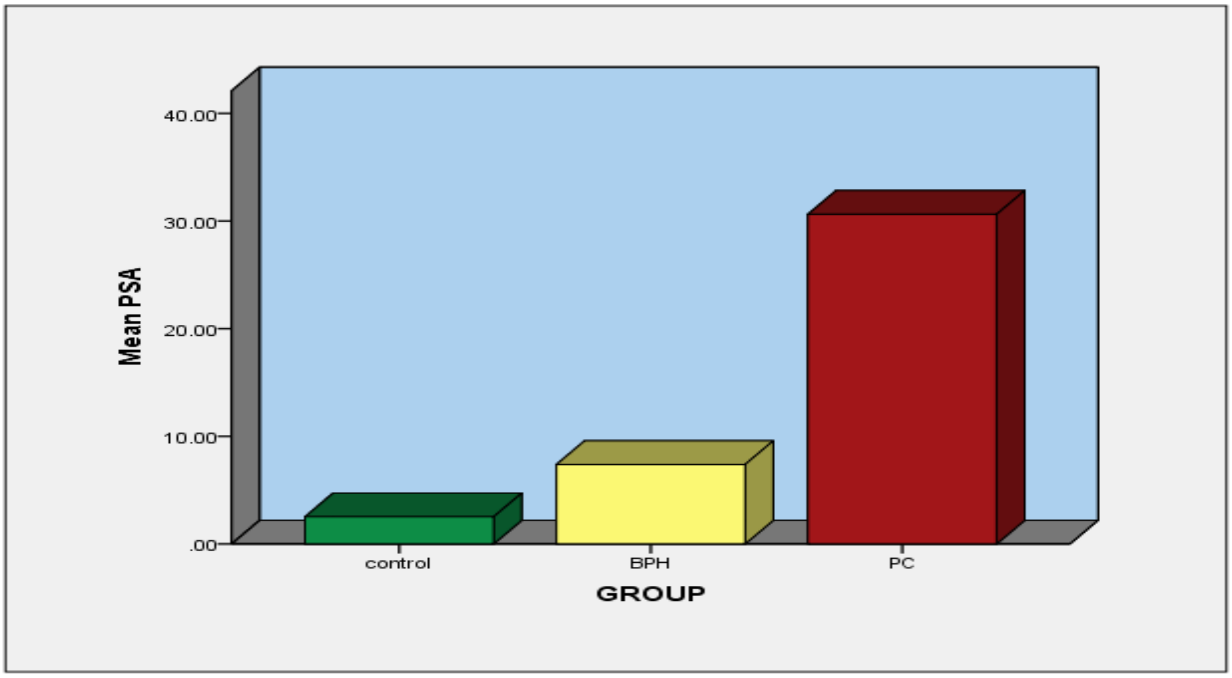

Figure (3.27) Concentration of PSA (ng/ml) in serum for BPH, PC, and matched with Healthy Men.

3.6 Correlation between AC with SPL

In this study it was been found that there is a negative correlation and significant between AC and SPL. This mean that the increase in AC leads to decrease the level and activity of SPL. That mean the level of S1P increase and hexadensal and ethanol aldehyde end products and low released out of cell to serum as shown in Figure (3.28).

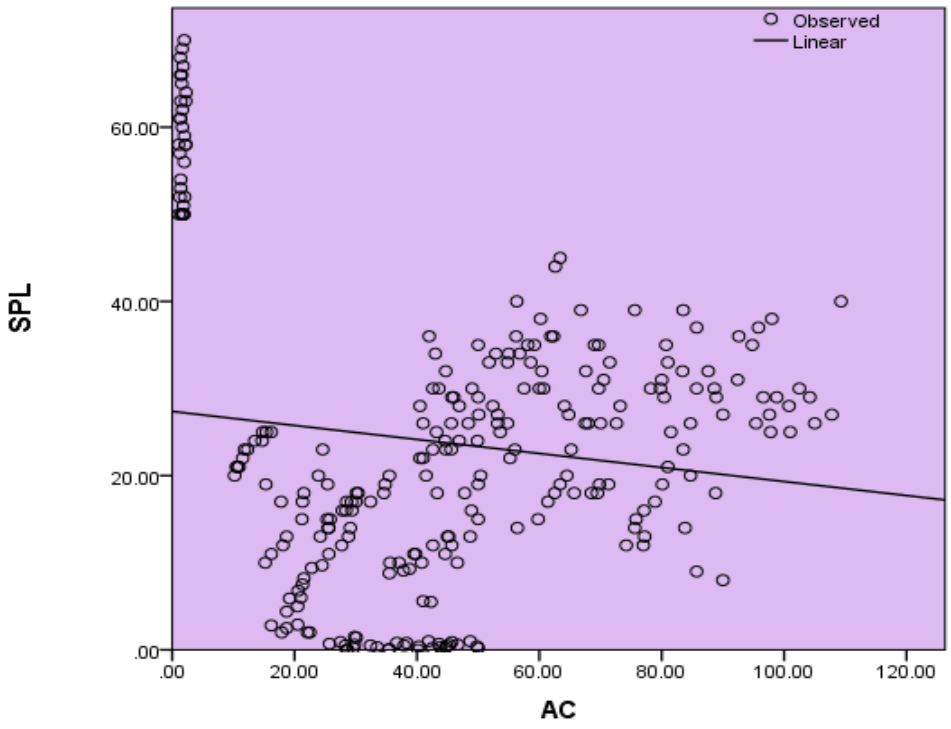

Figure (3.28) correlate between AC enzyme and SPL enzyme

3.7 Measurement of Protein in Tissue in BPH and PC

According to Brad ford assay was measured protein in tissue in patients with BPH and PC as figure (3.29).

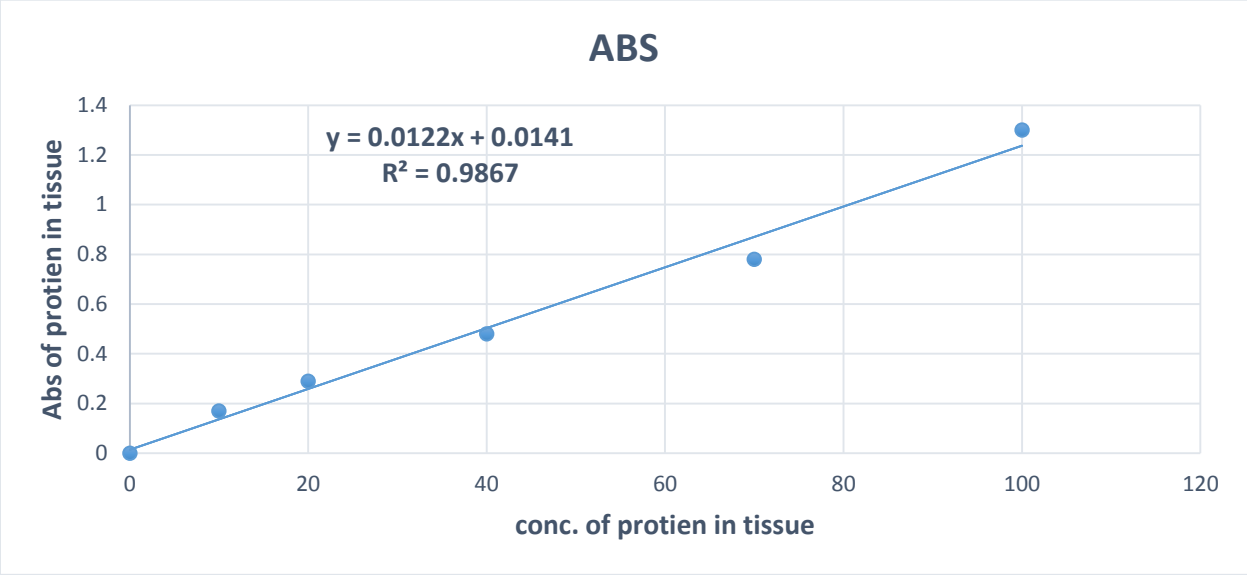

Figure (3.29) Standard curve of protein concentration

3.8 Effect of BMI with Prostatic Cancer:

BMI of three groups (control, BPH, and PC) the measurements of body fat based on height and weight show in figure (3.30) shows that BMI is higher in BPH group than other two groups.

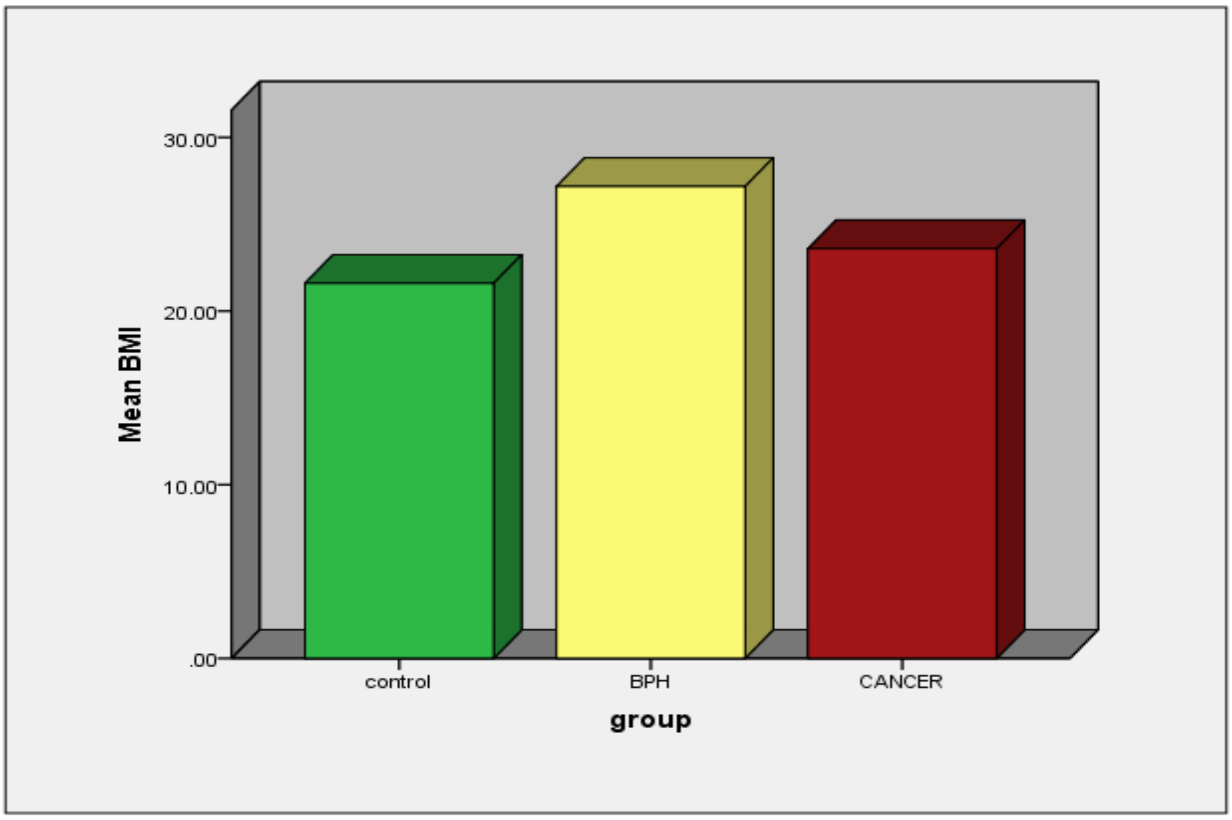

Figure (3.30) BMI for BPH, PC, and Control group

3.9 The Activity Measurement of SPL Enzyme in Serum and Tissue

Activity of SPL was measured by HPLC fluorescence detector in three groups (control, BPH and PC) in serum and tissue. When enzymatic activity was studying the optimal time of incubation (time) and velocity (nmoles x liter^{-1} x min^{-1}) was found the linearity up to 3h and enzymatic reaction was inhibited by drugs, chemotherapy and disease in figures (3.31), (3.32), (3.33), (3.34), (3.35), and figures (3.36) and (3.37) shows mixed noncompetitive inhibitors with enzyme in serum and tissue and data show in table (3-5), (3-6), (3-7), (3-8), and (3-9).

Table (3-5) Km and V_{max} of SPL enzyme By HPLC Fluorescence Detector for Healthy men

No.	[S] µl	V (µmoles / time)	1/ [S]	1/ V	-1 /Km	Km	1/Vmax	V max
1	100	25	0.010	0.04	-0.0005	0.0002	2000	5000
2	150	37.5	0.006	0.02				
3	200	50.1	0.005	0.02				
4	250	62.6	0.004	0.01				
5	300	75.1	0.003	0.01				
6	350	87.6	0.002	0.01				

Table (3-6) Km and V_{max} of SPL enzyme By HPLC Fluorescence Detector for Serum BPH

No.	[S] µl	V (µmoles / time)	1/ [S]	1/ V	-1 /Km	Km	1/Vmax	V max
1	100	0.45	0.010	2.22	-0.0016	0.2	625	5
2	150	0.67	0.006	1.4				
3	200	0.9	0.005	1.11				
4	250	1.12	0.004	0.89				
5	300	1.35	0.003	0.74				
6	350	1.57	0.002	0.63				

Table (3-7) Km and V_{max} of SPL enzyme By HPLC Fluorescence Detector for Tissue BPH

No.	[S]	V (µmoles / time)	1/ [S]	1/ V	-1 /Km	Km	1/Vmax	V max
1	100	12.5	0.010	0.07	-0.0025	400	0.013	76
2	150	18.75	0.006	0.053				
3	200	25	0.005	0.04				
4	250	31.2	0.004	0.032				
5	300	37.5	0.003	0.026				
6	350	43.7	0.002	0.023				

Table (3-8) Km and V_{max} of SPL enzyme By HPLC Fluorescence Detector for Serum PC

No.	[S] µl	V (µmoles / time)	1/ [S]	1/ V	-1 /Km	Km	1/Vmax	V max
1	100	9	0.010	0.11	-0.0012	0.01	833	100
2	150	13.5	0.006	0.07				
3	200	18	0.005	0.06				
4	250	22.5	0.004	0.05				
5	300	27	0.003	0.04				
6	350	31.5	0.002	0.03				

Table (3-9) Km and V_{max} of SPL enzyme By HPLC Fluorescence Detector for Tissue PC

No.	[S] µl	V (µmoles / time)	1/ [S]	1/ V	-1 /Km	Km	1/Vmax	V max
1	100	5	0.010	0.2	-0.002	0.015	500	66
2	150	7.5	0.006	0.13				
3	200	10	0.005	0.1				
4	250	12.5	0.004	0.08				
5	300	15	0.003	0.07				
6	350	17.5	0.002	0.06				

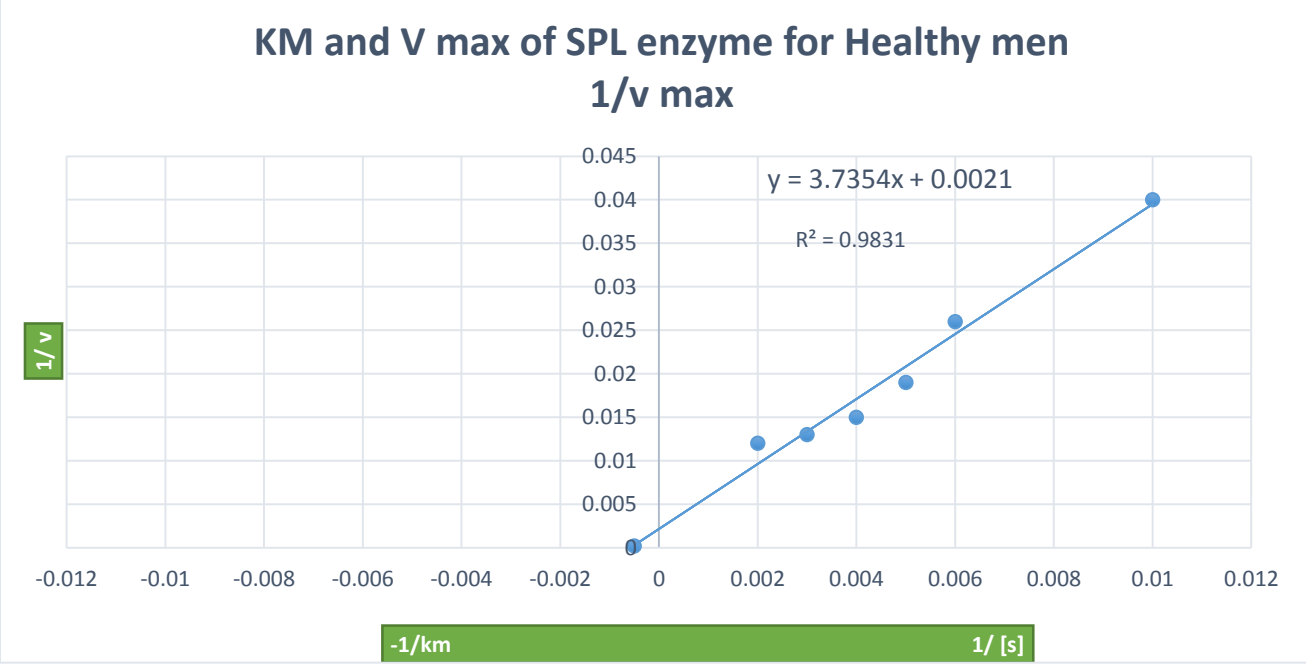

Figure (3.31) Line weaver-Burk Plot show Activity SPL in serum healthy Men

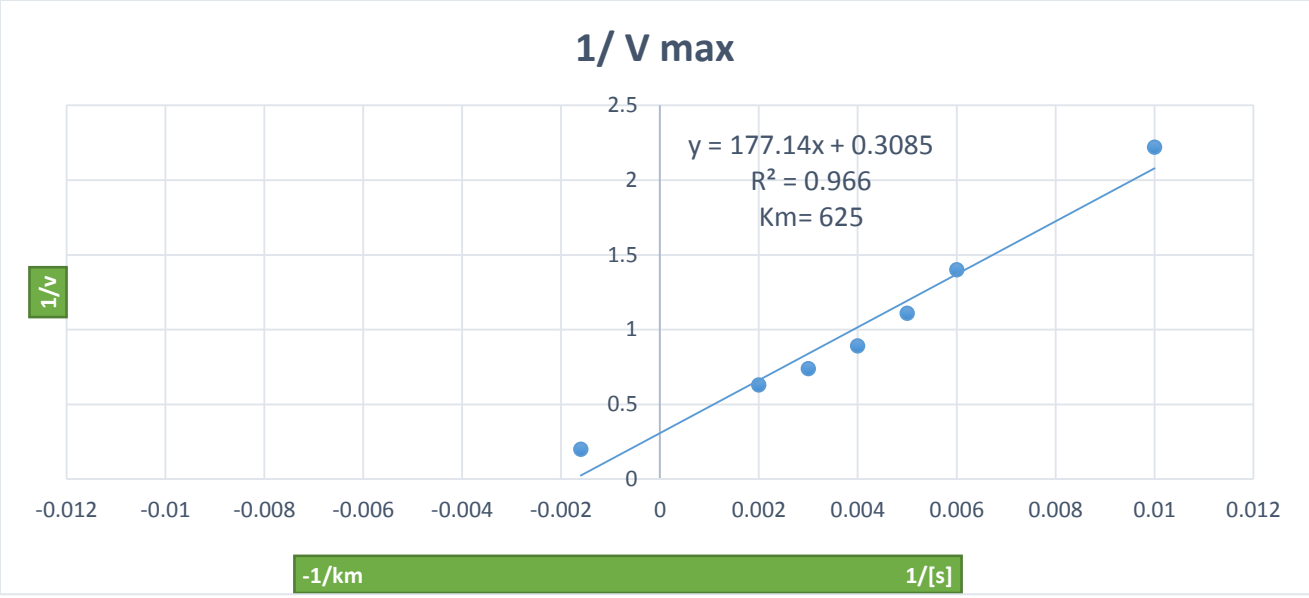

Figure (3.32) Line weaver-Burk Plot show Activity SPL in serum BPH

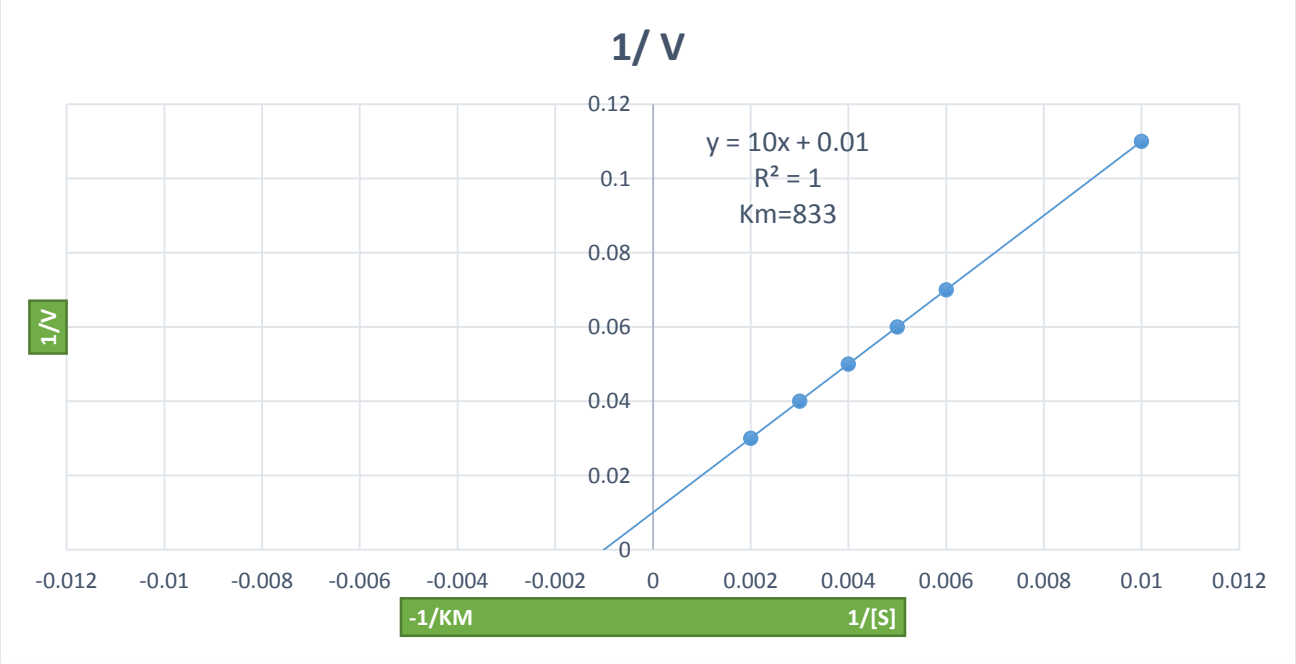

Figure (3.33) Line weaver-Burk Plot show Activity SPL in serum Prostatic cancer

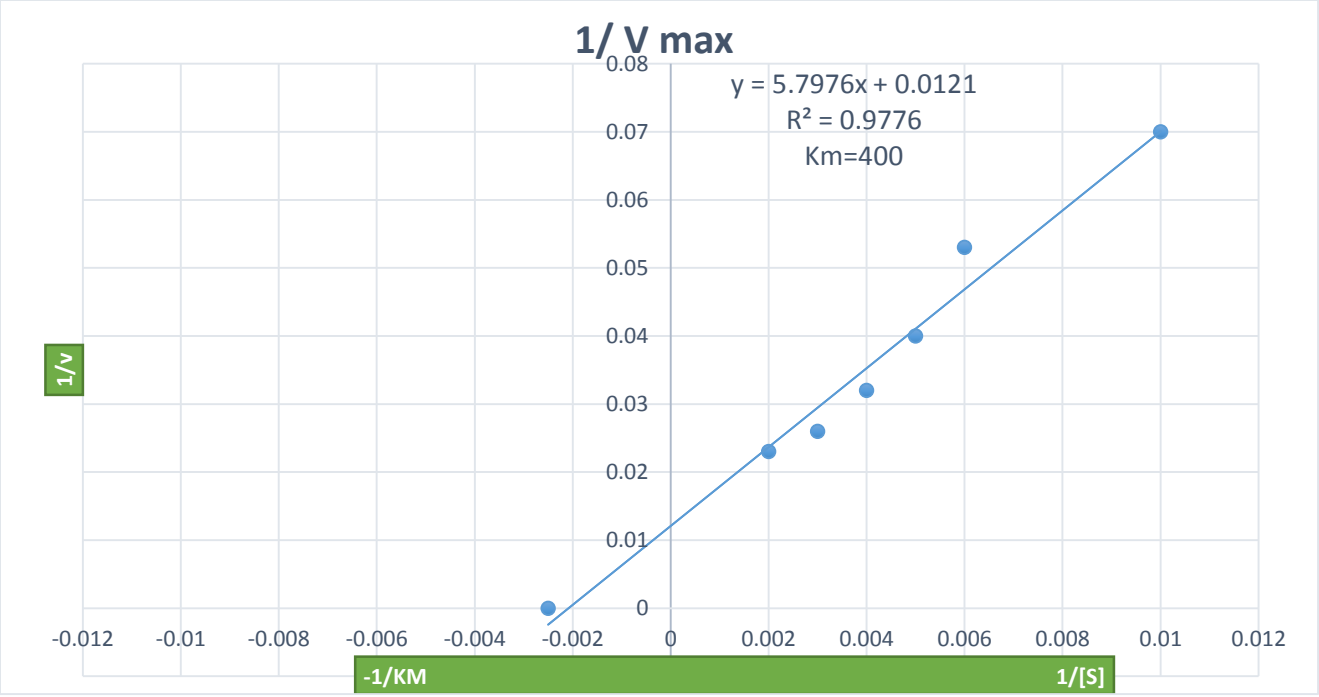

Figure (3.34) Line weaver-Burk Plot show Activity SPL in Tissue BPH Prostate

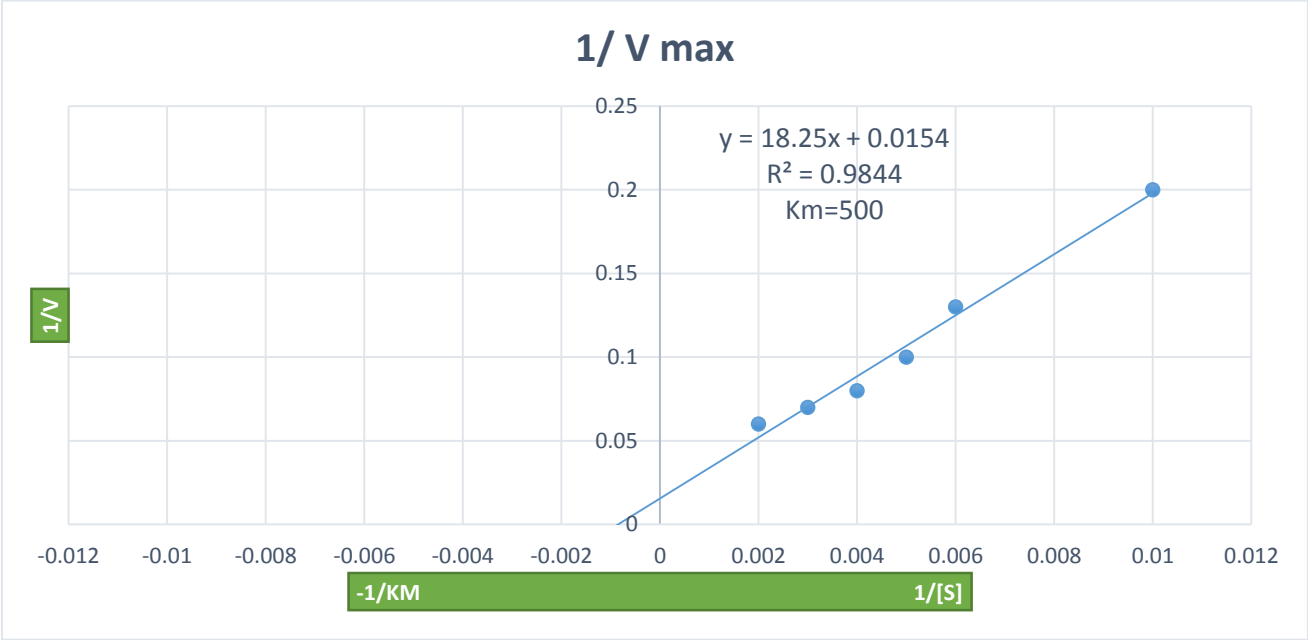

Figure (3.35) Line weaver-Burk Plot show Activity SPL in Tissue Prostatic cancer

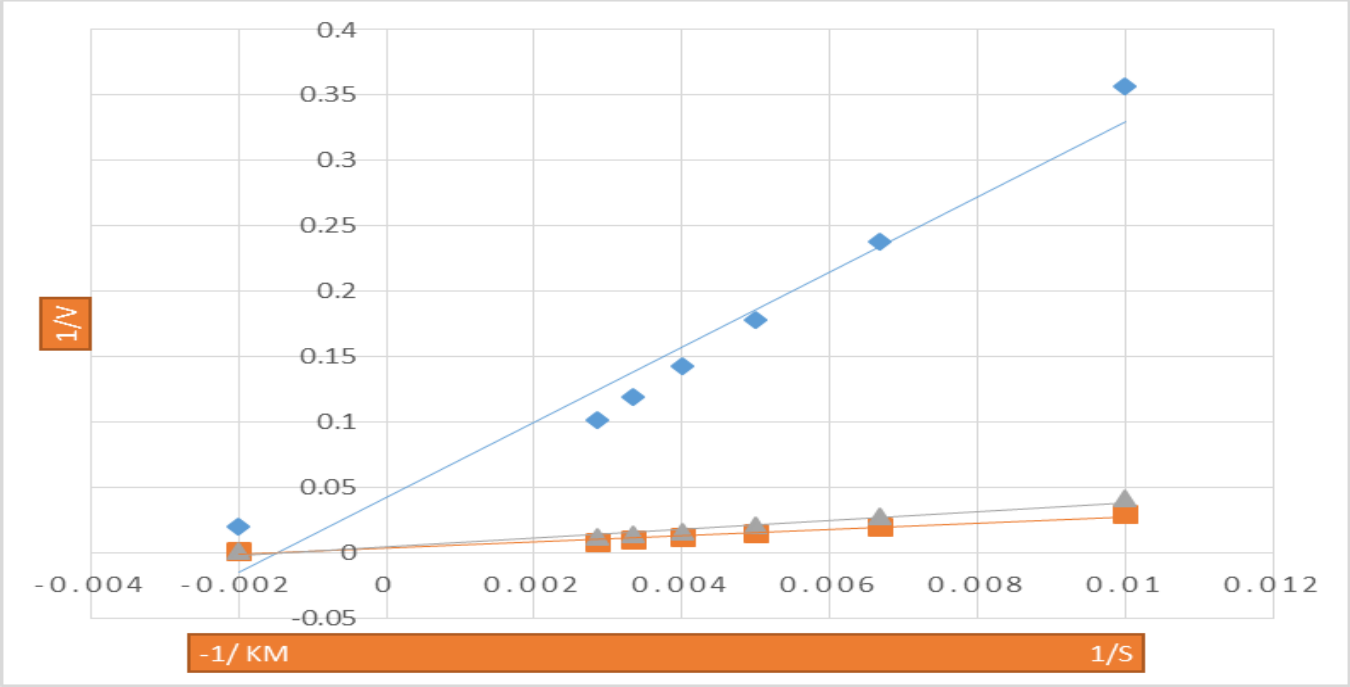

Figure (3.36) Line weaver-Burk Plot show activity SPL mixed noncompetitive inhibition in serum (control, BPH, and PC).

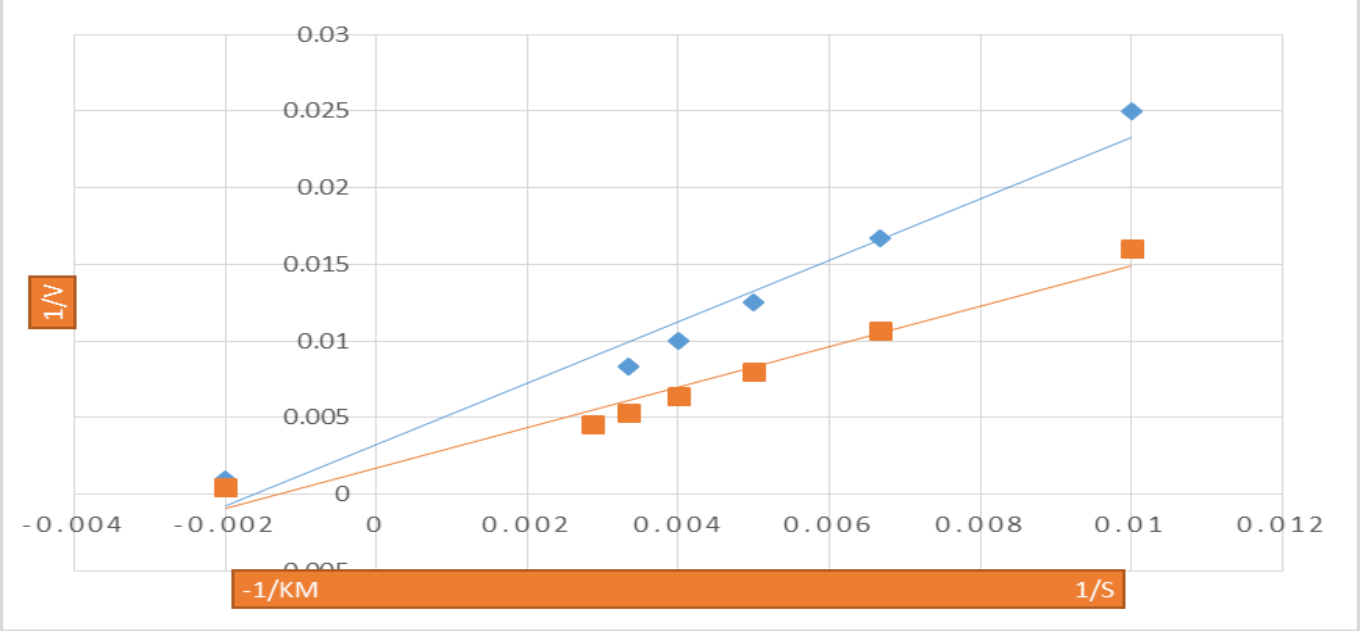

Figure (3.37) Line weaver-Burk Plot show activity SPL mixed noncompetitive inhibition in tissue (BPH and PC).

3.10 Measurement Activity of AC Enzyme in Serum and Tissue

Activity of AC was measured by HPLC fluorescence detector in serum healthy men, BPH and Prostatic cancer in serum and tissue. When enzymatic activity was studying the optimal time of incubation (time) and velocity (ppb) was found the linearity up to 4 h and enzymatic reaction did not affect the production of HPLC fluorescence but the enzymatic reaction was inhibited by drugs, chemotherapy and disease as figures (3.38), (3.39), (3.40) ,(3.41), (3.42), and figures (3.43) and(3.44) shows mixed noncompetitive inhibitors with enzyme in serum and tissue and data show in table (3-10),(3-11),(3-12),(3-13),and (3-14).

Table (3-10) Km and V_{max} of AC enzyme By HPLC Fluorescence detector for serum Healthy men

No.	[S] µl	V (µmoles / time)	1/ [S]	1/ V	-1 /Km	Km	1/Vmax	V max
1	100	1.0	0.010	1.000	-0.0018	0.06	555.6	16.7
2	150	1.4	0.006	0.714				
3	200	1.8	0.005	0.555				
4	250	2.2	0.004	0.454				
5	300	2.6	0.003	0.384				
6	350	3.0	0.002	0.333				

Table (3-11) Km and V_{max} of AC enzyme By HPLC Fluorescence detector for serum BPH

No.	[S] µl	V (µmoles / time)	1/ [S]	1/ V	-1 /Km	Km	1/Vmax	V max
1	100	19.5	0.010	0.0512	-0.001	0.003	1000	333.3
2	150	29	0.006	0.0344				
3	200	39	0.005	0.0256				
4	250	48.7	0.004	0.0205				
5	300	58.4	0.003	0.0171				
6	350	68.1	0.002	0.0146				

Table (3-12) Km and V_{max} of AC enzyme By HPLC Fluorescence detector for tissue BPH

No.	[S] µl	V (µmoles / time)	1/ [S]	1/ V	-1 /Km	Km	1/Vmax	V max
1	100	32	0.010	0.0312	-0.00025	0.003	4000	333.4
2	150	48	0.006	0.0208				
3	200	64	0.005	0.0156				
4	250	80	0.004	0.0125				
5	300	96	0.003	0.0104				
6	350	112	0.0028	0.0089				

Table (3-13) Km and V_{max} of AC enzyme By HPLC Fluorescence detector for serum PC

No.	[S] µl	V (µmoles / time)	1/ [S]	1/ V	-1 /Km	Km	1/Vmax	V max
1	100	28	0.010	0.0357	-0.0008	0.001	1250	1000
2	150	42	0.006	0.0238				
3	200	56	0.005	0.0178				
4	250	70	0.004	0.0142				
5	300	84	0.003	0.0119				
6	350	98	0.002	0.0102				

Table (3-14) Km and V_{max} of AC enzyme By HPLC Fluorescence detector for tissue PC

No.	[S] µl	V (µmoles / time)	1/ [S]	1/ V	-1 /Km	Km	1/Vmax	V max
1	100	43.1	0.010	0.0232	-0.001	0.006	1000	1666.6
2	150	64.2	0.006	0.0155				
3	200	86	0.005	0.0116				
4	250	107.8	0.004	0.0092				
5	300	129.3	0.003	0.0077				
6	350	151	0.002	0.0066				

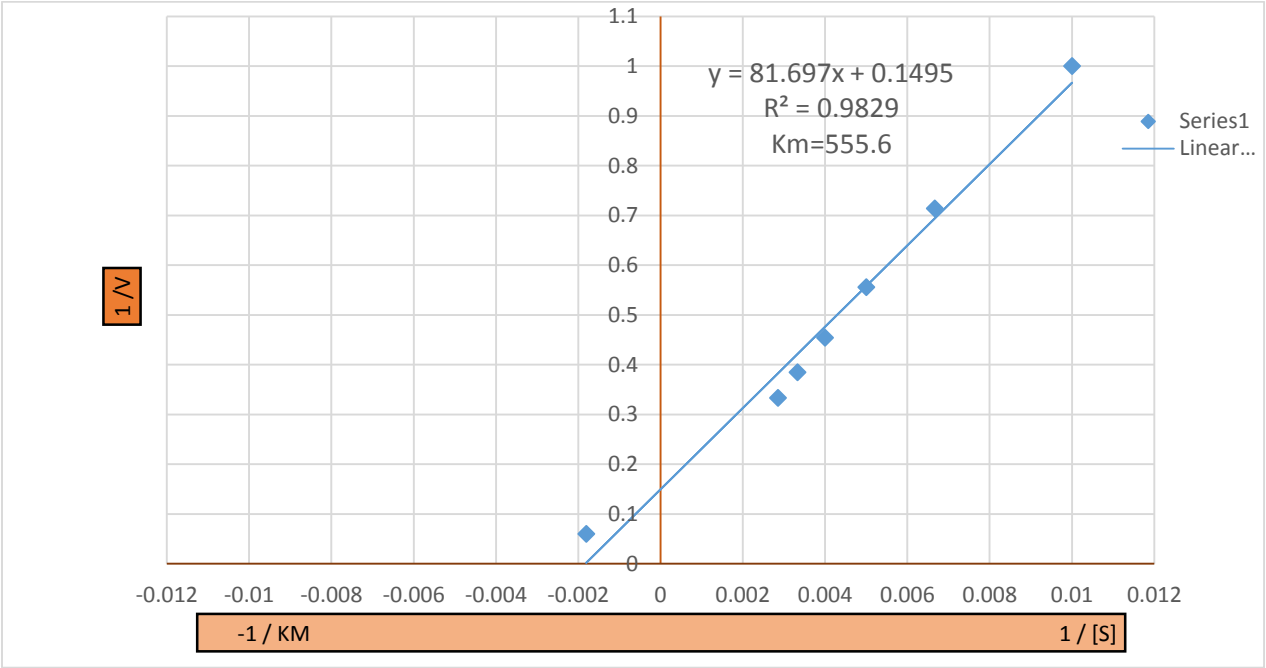

Figure (3.38) Line weaver-Burk Plot show Activity AC in serum healthy Men

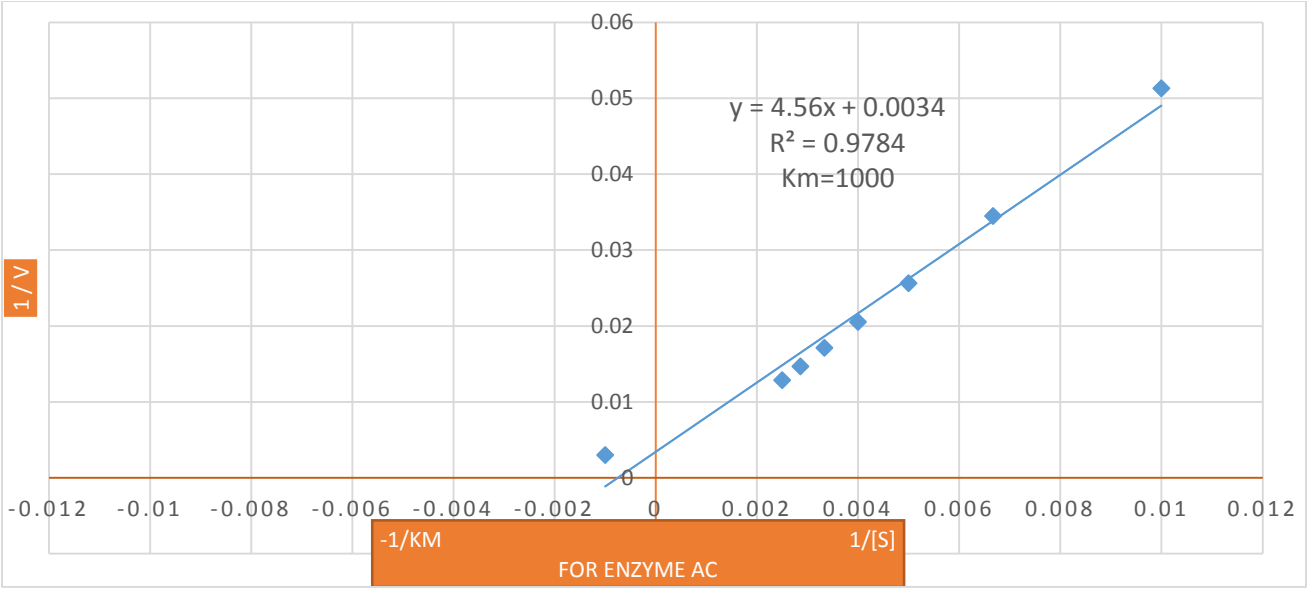

Figure (3.39) Line weaver-Burk Plot show Activity AC in serum BPH

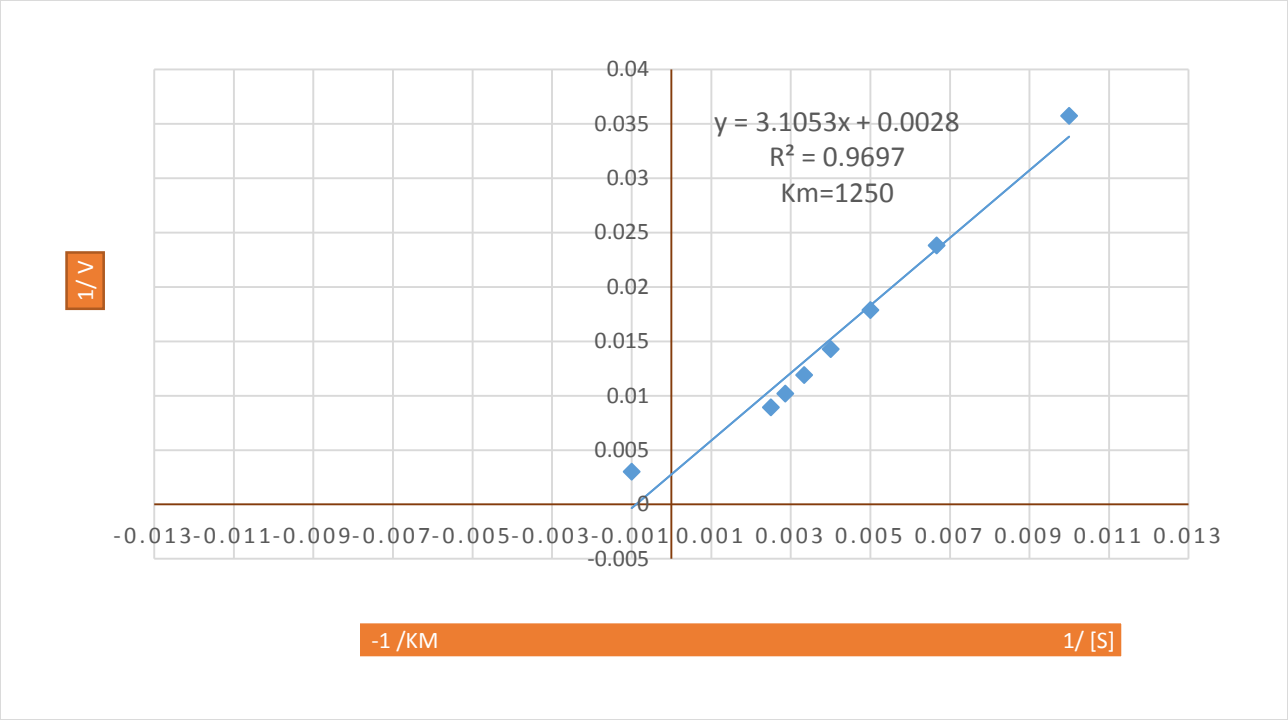

Figure (3.40) Line weaver-Burk Plot show Activity AC in serum Prostatic cancer

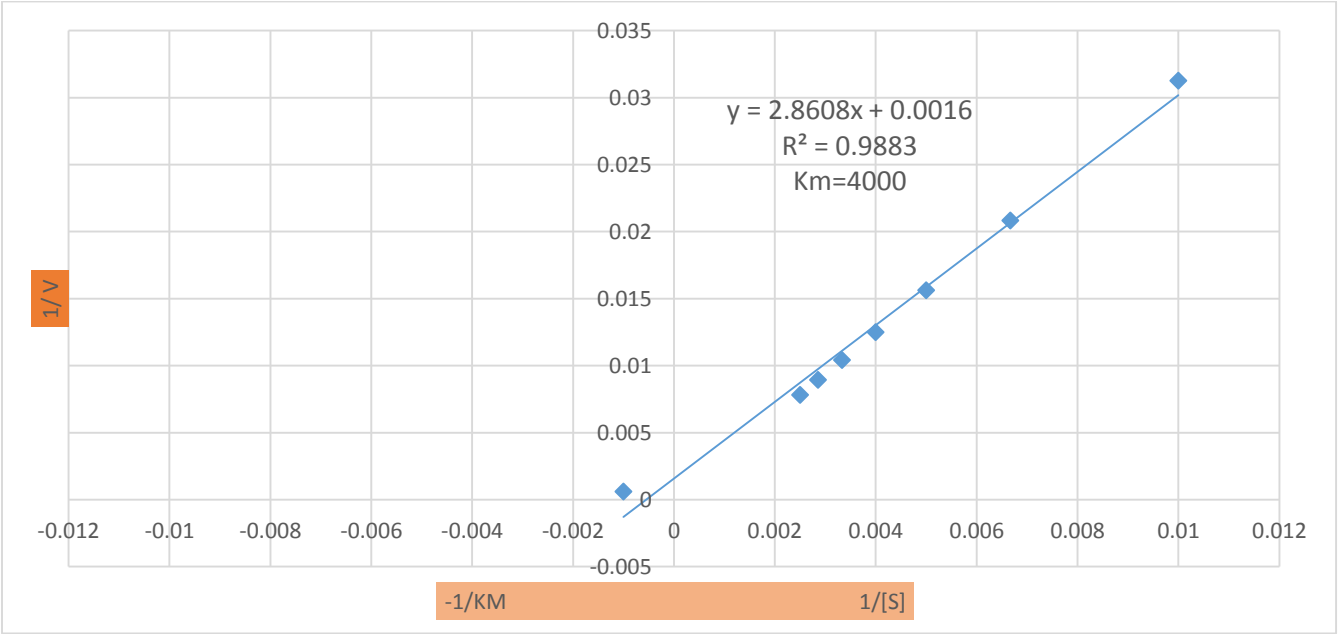

Figure (3.41) Line weaver-Burk Plot show Activity AC in tissue BPH

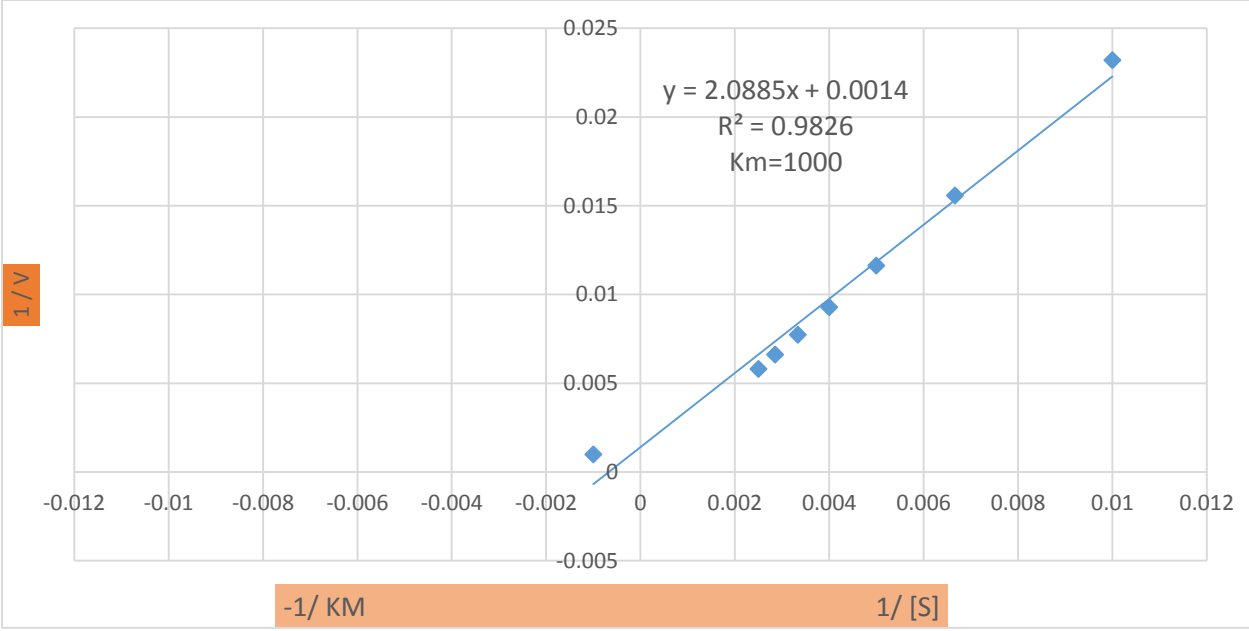

Figure (3.42) Line weaver-Burk Plot show Activity AC in tissue Malignant Prostate

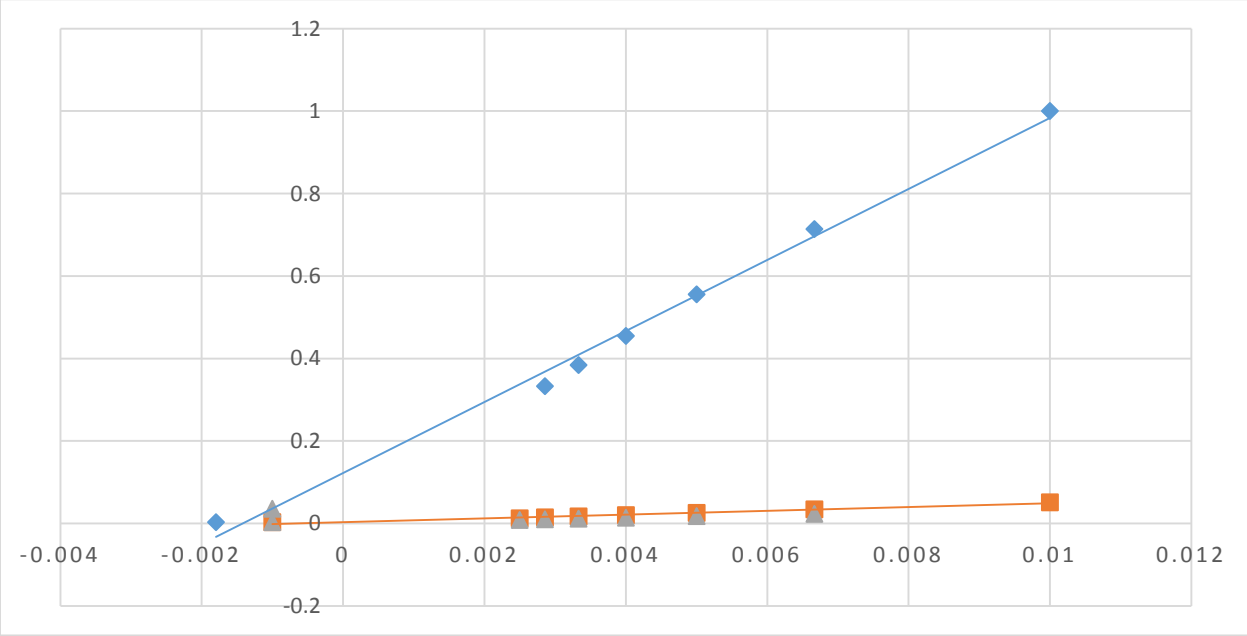

Figure (3.43) Line weaver-Burk Plot show activity AC mixed noncompetitive inhibition in Serum (control , BPH, and PC).

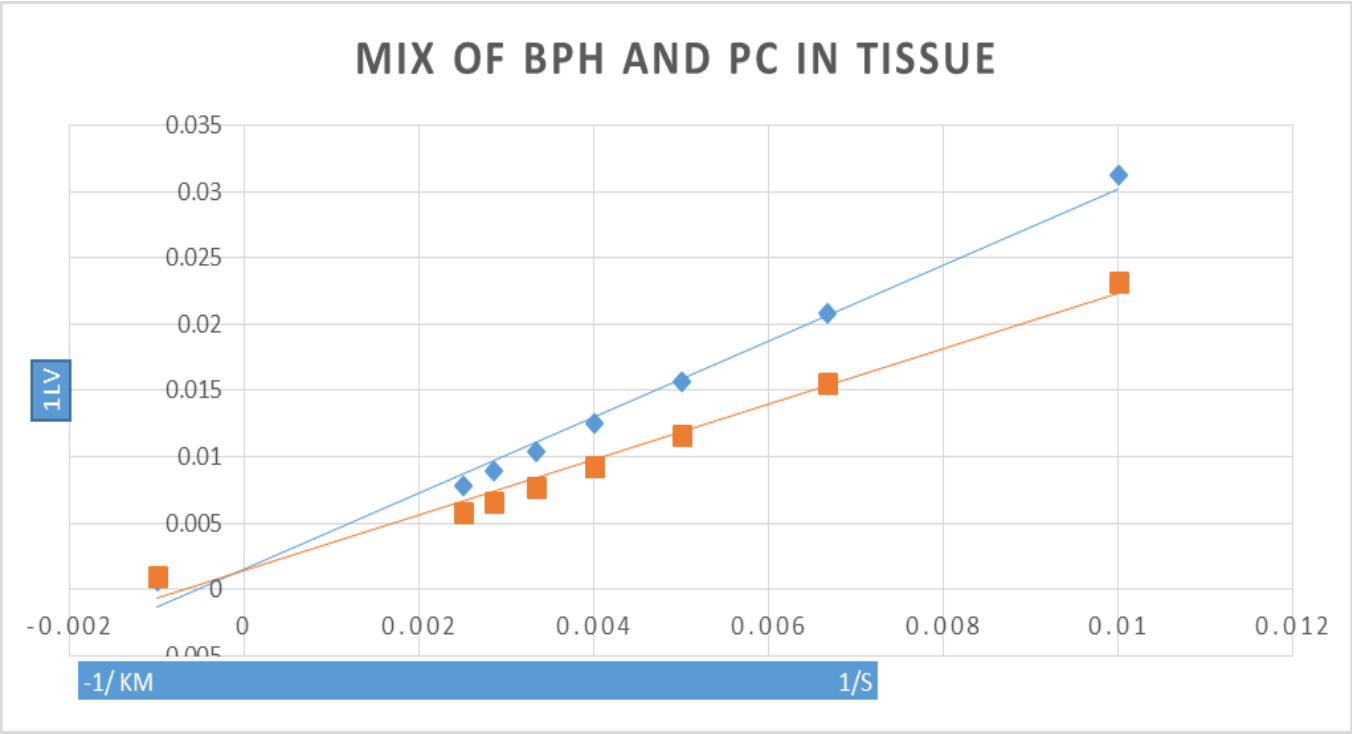

Figure (3.44) Line weaver-Burk Plot show activity AC mixed noncompetitive inhibition in tissue (BPH and PC).

In chapter four we were discussed these results with patients and controls.

Chapter Four

Discussion

4.1 Concentration of (Sphingosine 1 Phosphate Lyase) in Serum and Tissue

BODIPY as aromatic substrate using effectiveness of enzyme (SPL) was measured by HPLC fluorescence detector. The BODIPY- SPL is a stable compound that contains a fluorescent group attached to the terminal carbon of the long-chain base D- erythro-sphingosine (ω- BODIPY -SPL). The ω-linked BODIPY group would not substantially hinder the SPL reaction and that the products retaining the BODIPY label would be simple to monitor by HPLC and fluorescence detection. Accurate determination of SPL activity using the standard assay necessitates exposing an autoradiogram for 2 weeks to locate and scrape the tritium-labeled reaction products from a thin-layer chromatography plate (Zhang, Z.; Sun, 2012).

Additional time is often required to purify commercially available substrate, to avoid these difficulties, we have devised an SPL enzyme assay using a BODIBY-labeled fluorescent substrate that is effectively catabolized by SPL, yielding a fluorescent product that can be separated from substrate by lipid extraction and detected by separation with HPLC using a C18 column in a reaction that can be completed in 1 day this method makes a new and more potent fluorescent substrate are developed the SPL assay will be adapted to increase sensitivity and ability to employ substrates relevant to different species (Walsh, Ryan 2012). The mixture (materials, buffers and fluids of patients such as (serum and tissue) into three groups (control, BPH, and PC) were injected by HPLC fluorescence detector and appearance the many of peaks overlapping resulting from reaction of enzyme with substrate and Pyrodoixal 5- phosphate cleavage of phosphorylated sphingoid base at (C2 - C3) bond yielding a long chain aldehyde and ethanolamine products, the major phosphorylated sphingoid base in humans is S1P which is synthesized in

most cell types and circulates in lymph and blood at high concentrations (Venkataraman K, et al., 2008).

HPLC analysis of BODIPY labeled SPL products. Chromatogram showing separation peaks as of (1) BODIPY–sphingosine, (2) BODIPY–S1P, and (3) and (4) suspected product peaks of the SPL catalyzed. BODIPY–S1P and a small amount of unreacted BODIPY–sphingosine were detected in the final substrate preparation by HPLC with retention times of (7.0) and (7.5) min, respectively (padmavathi bandhuvula, et al., 2009).

Using this method the retention time was found to be (7.54) min. Usually (RT) has one peak, however two or more peaks may be obtained in some enzymatic reactions. These peaks findings indicate that one major product peak was observed in the fluorescence assay, with additional peak constituting a significantly smaller portion of the total product aldehyde and ethanolamine. This is accordance with results study in America by (Zaiguo Li ,et al., 2009) who found the final substrate preparation by HPLC with retention times of 7.0 and 7.3 min such as our study the final RT was (7.56 min) Similarly, Spain study found one suspected product peak (peak 1) was identified at 7.4 min (Alexander D. Borowsky, Padmavathi Bandhuvula, et al., 2012). These studies applied in human and mice prostate using HPLC fluorescence detector and cell line in RNA and DNA genetic studies to measure activity of SPL enzyme. The current study showed significant activity decreased in serum and tissue concentration of SPL enzyme in BPH without drugs and PC with low doses chemotherapy (Gault, C.R.2012). Accumulation of S1P in thymocyte from S1Plyase deficient of prostate and subsequent , however cannot prevent apoptosis induced by increased ceramide Therefore the inter conversion of S1P and ceramides may be more relevant for cell fate decision than the total amounts of these metabolites present in cells, and this may well be adapted by

cancer cells to increase survival levels then S1P lyase cannot converts S1P to ethanolamine phosphate , aldehyde and hexadecenal which then released in serum. The low concentration of SPL in serum PC patients with low doses of chemotherapy (zoladex and Taxotere) may be due to chemotherapy is recently been approved to treat metastatic hormone refractory PC and the level of S1P in tissue much more than realized in serum. In contrast, there was significant absence or decreased concentration of SPL in serum and tissue level in BPH taking drugs like prostate care (the drug used for the treatment of symptomatic BPH), because the action of this drug inhibition activity of SPL enzyme therefore less of released ethanolamine and aldehyde in serum (Eblen, S.T 2012). Also result shows low concentration of SPL in serum and tissue PC patients with high doses of chemotherapy because low level of androgen led to shrinking of prostate and low level of SPL enzyme in tissue and realized products in serum. Figure (3.2) shows concentration of SPL in serum of control and appear the high concentration and highly activity of SPL. Figure (3.3) shows concentration of SPL in serum of BPH without drugs (absence and decreased) concentration of SPL that due to accumulation high concentration of S1P in prostate cancer cell S1P to ethanolamine phosphate and aldehyde and hexadecenal then realized in serum. Figure (3.4) shows appear low concentration of SPL in BPH taking drugs because shrinking in volume of prostate and inhibition activity of SPL and less of realized ethanolamine and aldehyde in serum. Figure (3.5) shows low concentration of SPL in PC patients who take low doses of chemotherapy (zoladex and Taxotere) because chemotherapy is recently been approved to treat metastatic hormone refractory PC and the level of SPL in tissue much less than realized in serum. Figure (3.6) shows low concentration of SPL in serum PC patients with high doses of chemotherapy because low level of androgen led to shrinking of prostate and low level of SPL enzyme in tissue realized products in serum. Figure (3.7) shows

the low concentration of BPH patients in tissue with low dose drugs and Figure (3.8) shows low concentration of BPH patients in tissue with drugs to the same affected in Figure (3.3) and (3.4). Figure (3.9) show the low concentration of SPL levels in tissue PC with low doses of chemotherapy and figure (3.10) shows the low concentration of SPL level in tissue of PC patients with high doses of chemotherapy. Control comparing with these results considered as were very high results, also (mean and SD) there were significant difference between groups (control, BPH, and PC) in serum and tissue (Liu, J.; Ye, C.; 2013).

Figures (3.31),(3.32),(3.33),(3.34),and (3.35) shows the activity of SPL enzyme was measured in HPLC fluorescence detector in (serum and tissue) to three groups (control, BPH, and PC) using Line weaver-Burk equation applied mathematical alteration in the process by plotting double inverse of substrate concentration and reaction rate (velocity) and measured (average of 30 BPH, average of 30 PC, and average of controls) in serum and tissue for Km ,V max and rate reaction are liner that mean the an SPL enzyme amount of substrate converted to product per unit time under specific reaction conditions for pH and temperature these figures shows that high Km and Vmax (high affinity to binding enzyme with substrate and release product comparing with patients (BPH and PC) the results were found very low Km and Vmax (low affinity to binding enzyme with substrate due to low release product) and accumulation substrate as cancer cell. Figure (3.36) and (3.37) shows the mixed of three groups (control, BPH, and PC) in the same plot and appeared that inhibition of SPL enzyme by drugs in BPH and by chemotherapy in PC is known as noncompetitive inhibition, so the substrate and inhibitor bind to different parts of the SPL enzyme molecule. V max changes because effect enzyme is being removed from the reaction and Km was not changed this value is constant of effect enzyme concentration for 30 patients BPH in (serum and tissue) and in 30

patients PC in (serum and tissue). (P value) between control group and all groups were significant and (P value) between also two groups (BPH with and without drugs in serum and tissue) (PCa with high and low doses chemotherapy in serum and tissue) also founded significant (Hannun, Y.A,2014).

4.2 Concentration of Acid Ceramidase in Serum and Tissue

Based on the important roles of ceramide and sphingosine in regulating cell growth, AC is one of the key enzymes regulating sphingolipid metabolism. In the cell, AC is located in the lysosomes and functions metabolized ceramide which forming sphingosine and a fatty acid. Sphingosine can be phosphorylated by two different sphingosine kinases, SphK1 and SphK2 forming sphingosine 1-phospate (S1P). (Saddoughi SA, et al., 2008). Because Ceramide degradation is the only catabolic source of intracellular sphingosine (Rother J.et al., 1992). AC activity may be the rate-limiting step in determining the intracellular levels of sphingosine, and subsequently, S1P Contrary to SPL the RT of AC was found to have one peak (14.99 min). This is agreement with study of (Midori Yano, et al., 1998) and agreement with study of (Xingxuan He. Et al., 1999) in Israel but RT (7.83) different in our study RT (14.99) because the flow rate in this study was 1ml/min also in our study at flow rate (0.6) ml/min.

The study revealed significant high concentration of AC in serum and tissue for BPH patients without taking drugs and PC with low doses of chemotherapy. This is indicating that enlargements of prostate in both BPH and PC leads to direct proportion to enzyme activity, this is because depending on cell type, developmental stage and physiological state. Abnormal expression of AC has been reported in several human cancers; for example PC and melanomas. AC contribute to decrease the levels of ceramide and increase those of S1P, thereby resulting in resistance to cell death and enhancement of cell proliferation. (Gemma fabrias

doming and Gemma M.N., 2011). AC inhibition induced apoptosis. Many reports confirm the relationship between AC activity and radio or chemotherapy resistance, as well as the interest of AC inhibitor as anticancer drugs, either alone or in combination with other therapies. (Gemma fabrias doming and Gemma M.N., 2011). Moreover, since AC is one of the key enzymes contributed to regulate sphingolipid metabolism, many thesis focused on this enzyme, based on the fact that inhibitors of AC activity may lead to increased Cer levels and stimulate apoptotic cell death. ceramide and its bioactive metabolites, sphingosine and S1P, represent signaling molecules that act as regulators of cellular life and death. Ceramide signaling functions are catabolically muted by AC at lysosomal pH. Other previous studies have shown that AC is over expressed in 60% of patients with PC compared to patient-matched adjacent tissue. The percentage of tumors overexpressing AC increases with higher Gleason grades.12 The current study demonstrates that when elevated in tumor cells, AC provides a growth advantage for PCa cells, and contributes to the altered balance between proliferation and death leading to tumor progression (Antonio F., Saad1 William D, 2007). This differential regulation of ceramide levels may have important consequences on cell function. This is agreement with many studies which indicate that Ceramide metabolism in tumors is affected by a multitude of signaling mechanisms(Ogretmen et al.,2004), including stress via hypoxia, radiation, heat, viral infection, nutrient deprivation, chemotherapy and receptor-mediated signaling of a variety of growth factors and cytokines. When ceramide is elevated in the cell, it creates a pro-apoptotic phenotype by virtue of its ability to inhibit growth through cell cycle arrest and/or induction of apoptosis.(Norris .J S. 2006).

In contrast there was a decrease in concentration of AC in serum and tissue for BPH patients with drugs and PC with high doses of chemotherapy. This result

because the action of drugs as inhibitors to the enzyme AC. Several studies have suggested that the inhibition of AC activity may serve cell apoptosis in response to various stressful stimuli. In Figure (3.13) shows concentration of AC in control that is mix BODIPY- Ceramide with buffer and serum of control. Figure (3.14) shows high concentration of AC in BPH patients (serum) without taking drugs but Figure (3.15) shows low concentration of AC in BPH patients (serum) with drugs that is mean Acid Ceramidase increased convert Ceramide to S1P in prostate. Figure (3.16) shows high concentration of AC in PC with low doses of chemotherapy because enlargements of prostate cancer and AC response to increase convert Ceramide to sphingosine 1 phosphate and realized in serum but Figure (3.17) shows low concentration of AC in PC with high doses of chemotherapy because AC is response of tumor therapy and progression of cancer. Figure (3.18) shows high concentration of AC in BPH tissue without drugs and Figure (3.19) shows low concentration of AC in BPH tissue with drugs show that AC was appeared in cell lines cancer tissues to contribute to decreased the levels of Ceramide and increased of S1P thereby resulting to cell cancer death and enhancement of cell proliferation. Figure (3.20) shows high concentration of AC in tissue with low doses of chemotherapy and Figure (3.21) shows low concentration of AC in tissue with high doses of chemotherapy to the same reason in figure (3.18 and 3.19).

The area under peak for serum and tissue for three groups (control, BPH, and PC) at same conditions of standard show mean and SD were significant. Figures (3.38),(3.39),(3.40),(3.41),and (3.42) shows the activity of AC enzyme was measured in HPLC fluorescence detector in (serum and tissue) to three groups (control, BPH, and PC) using Line weaver-Burk equation applied mathematical alteration in the process by plotting double inverse of substrate concentration and reaction rate (velocity) and measured and measured (average of 30 BPH, average

of 30 PC, and average of controls) in serum and tissue for Km ,V max and rate reaction are liner that mean the an AC enzyme amount of substrate converted to product per unit time under specific reaction conditions for pH and temperature. Figure (3.43) and (3.44) shows the mixed of three groups (control, BPH, and PC) in the same plot and appeared that inhibition of AC enzyme by drugs in BPH and by chemotherapy in PC is known as noncompetitive inhibition, so the substrate and inhibitor bind to different parts of the AC enzyme molecule. V max changes because effect enzyme is being removed from the reaction and Km was not changed this value is constant of effect enzyme concentration for 30 patients BPH in (serum and tissue) and in 30 patients PC in (serum and tissue). These are result is accepted with other studies in America and Spain that use AC as inhibitors induced apoptosis as anticancer drugs or in combination with other therapies. (P value) between control group and all groups were significant and (P value) between also two groups (BPH with and without drugs in serum and tissue) (PCa with high and low doses chemotherapy in serum and tissue) also founded significant. Many tumor types express high levels of acid ceramidase (AC).

Specifically, the expression levels of AC in prostate cancer have been reported to be elevated relative to normal prostate tissue Prostate cancer (PC) is the most prevalent neoplasia in men in industrialized nations. Although PC is frequently initially sensitive to hormonal deprivation therapies and follows indolent clinical courses, a significant proportion of cases eventually become resistant to such therapeutic approaches, accompanied with aggressive growth, establishment of metastasis, and tumors that are highly resistant to conventional chemotherapeutic regimes. Two major challenges in PC are to find predictive markers that identify those tumors most likely to follow a hormone independent, aggressive clinical course as aids to decide early intervention and to identify molecular targets for

improved therapies of castration resistant cases that respond poorly to conventional chemotherapeutic regimes. Here, we provide new evidence to reinforce the notion that the acid ceramidase ASAH1 is a valid therapeutic target in advanced prostate cancer, and we characterize new potent and specific inhibitors of AC (Yap T. A., Zivi A., Omlin A., de Bono J. S. 2011).

4.3 Concentration of Sphingosine-1- Phosphate (S1P) in Serum and Tissue

ELISA technique was employed to measure serum and tissue levels of S1P. Mean serum and tissue level of S1P was shown to significant increase in patient BPH without drugs and PC with low doses chemotherapy and decrease in patients with drugs and high doses chemotherapy. The S1P concentrations are highest in plasma than tissue for all groups, this is due to the activity of the S1P-lyase which is predominantly expressed by tissue cells. This is agreement with Christina-Maria Reimann et, al 2015) which demonstrated The differences produce concentration gradients between the circulatory system and peripheral tissues which are important to induce lymphocyte egress from lymphoid organs and to maintain lymphocyte circulation (Gräler MH, 2004). The S1P levels are regulated by the relative complement of enzyme activities in a cell's sphingolipid metabolic pathway. Even S1P is formed in most cells, but it is then degraded irreversibly by intracellular SPL or dephosphorylated by S1P kinase. The erythrocytes play role in contributed the high plasma level of S1P. Free S1P or S1P bound to serum albumin is more susceptible to degradation than S1P bound to lipoproteins such as high-density lipoprotein (HDL). This indicates that various serum protein partners might have a role in determining the uptake and intracellular degradation of S1P thereby regulating serum levels of S1P. Inhibition of SPL activity results in a marked

increase in the level of S1P, particularly in the tissues (Pappu R, et al 2007). This study together with references 5 and 34, shows the dependency of lymphocyte egress into blood on a gradient of S1P from tissue (low) to blood (high) and on lymphocyte S1PR1. It also shows that plasma S1P is mostly generated by erythrocytes. The role of S1P in angiogenesis (the formation of new blood vessels) is another key area. This involves endothelial-cell differentiation and increased motility}mitogenesis of smooth muscle cells, which may be influenced by lipoproteins, such as oxidized LDL (Rathinasamy, A., N.et al.,2010). Several reports of the angiogenic role of S1P have been published. Leucocyte migration is also affected by S1P, which may play a role in inflammation. For example, S1P inhibits neutrophil motility and modulates adhesion molecule expression.

Additionally, the relative levels of sphingosine and S1P in mast cells may determine, in part, their allergic responsiveness. Sphingosine 1-phosphate (S1P) acts as a chemoattractant in the blood and lymph at concentrations in the hundred-nanomolar range (Rosen, H., and E. J. Goetzl. 2005). S1P promotes lymphocyte egress from lymphoid organs (Czeloth, N., G.2005) and, as revealed through studies using S1P agonists and S1P receptor knockout mice, regulates migration of mature DCs from skin or lung to draining lymph nodes in vivo (Gollmann, G., H 2008). Subsequently S1P and ceramides as the closest sphingosine metabolites came into the focus as potential mediators of sphingosine related effects (although sphingosine itself still has unique physiological functions apart from ceramide and S1P signaling. Ceramide evolved as a proapoptotic molecule, while S1P was regarded as a prosurvival factor. These opposing physiological functions of the two closest metabolites of sphingosine shaped the concept of the sphingolipid rheostat. It postulates that the intracellular balance of ceramide and S1P generation determines cell fate. Increased ceramide production would lead to apoptosis while

increased S1P production would promote cell survival(Bode C, Berlin M, Röstel F, et al, 2014). S1Pinduced autophagy was later on identified as a possible mechanism to promote cell survival and to avoid ceramide induced apoptosis . Cellular production of S1P by SphKs appears to be a key event of this hypothesis, and indeed many publications report increased expression particularly of SphK1 in different tumors. On the other hand, accumulation of S1P in thymocytes of S1Plyase deficient mice did not prevent ceramide induced apoptosis and thymus atrophy, indicating that the sole presence of high amounts of intracellular S1P was not sufficient to rescue them from cell death. In addition specific SphK inhibitors failed to inhibit tumor cell growth and viability, also questioning the relevance of the sphingolipid rheostat concept in cancer. Since intracellular targets of both S1P and ceramide are still not worked out very well, defining relevant intracellular signaling processes for both metabolites will be required to understand this system more thoroughly and to potentially use it for medical interventions (Safarian F, Khallaghi B, Ahmadiani A, et al, 2015).

4.4 Concentration of Ceramide kinase (Cer) as assay to Measure Cellular Levels of Ceramide in Serum and Tissue

In this study it has been developed through examination to assess the Ceramide kinase as assay to measure concentration of ceramide in Serum and Tissue into three groups (control, BPH, and PC) of patients by using Elisa Kit and this Figure (3.25) shows standard curve of Ceramide in serum and tissue, and The (Mean ± SD) of Cer in serum and tissue had shown an increase in the patient with BPH and malignant prostatic cancer in comparing to with control group as in Table (3-4), while appendix (5.4) reverted to the different in Ceramide Concentration between groups of patient themselves-according to division. Membrane sphingolipid, regulators for cell growth, death, senescence, adhesion, migration, angiogenesis,

inflammation, and intracellular trafficking, are bioactive metabolites including sphingosine, ceramide, sphingosine-1-phosphate (S1P), and ceramide-1-phosphate (C1P) (Obeid, L.M. 2013).

Approximately 20% of the population in Western countries suffers from chronic pain syndrome there is a major need for renewed focus on novel targets that will be effective in both neuropathic and inflammatory pain. Compelling evidence implicates Ceramide-to-sphingosine 1-phosphate (S1P) pathways as contributors to pain of diverse etiologies. S1P and its receptors are emerging as important neuronal and immune cell regulators interacting at several sites in the pain pathway. Timely and important to critically evaluate the pharmacological basis for targeting the ceramide-to-S1P pathway as an approach to pain management (Braak M,et al, 2009). (P value) between control group and all groups were significant and (P value) between also two groups (BPH with and without drugs in serum and tissue) (PCa with high and low doses chemotherapy in serum and tissue) also founded significant.

4.5 Concentration of Prostatic Specific Antigen (PSA) in Serum and Correlation with SPL and AC

A relationship between PSA analysis and the Sphingolipids as the levels rise with the onset of the disease when the tumor is benign and patients with malignant prostatic cancer as figure (3.27) and significant. Note that the high concentrations levels of enzymes SPL and AC is much higher in patients were suffering benign and malignant prostatic cancer and these enzymes SPL and AC were sensitive when using HPLC and results were higher than compared with PSA used immunosorbent Elisa Technology.

4.6 BMI Effect with Prostatic Cancer:

Body mass index (BMI). It is widely accepted that a high BMI is associated with an increased risk to health, in particular with regard to hypertension, diabetes mellitus, and ischemic heart disease. There is also growing evidence for a link between body weight and cancer risk. Called and co-workers for example showed a positive linear trend in death rates for all cancers with increasing BMI. Among western men the three most common cancers in order of incidence are prostate, lung, and colon/rectum, these cancers are all considered to be related to body weight, but there is not a clear consensus yet. Obese men have been reported to be at higher risk of colorectal cancer in most but not all studies. Figure (3.30)shows three groups of (control, BPH, and PC) different value of BMI and appear the control and PC is normal value of BMI but BPH much more them and that may risk for prostate cancer previous surveys on the relationship between incident prostate cancer and BMI have been controversial, predominantly no association, or a small increased risk among heavy men was found, the few mortality data, on the other hand, showed an increased risk an elevated risk of prostate cancer associated with lower levels of BMI has been reported in a number of studies. Evidence on body weight and cancer is not straightforward because the cancer process itself may cause loss of weight, even before the cancer is evident (Manson et al. 1987) proposed several approaches to minimize this effect, i.e. careful screening of the patient population at baseline, exclusion of subjects experiencing substantial weight loss in the previous year, and to disregard mortality within the first few years of follow-up. On the other hand, analytic and simulation work by Allison (Elizabeth R. et al, 2015).

4.7 Correlation between AC with SPL

Figure (3.28) shows that found that is negative correlation between AC and SPL that mean even rise level of AC that low level of product of S1P at last rise activity of SPL and lyses of S1P to hexadensal and ethanol aldehyde end products and released out of cell to serum correlation between AC with SPL negative that mean SPL is independent on AC when the activity of SPL is dependent on level of S1P (Clin. In. 2004).

4.8 Measurement Protein in Tissue BPH and PC

High levels of protein in tissue in PC and BPH were shown in figure (3.29) the standard curve of protein in tissue for BPH and PC patients. Prostate cancer is a leading cause of cancer-related death in males and is second only to lung cancer. Result shown S1P functions primarily by activating a sub group of the endothelial differentiation gene (EDG) family of G-protein coupled cell surface receptors now referred to as S1P (1-5) with high affinity and also to dihydro-S1P with similar or slightly lower affinity (Kihara, A., Mitsutake, S., et al(2007). Although effective surgical and radiation treatments exist for clinically localized prostate cancer, metastatic prostate cancer remains essentially incurable. Through gene expression profiling, that the polycomb group protein enhancer of zest homolog 2 (EZH2) is overexpressed in hormone-refractory, metastatic prostate cancer (Doll, F.; Pfeilschifter, J.;2005).

4.9 Measurement Albumin and Creatinine in Serum and Urine Patients with BPH and PC

We were taken selecting patients without kidney problems and depending on their results of Albumin in serum and urine were normal and results of Creatinine in serum and urine were normal.

4.10 Measurement Glucose and HbA1C in Serum Patients with BPH and PC.

We were taken patients without Diabetic problems and depending on their results of Glucose were normal and results of HbA1C in serum were normal.

Chapter Five

Conclusion, Recommendation, Appendix & References

Conclusions:

1. This study suggests the benefit of measuring serum AC and SPL activity to assess BPH and PC by HPLC fluorescence detector. Simply measuring AC and SPL activity along with the cost-effectiveness of giving additional feature to consider AC and SPL as a sign of the tumor marker in the Prostatic cancer.

2. A significant inverse correlation between total SPL and Acid Ceramidase levels with BPH and Prostatic cancer.

3. A significant inverse correlation between total S1P and Ceramide levels with BPH and Prostatic cancer.

4. The study concludes that increased incidence of Prostatic cancer with BMI.

5. Increase the concentration of protein in tissue with BPH and PC and as well as being a marker that distinguishes indolent prostate cancer from those at risk of lethal progression.

6. AC and SPL are mixed noncompetitive inhibition in serum and tissue have same result of (Km) and different result of (V max) for each groups.

Recommendations:

1. Study the relationship between prostate cancer and / or bladder, urethra cancers, especially in severe cases of prostate cancer.

2. Genotyping study of SPL and AC responsible gene to correlate with protein expression with study (EZH2) protein enhancer of zest homolog 2 and (FAPP2) sphingosine transfer protein as a biomarker for prostatic cancer.

3. A follow-up study needs to document that IgE level increases after cancer resection.

4. Cell culture study to assess the usefulness of using Cu, Zn, Se elements as treatment cancer or giving the patient chemotherapy containing selenium.

5. Measure other antioxidant parameter, and study HDL parameter because low level of HDL is related with increase probability of prostatic cancer.

6. Establishment of a specialized center for the detection of early stage prostate cancer and increase weigh.

7. Hormone-related cancers which are numerically the most important, namely, breast, prostate, endometrium, and ovary. Follow- up needs to document that FSH, LH, T3, T4, and TSH increase after cancer reaction, epidemiological evidence also suggests a hormonal role in the pathogenesis of testis cancer, thyroid cancer, and osteosarcoma. We believe that the primary prevention of all these cancers will probably depend on modification of the factors which affect the secretion and metabolism of the responsible hormones rather than on control of exposure to classical exogenous initiators

Appendixes:

Procedures for all Analysis

1- Determination of glucose in serum by using spectrophotometer

PRINCIPLE Glucose is determined after enzymatic oxidation in the presence of glucose oxidase. The hydrogen peroxide formed reacts, under catalysis of peroxidase, with phenol and 4-aminophenazone to form a red - violet quinoneimine dye as indicator.

REACTION GOD Glucose + 02 + H20 gluconic acid + H2O2 POD 2H202+ 4-aminophenazone+phenol quinoneimine + 4H2O.

Sample Blood, serum, heparinized plasma, EDTA plasma. Glucose is stable for 24 hours at +2 to +8 °C if the serum or plasma is prepared within 30 min of collection.

STABILITY AND PREPARATIONS OF SOLUTIONS

1. Buffer Contents ready for use. Stable up to the expiry date when stored at +2 to +8oC.
2. GOD-PAP Reagent Reconstitute the contents of one vial of Reagent 2 with a portion of Buffer 1 and then transfer entire contents to bottle 1, rinsing bottle 2 several times. The working reagent is stable for 3 months at +2 to +8oC or 5 days at +15 to +25oC.
3. Standard Contents ready for use. Stable up to the expiry date at +2 to +8oC.

PROCEDURE FOR GLUCOSE GOD-PAP ASSAY Wavelength:

Wavelength:	**500 nm, Hg 546 nm**
Cuvette:	**1 cm path length**
Temperature:	**15-25oC or 37oC Measurement: against reagent blank**

Pipette into test tubes :

	Blank	Standard	Sample
Standard	**……**	**10 µL**	**……**
Sample	**……**	**…….**	**10 µL**
Reagent buffer	**1000 µL**	**1000 µL**	**1000 µL**

Mix, incubate for 25 min at 15-25oC or 10 min at 37oC. Measure the absorbance of the standard (A standard) and the sample (A sample) against the reagent blank within 60 minutes

CALCULATION :

Glucose concentration (m.mol/l) = (ABS of sample / ABS of standard) × 5.55

Glucose concentration (mg /l) = (ABS of sample / ABS of standard) × 100

2- Determination of urea in serum by using spectrophotometer

PRINCIPLE

The method is based on the following reaction:- urease Urea + H2O 2NH3 + CO2 Salicylate and hypochlorite in the reagent react with the ammonium ions to form a

green complex (2.2 dicarboxylindophenol). SAMPLE Serum, EDTA, citrated or heparinized plasma. Urine diluted 1+20 in distilled water. Multiply the result by 21.

REAGENT COMPOSITION

Contents	Initial Concentrations of Solutions
R1a. Urease	≥ 5000 U/l
R1b. Phosphate Buffer	120 mmol/l, pH 7.0
Sodium Salicylate	63.4 mmol/l
Sodium Nitroprusside	5.00 mmol/l
EDTA	1.5 mmol/l
R2. Sodium Hypochlorite	18 mmol/l
Sodium Hydroxide	750 mmol/l
CAL. Standard	8.33 mmol/l (50 mg/dl)

PROCEDURE

Wavelength:	600 nm, (Hg 578 nm - Hg 623 nm)
Cuvette:	1 cm path length
Temperature:	15-25oC or 37oC Measurement:
Measurement:	against reagent blank

Pipette into test tubes:

	Blank	Standard	Sample
Standard	**……**	**10 µL**	**……**
Sample	**……**	**…….**	**10 µL**

Working Reagent R1	**1000 µL**	**1000 µL**	**1000 µL**

Mix. Incubate for at least 3 min at 37°C or 5 min at 20- 25°C.

R 2	200 µL	200 µL	200 µL

Mix. Incubate for at least 5 min at 37°C or 10 min at 20-25°C. Measure the absorbance of the standard (A standard) and the absorbance of the sample (A sample) against reagent blank within 2 hours.

CALCULATION :

Urea concentration (m.mol/l) = (ABS of sample / ABS of standard) × 8.33

Urea concentration (mg /l) = (ABS of sample / ABS of standard) × 50

3- Determination of creatinine in serum by using spectrophotometer

PRINCIPLE

Creatinine is derived from creatine and creatine phosphate in muscle tissue and may be defined as a nitrogenous waste product. Creatinine is not reutilised but is excreted from the body in the urine via the kidney. It is produced and excreted at a constant rate which is proportional to the body muscle mass. As a consequence of the way in which creatinine is excreted by the kidney, creatinine measurement is used almost exclusively in the assessment of kidney function. Creatinine is regarded as the most useful endogenous marker in the diagnosis and treatment of kidney disease. Creatinine is measured primarily to assess kidney function and has certain advantages over the measurement of urea. The plasma level of creatinine is relatively independent of protein ingestion, water intake, rate of urine production and exercise. Since its rate of production is constant, elevation of plasma creatinine is indicative

of under-excretion, suggesting kidney impairment. Depressed levels of plasma creatinine are rare and not clinically significant. Creatinine in alkaline solution reacts with picric acid to form a coloured complex. The amount of the complex formed is directly proportional to the creatinine concentration.

SAMPLE COLLECTION AND PREPARATION :

Serum or plasma and Urine: diluted 1 + 49 with double distilled water

PREPARATION OF WORKING REAGENT

Mix equal volumes of Solutions 2 + 3. Stable for 3 days at +15 to +25°C.

REAGENT COMPOSITION:

Contents	Initial Concentrations of Solutions
1. Standard	177 mmol/l (2 mg/dl)
2. Picric acid	35 mmol/l Surfactant
3. Sodium hydroxide	0.32 mol/l

Wavelength:	492 (490-510 nm)
Cuvette:	1 cm path length
Temperature:	15-25oC or 37oC Measurement:
Measurement:	against reagent blank

Pipette into test tubes:

	Blank	Standard	Sample
Standard	**……**	**100 µL**	**……**
Sample	**……**	**…….**	**100 µL**
Working Reagent	**1000 µL**	**1000 µL**	**1000 µL**

Mix and after 30 seconds read the absorbance A1 of the standard and sample. Exactly 2 minutes later, read absorbance A2 of standard and sample.

CALCULATION :

A2 - A1 = DA sample or DA standard

Concentration of creatinine in serum

Creatinine concentration = (ABS of sample / ABS of standard) × 177 = µmol/l

Creatinine concentration = (ABS of sample / ABS of standard) × 2 = mg/dl

Concentration of creatinine in urine

Creatinine concentration = (ABS of sample / ABS of standard) × 8.85 = µmol/l

Creatinine concentration = (ABS of sample / ABS of standard) × 100 = mg/dl

4- Determination of Albumin in serum by using spectrophotometer

PRINCIPLE :

In buffered solution at pH 4.2, bromocresol green binds albumin to form a coloured compound which absorbance, measured at 630 nm (620-640) is proportional to the albumin concentration in the specimen.

REAGENT COMPOSITION:

Contents	Initial Concentrations of Solutions
1.Standard(Bovine albumin)	725 µmol/l (5 g/dl)
2.Succinic acid	**83 mmol/L**
3.Bromocresol green (BCG)	**167 µmol/L**
4. Sodium hydroxide	**50 mmol/L**
1. Polyoxyethylene monolauryl ether Preservative	**1.00 g/L**

Wavelength:	630 (620-640 nm)
Cuvette:	1 cm path length
Temperature:	15-25oC or 37oC Measurement:
Measurement:	against reagent blank

Pipette into test tubes:

	Blank	Standard	Sample
Standard	**……**	**10 µL**	**……**
Sample	**……**	**…….**	**10 µL**
BCG Reagent	**2000 µL**	**2000 µL**	**2000 µL**

Mix well. Record absorbance at 630 nm (620-640) within 3 minutes against reagent blank or better after exactly 1 minute.

CALCULATION :

Albumin concentration (g/dl) = (ABS of sample / ABS of standard) × 5

Albumin concentration (µmol /l) = (ABS of sample / ABS of standard) × 725

5- Determination of Albumin in urine by using : Solid phase, sandwich-format, immunometric assay using a gold-antibody conjugate.

Micro albuminuria is defined as a persistent elevation of the **urinary albumin excretion** to 20-200 µg/min or 20-200 mg/L when using early morning urine. Studies have demonstrated that **micro albuminuria** independently predicts cardiovascular morbidity and all-cause mortality in essential hypertension. Monitoring of **micro albuminuria** is worth-while in order to monitor the effect of

anti-hypertensive treatment on target-organ damage Low-protein diets, lowering blood pressure and the use of anti-hypertensive therapy have all been reported to have a positive effect on decreasing the urinary albumin excretion . The DCCT Research Group has shown the importance of strict glycaemic control in preventing **micro albuminuria. NyoCard U-Albumin** is simply quick and convenient - within only 3 minutes aquantitative test result is obtained using the NycoCard® READER

Characteristics

Measuring range: 5-200 mg/L albumin

Sample Material: 50µl urine

Rest principle: Solid phase, sandwich-format, immunometric assay using a gold-antibody conjugate.

Precision: In controlled laboratory testing, a coefficient of variation(CV) of 5-8% is usually obtained.

6- Determination of HbA1C by using : Solid phase, sandwich-format, immunometric assay using a gold-antibody conjugate.

The importance of good metabolic control Several studies have shown the importance of good metabolic control in preventing – and slowing – the progression of diabetic late complications - for person with type I (DCCT study) and type II (UKPSD) diabetes. Measurement of glycated hemoglobin has proven to be an important tool in determining the quality of the metabolic control.

Time-saving for you and your patients

Nyco Card® HbA1c is rapid and provides an accurate HbA1c result within 3 minutes during the patient consultation - making a revisit to alter the treatment regime no longer necessary.

Standardization

The International Federation of Clinical Chemistry (IFCC) has established a working group on glycol hemoglobin standardization. This will be based on HbA1c, as it is the easiest component to define. Methods that measure total hemoglobin, such as affinity methods, can also be standardized against HbA1c, as their respective values correlate well. NycoCard® HbA1c is standardized according to the recommendation of the ERL laboratory (European Reference laboratory) at DCCT-level and is certified in accordance with the ERL Check-Up Protocol.

Characteristics

Measuring range: 3-18% HbA1c

Reference range: 4.5-6.3% HbA1c

Sample material: 5µl Whole blood

Test result within 3 minutes No interference of hemoglobin variants of derivatives

7- **determination concentration of Prostatic Specific Antigen (PSA) in Enzyme Linked Immunosorbent Assay (ELIZA) Principle of the Assay**

The PSA (Human) ELISA Kit is based on the principle of a solid phase enzyme-linked immunosorbent assay. The assay system utilizes a goat anti-PSA antibody directed against PSA for solid phase immobilization (on the microtiter wells). A monoclonal anti-PSA antibody conjugated to horseradish peroxidase (HRP) is in the antibody-enzyme conjugate solution. The test sample is allowed to react first with the immobilized goat antibody at room temperature for 60 minutes. The wells are washed to remove any unbound antigen. The monoclonal anti-PSA-HRP conjugate is then added and allowed to react with the immobilized antigen for 60minutes at room temperature resulting in the PSA molecules being sandwiched between the solid phase and enzyme-linked antibodies. The wells are washed with water to remove unbound-labeled antibodies. A solution of TMB Reagent is added and incubated at room temperature for 20 minutes, resulting in the development of a blue

color. The color development is stopped with the addition of Stop Solution changing the color to yellow. The concentration of PSA is directly proportional to the color intensity of the test sample. Absorbance is measure spectrophotometrically at 450 nm.

Assay Procedure

1. Secure the desired number of coated wells in the holder.
2. Dispense 50 µl of standards, specimens, and controls into appropriate wells.
3. Dispense 50 µl of Zero Buffer into each well.
4. Thoroughly mix for 30 seconds. It is very important to have a complete mixing in this setup.
5. Incubate at room temperature (18-25°C) for 60 minutes.
6. Remove the incubation mixture by emptying plate contents into a waste container.
7. Rinse and empty the microtiter wells 5 times with distilled or deionized water. (Please do not use tap water.)
8. Strike the wells sharply onto absorbent paper or paper towels to remove all residual water droplets.
9. Dispense 100 µl of Enzyme Conjugate Reagent into each well. Gently mix for 10 seconds.
10. Incubate at room temperature (18-25°C) for 60 minutes.
11. Remove the incubation mixture by emptying plate contents into a waste container.
12. Rinse and empty the micro titer wells 5 times with distilled or deionized water. (Please do not use tap water.)
13. Strike the wells sharply onto absorbent paper to remove residual water droplets.
14. Dispense 100 µl of TMB Reagent into each well. Gently mix for 10 seconds.
15. Incubate at room temperature for 20 minutes.
16. Stop the reaction by adding 100 µl of Stop Solution to each well.

17. Gently mix for 30 seconds. It is important to make sure that all the blue color changes to yellow color completely.

18. Using a microtiter plate reader, read the optical density at 450nm within 15 minutes.

Preparation of Reagents:

1. **Dithio theritol (DTT):**

   A weight (of o.o4 g) of DTT were dissolved in 100 ml di ionized water

2. **Ethylene di amine tetra acetate EDTA**

   A weight of (0.223 g) of EDTA were dissolved in 100 ml di ionized water

3. **Sucrose**

   A weight of (12 g) of sucrose were dissolved in 50 ml di ionized water

4. **Sodium fluoride (NaF)**

   A weight (1.5 g) of NaF were dissolved in 100ml di ionized water

Pyrodoixal 5' –phosphate

A weight (0.09) of Pyrodoixal 5' -phosphate were dissolved in 100 ml di ionized water.

5. **Potassium phosphate buffer**

   A total of 136 g of mono basic ($KH_2 PO_4$) MW 136 was added to 750 ml water after it has gone into solution bring it to a final volume of 1 L. and a weight of 174 g of di basic (K_2HPO_4) MW 174 was added to 750 ml water. The volume was the adjusted to (1L). the two solution (1 M of dibasic solution and 1 M of monobasic solution) were mixed to obtain potassium phosphate buffer pH=6.5.

500 ml of this buffer was added to 0.4 Triton-X100

6. Di ammonium hydrogen phosphate ($(NH_4)2H_2PO_4$

A weight (13.4 g) of $(NH_4)2\ H_2PO_4$ were dissolved in 100 ml D.W.

7. Phosphate Buffered Saline (PBS) buffer method

PBS buffer was used to kept enzyme in tissue from damage for long period.

Phosphate buffer 0.01 M pH (7.4) containing 0.9 % NaCl Di Sodium hydrogen phosphate ($Na_2\ HPO_4\ .\ 2H_2O$)= 2.76 g, Sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$)=0.35 g , Sodium chlorid (NaCl) =9.0 g were Dissolved in 1000 ml D.W adjust PH to 7.4. (Sam brook, Fritsch, and Maniatis (1989).

8. Preparation of Reagents Citrate-Phosphate Buffer:

Stock solutions (A) 0.1 M solution of citric acid (19.21 g in 1L) and (B) 0.2 M solution of dibasic sodium phosphate (53.65 g of $Na_2HPO_4.7H_2O$) were prepared in (1L) then was taken 26.7 ml from (A) and 23.3 ml from (B) diluted to a total of 100 ml PH 4.5.

Questionnaire Paper :-

Name patient:

Age

Medical history

Education

Occupation

Smoker Nonsmoker exposure of smoker

Drugs history

Other investigation

Other problems

Blood Pressure Diabetes

Size

Stage of tumor

Benign (BPH)

Low doses of drugs

High doses of drugs

Malignant

Low doses of chemotherapy

High doses of chemotherapy

Control

Mobile phone

Name doctor

Signature :……..

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Al-Nahrain University / college of Medicine

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Bioactive Sphingolipids and metabolizing enzymes in patients with benign prostate hyperplasia and malignant prostate cancer

A thesis

Submitted to College of Medicine/ Al- Nahrain University in partial Fulfillment of the Requirement for the Degree of Master in Medical chemistry

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We, the examining committee, certify that we have read this thesis we have examined the student **"Raghdah Abdul-Monaam Razoqi"** in its context, find it adequate with standing **"Excellent"** as a thesis for the degree of Master Science in Medical chemistry.

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As a thesis for the degree of

Master science

In

Medical chemistry

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سُورَةُ الفَتْحِ
آياتها ٢٩
ترتيبها ٤٨
بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ
إِنَّا فَتَحْنَا لَكَ فَتْحًا مُّبِينًا ﴿١﴾ لِّيَغْفِرَ لَكَ اللَّهُ مَا تَقَدَّمَ مِن ذَنبِكَ
وَمَا تَأَخَّرَ وَيُتِمَّ نِعْمَتَهُ عَلَيْكَ وَيَهْدِيَكَ صِرَاطًا مُّسْتَقِيمًا ﴿٢﴾

سورة الفتح

Dedications

To the Candle of my life

Who supporting me all times

My father and mother

To my eyes and soul

Yousef and zainab

The strength source and the loving partner of my life my Husband Omer

To my sisters, and brother

To my all family, friends

And

To all who suffered and still suffering

To my motherland, IRAQ

Acknowledgement

First of all, I'm so far thankful and grateful to **almighty ALLAH** for this merciful support, help and his willing to make this study possible.

A very special note of appreciation and gratitude, heartfelt and deep, is extended to my supervisors, **Dr. Raid J. Al-Timimi and Dr. Usama S. Al- Nasiri,** for their great help, kindness, guidance and encouragement and without their support, this study would never see the light.

I am deeply indebted and sincerely grateful to Professor **Dr. Omar F. Abdul Rasheed** Head of Department of Chemistry and Biochemistry and all my Doctors especially Professor **Dr. Najat Abdul-Razaq** also **Professor Ferial Hassan Abdul-Jalil**, and Faculty members in the department of Biochemistry, Al-Nahrain College of Medicine, for their continuous help and valuable notes to fulfill this work as perfect as possible.

I would like to express my deepest appreciation to membranes in Research and Technology Environment and Water Department of the Ministry of Science and Technology Especially (Msc.chemical Ali Mohammed Jawad Al-Tamimi, Dr.Ahaar A. Kamel and Riyadh Raheem) for their continuous encouragement, patience and continuous support during my study.

I would like to especially thankful Ph.D. Dr. Kasim Sharhan Haraj Head of Department of Research

Finally, my deep grateful for my best friend Msc. Chemist Nadia Saad and to everyone who helped me through my study, to the ones whom truly sacrificed their rest and time to inspire me, to the ones whom have delighted my way to achieve this study ….

Raghdah….

Summary

Bioactive Sphingolipids an important molecules of structural and signaling lipids. In this study was highlight on new biomarkers uses to investigate prostate cancer by using new method HPLC fluorescence detector to measure concentration and activity enzymes sphingosine-1-phosphate lyase enzyme (SPL) and Acid Ceramidase enzyme (AC) by used new material (BODIPY) aromatic structural is binding with enzymes and give fluorophore product sensitive by HPLC fluorescence detector in patients have BPH and PC in serum and tissue and comparing with healthy men as (control). Eliza method was used to measured sphingosine-1-phosphate and Ceramide in patients (BPH and PC) in serum and tissue with controls in serum. Sphingolipids metabolites pathological processes, are established regulators of myriad cellular and sphingolipids research is intricate due to the role of these molecules in vastly different biologics, interconnected metabolic pathways and structural properties. Ceramide and sphingosine-1-phosphate (S1P), SGPL and AC have been defined as reciprocal regulators of cellular fate, and not surprisingly have been targeted for their role in cancer and their therapeutic potential, sphingolipid metabolic enzymes and lipids are metabolically interconnected and highlight recent findings to support the reciprocal role of Ceramide and S1P in cellular processes and in cancer. Studies show increase levels markers in serum and tissue in BPH and PC with healthy men and different Km and V max in AC and SPL between healthy, BPH and PC patients also this reaction was noncompetitive inhibition with enzyme because V max was change and Km was not change in all cases these results were accepted with studies in America and Spain (Liu, Y.Y.; Patwardhan, G.A.;et al. 2011).

Aim of study

1- The main aim of this study is found a new sensitive and specific bio -1 markers for diagnosis prostatic cancer And other goals of conducting this study are:

2- To measure activity of Acid ceramidase and Sphingosine-1-Phospate Lyase in sera and prostate tissue of patients with benign prostatic hyperplasia and prostate cancer and compare these activities of enzymes with normal controls.

3- To determine the concentration of bioactive Sphingolipids Ceramide and Sphingosine-1- phosphate in tissues and sera of the patients and control.

4- To correlate the level of bioactive lipids with serum PSA concentration to detect their use as additional biomarker for diagnosis and follow up of patient with prostate cancer.

Methods: A case-control study design was employed in this study. On a total of 30 patients with confirmed PC, 30 patients with BPH and age matched 30 healthy men. Prostate tissue samples were obtained for patients were serum and urine samples were collected for patients and controls. Enzyme linked immune assay (Elisa) was used to measure S1P and Ceramide concentration in serum and tissue, while HPLC fluorescence detector was used to estimate activities of SPL and AC and measured Km and V max. Urine and serum samples were undergone biochemistry analysis. HbA1c was used to measure cumulative sugar for three groups. Protein in tissue was measure by brad ford method to PC and BPH patients.

Results: The results showed that increase levels of Sphingosine 1 phosphate lyase and Acid ceramidase in serum and tissue in BPH and Malignant cancer in men these levels were different result in patient were

taken drugs or not and patients were taken chemotherapy or not and activity, Km and Vmax of enzyme SPL and AC were different in BPH and PC that men is inhibition enzymes was noncompetitive it had same Km but different data of control, BPH and PC men in tissue and serum.

The concentration of S1P and Ceramide were different levels with control, and high levels in patients without any drugs or chemotherapy while levels of these markers still low with patients were taken drugs or chemotherapy and level of protein in tissue were raised. Results of biochemistry analysis still normal because we take patients without any D.M., renal failure, and hypertension.

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List of Abbreviations

ANOVA	Analysis of Variance
ABS	Absorbance
AC1	N-acylsphingosine deacylase
ASA	Arylsulfatase A
ASAH1	Acid ceramidase
ASM	Acid sphingomyelinases
BMI	Body Mass Index
BODIPY FL C5-Ceramide	C5-Ceramide Acid Ceramidase substrate enzyme
BODIPY–sphingosine	Sphingosine 1phosphate substrate sphingosine 1-Phosphate Lyase (SGPL)
BOO	Bladder outlet obstruction
BPH	Benign Prostatic Hyperplasia
B.U	Blood urea
Cer	Ceramide
Cer1P	Ceramide 1-phosphate
CERK	Ceramide kinase
CERS	Ceramide synthase
CERT	Ceramide transfer protein
CGT	Ceramide galactosyl transferase
CK	Ceramide kinase

CR	Creatinine
CST	Cerebroside sulfotransferase
D.W	Distilled water
DAG	Diacylglycerol
DEGS1/2	Dihydroceramide desaturase 1 or 2
DES	Dihydroceramide desaturase
DRE	Digital rectal exam
DTT	Dithiothreitol
EDG	Endothelial differentiation gene
EDTA	Ethylene di amine tetra acetate
ELIZA	Enzyme Linked Immunosorbent Assay
ER	endoplasmic reticulum
ERK	Extracellular regulated kinase
EZH2	Protein enhancer of zest homolog 2
FAPP2	Sphingolipids transfer protein
GCase	Glucosyl Ceramaidase
GCS	Glucosylceramide synthase
GLU	Glucose
GOD	Glucose peroxidase
HbA1C	Glycated haemoglobin
HDAC 1 & 2	Histone deacetylase 1 and 2

HDL	High density lipoprotein
HPLC	High Performed Liquid Chromatography
HPR	Horseradish peroxidase
IL-6	Interleukin-6
IP3	Inositol triphosphate
KDS	3-ketosphinganine reductase
3KdhSph	3-keto dihydro sphingosine
Km	Michaelis–Menten kinetics
LacCer synthase	Lactosyl ceramide synthase
LUTS	Lower urinary tract symptoms
MAMs	Mitochondria associated membranes
MMP2 and 9	Matrix metalloproteinase 2 and 9
NaF	Sodium fluoride
NSM	Neutral sphingomyelinases
O.D	Optical density
PBS	Phosphate Buffered Saline
PC	Phosphatidylcholine
PCa	Prostate cancer
PI3K	Phosphatidylinositol 3' kinase
PKC	Protein kinase C
PSA	Prostate specific antigen

PSGR	Prostate specific G protein coupled receptor
PTEN	Phosphatidylinositol 3' phosphatase
RT	Retention time
Rock	Rho-associated protein kinase
S	Substrate
S.E.	Standard error
S1P	Sphingosine 1 Phosphate
SGPP1/2	Sphingosine 1-phosphate phosphatase 1 or 2
S1P1–5	S1P receptors 1 to 5
SGPL	Sphingosine 1-phosphate lyase enzyme
siRNA	Small interfering RNA
SLs	Sphingolipids
SM	Sphingomyelin
SMS	Sphingomyelin synthase
Sph	Sphingosine
SPHK1	Sphingosine kinase 1
SPPase	Sphingosine phosphate phosphatase
SPT	Serine palmitoyl transferase
SRF	Serum response factor
TBAP	Tetra butyl ammonium dihydrogen phosphate
TNM	Tumor nods metastasis

TRAF2	TNF receptor associated factor 2
UV	ultra violet light
Vi	Initial velocity
V max	Maximum velocity

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جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة النهرين /كلية الطب

قسم الكيمياء والكيمياء الحياتية

دراسة الدهون الأسفنجية النشطة بايولوجيا" والأنزيمات الأستقلابية في كل من تضخم البروستات الحميد وسرطان البروستات الخبيث

الرسالة مقدمة الى

كلية الطب/جامعة النهرين كجزء من متطلبات نيل درجة الماجستير

في الكيمياء الطبية

من قبل

رغده عبد المنعم رزوقي

بكالوريوس علوم /قسم الكيمياء /جامعة بغداد *2006*

المدرس الدكتور

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فرع الكيمياء والكيمياء الحياتية

الأستاذ الدكتور

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رئيس المجلس العراقي للأختصاصات الطبية

أستشاري أمراض الجهاز التناسلي والبولي

أذار/2017 م

جمادي الأخر/ 1438 هـ

الخلاصة

المقدمة:

البروستات هي غدة خارجية الإفراز من الجهاز التناسلي الذكري و تتكون من الأنسجة الغدية والعضلات . انها تقع مباشرة تحت المثانة و أمام المستقيم (الجزء السفلي من الأمعاء) .حجم البروستات حوالي حجم حبة الجوز و يحيط جزء من مجرى البول و غدة البروستات هي التي يصنع فيها السائل الذي هو جزء من السائل المنوي.

سرطان البروستات هو أكثر أنواع السرطان انتشارا لدى الرجال ويأتي ثاني أخطر أنواع السرطان في العالم لدى الرجال بعد سرطان الجلد، حيث يبلغ متوسط العمرعند 68 عاما ، أن ثلثي الوفيات الناجمة عن سرطان البروستات يحدث في الرجال الذين تتراوح أعمارهم > 75 عاما وفي العراق كانت نسبة المصابين بورم البروستات هي 5,06 % حسب احصائية وزارة الصحة العراقية لسنة 2011 ، كما أن الرجال الكبار في العمر تكون لديهم مستوى الورم ودرجته اعلى من الرجال المرضى الاصغر سنا. وعليه يجب اخذ قرارالعلاج لتجنب خطر الموت من السرطان عند كبار السن من الرجال المعرضين لخطر الموت من سرطان البروستات (التي تعتمد على درجة و مرحلة الورم) ، والآثار الضارة المحتملة من العلاج ، ومع ما يفضله المريض. سرطان البروستات يكون على نوعين النوع الاول وهو ورم البروستات الحميد والثاني الذي يكون سرطان البروستات الخبيث وفي دراستنا هذه تم التطرق لهذين النوعين من الاورام ولهذه الاورام علاقة كبيرة مع الانزيمات الاسفنجية الدهنية الاستقلابية و النشطة بيولوجيا والتي تمثل العائلة المهمة من الدهون الهيكلية والدهون التي تشترك في ارسال الاشارات . وقد زاد التركيز على هذه الدهون الاسفنجية في البحوث والدراسات أضعافا مضاعفة منذ ان تم التعرف عليها ومعرفة كونها انزيمات نشطة بيولوجيا لأول مرة منذ أكثر من عقدين من الزمن. الأيض الإسفنجية هو المنظم لكل من العمليات الخلوية والمرضية التي لا تعد ولا تحصى. البحث في أنزيمات الدهون الإسفنجية هو معقد نظرا لدورالجزيئات المختلف لحد كبير في العمليات البيولوجية ،وايضا بسبب الخصائص الهيكلية المتميزة والمسارات الأيضية المترابطة السيرامايد ,السفنغوسين -1-فوسفيت , انزيم سفنغوسين -1-فوسفيت الحالة (s1p lyase) وانزيم حامض السيرامايديز (AC) التي تعبر كمنظمات متبادلة في النظام الخلوي، وليس من غير البعيد استهداف دورهم في السرطان وإمكاناتها العلاجية. في هذا البحث سوف نشرح طرق محددة الأنزيمات والدهون الأيض الشحميات السفينغولية مترابطة مع عملية الأيض وإبراز النتائج الأخيرة لدعم دور متبادل من ميلنيوم و S1Pفي العمليات الخلوية والسرطان. وتشير الدراسات إلى زيادة مستوياتها في الدم والأنسجة في الورم الحميد

وسرطان البروستات الخبيث بالنسبة للرجال الأصحاء كما تم دراسة الفعالية لهذه الانزيمات والسرعة القصوى لها في تفاعلها ومنافستها على الماده الموجوده في الخلية مع الدواء والعلاج الكيمياوي.

أهداف البحث

الهدف الرئيسي من البحث هو ايجاد طرق تشخيصية جديده وانزيمات جديده لتشخيص ورم البروستات وبوقت مبكر لان التحليل الحالي هو فقط PSA المستضد النوعي للبروستات والذي يعطي في احيان كثيرة نتائج غير دقيقة ممايسبب أرباك للطبيب وللمريض في بعض الاحيان.

1- دراسة فعالية الانزيمات الدهنية الاسفنجية ودراسة مستويات فعاليتها في الدم وفي نسيج البروستات في كلا من الورم الحميد وسرطان البروستات الخبيث ومدى الاختلاف في مستوى الفعالية بينها وبين الانزيمات عند الرجال الاصحاء.
2- تقدير الدهون الاسفنجية النشطه بايلوجيا في السيرم وفي النسيج ومدى الاختلاف بين الرجال الاصحاء والمرضى .
3- العلاقة في مستويات الدهون النشطة بايلوجيا والانزيمات الاستقلابية مع مستوى تحليل الخاص بالبروستات PSA واعتبارها كتحاليل تستخدم لمعرفة مستوى هذا الانزيمات في الدم والنسيج عند المرضى المصابين بسرطان البروستات.

خطة البحث وطرائق العمل

شملت هذه الدراسة 60 مريضا يعانون من ورم البروستات الحميد وسرطان البروستات الخبيث ضمن مراحل مختلفة وكانت اعمارهم تتراوح بين (50-70) عاما من الذين يجرون عمليات في مستشفى التمرض الخاص، مستشفى غازي الحريري في مدينة الطب ، مستشفى السعدون الاهلي، مستشفى الهلال الاهلي، مدينة الامامين الكاظميين (ع) ، مستشفى اليرموك التعليمي، وكذلك المرضى الذين يراجعون مستشفى الاورام التعليمي في مدينة الطب ومركز الجواد للاورام في مدينة الامامين الكاظميين (ع). خلال الفترة من كانون الاول 2015 الى حزيران 2016 وتم أستبعاد المرضى الذين يعانون من امراض السكري وامراض الكلى ,ارتفاع ضغط الدم ,المدخنيين والذين لديهم تاريخ وراثي لهذا المرض. اضافة الى مجموعة مؤلفة من 30 شخصا اصحاء مطابقين لمجموعة المرضى في العمر ودالة كتلة الوزن كما تم حساب دالة كتلة الوزن (BMI) حسب المعادلة :

دالة كتلة الوزن = الوزن كغم / مربع الطول بالمتر

النتائج

أظهرت النتائج أرتفاع في مستوى الانزيمات الاسفنجية مقاسة بوحدة جزء من البليون (PPb) والدهون النشطة بايلوجيا عند المرضى المصابين بسرطان البروستات الورم الحميد والخبيث الذين لم يأخذوا اي علاج كيمياوي او دواء حيث كانت النتائج للانزيمات النشطه بايلوجيا والتي ظهرت عند الاصحاء ان مستوى أنزيم سفنغوسين 1 فوسفيت الحال (SPL) هي الاكثر (59.20 ± 6.53) بينما كانت النتائج عند المرضى المصابين بالورم الحميد الذين لم يتناولوا اي علاج في مصل الدم(0.39 ± 0.54) وهي قيمة تظهر غياب فعالية الانزيم او ظهوره بنسبة ضئيلة جدا" مما يدل على قلة فعاليته , بينما كانت النتائج مستوى الانزيم اكثر عند المرضى الذين يتناولون الدواء في مصل الدم (14.71 ± 9.04) أما النتائج للمرضى المصابين بسرطان البروستات الخبيث والذين تناولوا جرع كيمياوية عالية (32.94 ± 5.19) أرتفع مستوى الانزيم في مصل الدم عن المرضى الذين تعرضوا الى جرع كيمياوية قليلة (4.46 ± 13.37)

بينما في النسيج ظهر مستوى الانزيم بمستوى اعلى من مصل الدم اذ كانت النتائج للمرضى المصابين بسرطان الورم الحميد و الذين تناولو جرع عاليه من الدواء(3.91 ± 30.43) اما الذين تناولوا جرع اقل كانت النتائج لديهم (4.73 ± 16.87).

اما النتائج للذين يتعرضون للعلاج الكيمياوي وبجرع عالية كان مستوى الانزيم لديهم(4.49 ± 30.75) بيمناانخفضت النتائج للذين يتعرضون لجرع قليلة (3.95 ± 17.49) . ومن الملاحظ في النتائج ان المرضى المصابين بالسرطان الحميد و الخبيث قد حدث لديهم تثبيط في فعالية وعمل الانزيم عن الرجال الاصحاء بسبب العلاج.

وفي انزيم (Acid Ceramidase) كانت النتائج مغايرة اذ ارتفعت عند المرضى الذين لم يتناولوا اي علاج والمرضى الذين تناولوا كميات قليلة من الدواء والعلاج الكيمياوي مع الذين تعرضوا لجرع عالية من الدواء و العلاج الكيمياوي في مصل الدم والنسيج وكانت نتائجهم (37.85±7.551 vs 17.53 ±4.777)
(52.38± 9.627 vs 32.62±10.21).

وفي الدهون الاسفنجية سفنغوسين 1 فوسفيت (S1P) ايضا ظهرت النتائج مختلفة حيث ارتفعت عند المرضى المصابين بسرطان البروستات الخبيث في كل من مصل الدم والنسيج وتحديدا عند المرضى الذين تعرضوا لعلاج كيمياوي قليل او تناولوا جرع دواء قليلة بينما انخفضت النتائج عند المرضى في حالة تناولهم لكميات عالية من الدواء وتعرضهم لعلاج كيمياوي عالي لفترات زمنية طويلة حسب النتائج في مصل الدم (193.5 ± 76.48 vs 157.7± 84.10) وفي النسيج كانت النتائج (224.05 ± 55.63 vs 212.8±32.59)

وفي السيرامايد (Ceramide) كانت النتائج في مصل الدم (690.9 ±357.9 vs 463.68± 265.3) وفي النسيج (1330.4± 780.2 vs 703.8±180.5).

كما أظهرت هذه الدراسة أن هنالك علاقة عكسية بين كل من أنزيمي AC و SPL P value < 0.001 وكانت قيمة r=0.835 وكذلك العلاقة طردية بين كل S1P وCeramide كما كانت القيمة r=0.233 فبالاظافة الى ان هنالك علاقة بين دالة الوزن مع الانزيمات النشطة بايولوجيا والدهون الاسفنجية حيث كانت النتائج مغايرة عند الاصحاء (21.60±2.931) أما المصابين بورم البروستات الحميد (27.17± 3.168) والمصابين بسرطان البروستات الخبيث (23.59±3.793) وكانت P < 0.001 .

كما تم دراسة فعالية الانزيمات لكل المرضى المصابين لسرطان البروستات الخبيث والورم الحميد في مصل الدم والنسيج لكل من المجاميع المذكورة اعلاه في جهاز HPLC وظهرت نتائج V max مختلفة ولكن ثبوت قيمة Km كما ان التفاعل لكل من انزيمي AC و SPL هي تفاعلات غير انعكاسية حيث أظهرت الدراسة حدوث تنافس بين الادوية المثبطة لسرطان البروستات مع المواد الموجوده في الخلية على الانزيمين.

الأستنتاج :

هناك ترابط كبير بين الدهون الاسفنجية والانزيمات النشطه بايلوجيا مع سرطان البروستات الخبيث وورم البروستات الحميد وعليه فقد أقترح في هذه الدراسة أن يتم قياس هذه الانزيمات بوساطة تقنية الاداء العالي للفصل لكل من انزيمي السفنغوسين 1 فوسفيت لاييز وحامض السيرامايدز لكونها أكثر حساسية ودقة أذ أنها تتحسس وجود الخلايا المسرطنه والورم بنسب جدا قليلة في النسيج وفي مصل الدم. كما يمكن قياس فعالية هذه الانزيمات وبكلفة اقل وباجهزة متوفرة في العراق وهي تقنية الفصل ذات الاداء العالي وباستخدام الفلورة fluorescence detectors.

لوحظ وجود علاقة عكسية بين عمل هذين الانزيمين فكلما أرتفع عمل الحامض AC وتحول السيرامايد الى السفنغوسين الذي بدوره يتحول الى سفنغوسين 1 فوسفيت بواسطة انزيم سفنغوسين كايينيز و كلما أرتفعت كما ان فعالية عمل سفنغوسين 1 فوسفيت لاييز وتكسر السفنغوسين 1 فوسفيت الى مركبات اخرى وهي ethanolamine phosphate + hexadecenal وذلك للمحافظه على الخلايا من تراكم مادة S1P والتي تعد غذاء للخلايا السرطانيةكما ان عمل انزيم الاسد سيرامايديز مهم جدا لتحويل السيرامايديز الى سفنغوسين ويعمل بطريقة عكسية للمحافظه على مستوى السيرامايد بالخلية لكي يقلل من تراكم s1p في الخلية ويحافظ على الخلية من من الخلايا السرطانية وبالتالي تستخدم هذه الانزيمات كطرق تشخيصية لوجود السرطان اظافة الى PSA وعلية فان العلاقة بين السيرامايد والسفنغوسين 1 فوسفيت ايضا طردية.

ارتفاع مستوى البروتينات PCG protein وكذلك EZH2 في النسيج في حالة سرطان البروستات .
وأخيرا فان الفعالية الانزيمية لكل من AC , S1P L مع الدواء والعلاج الكيمياوي هي تفاعلات غير أنعكاسية لكونها تملك KM واختلاف قيم V max . كما أن هذه نتائج هذه الدراسة مطابقة لنتائج الدراسات في امريكا وفي اسبانيا وفي مصر ويمكن اعتبار هذه الانزيمات هي ايضا كعلاج لهذا الورم والاورام الاخرى مثل سرطان الرئة والعنق والدماغ و الامعاء وايضا في مرض الزهايمر وليس كطرق تشخيصية فقط ولكن كعلاج ايضا لانها افضل من التعرض للعلاج الراديوي (الاشعاعي) الذي يسبب تسمم للانسجه الطبيعية التي لم يحدث لها اي مرض.