

## **Determination of some polyamines in serum of breast cancer patients**

تقدير بعض متعدد الأمين في مصل مرضى سرطان الثدي

Ahmed Fadhil kadhier Al-Sultany

Dept. of chemistry , College of Science, Karbala University

### **Abstract:**

In this study, serum polyamine (spermidine & spermine) levels were measured in 28 breast cancer patients and compared with 25 healthy controls. Blood samples obtained from two groups (control & patients) and quantitative determination of polyamine were determined by using High Performance Liquid Chromatography (HPLC). It was found that spermidine is significantly increased ( $p < 0.05$ ) in patients levels was observed when breast cancer patient compare with normal subjects, and significantly increase than the healthy control population to the spermine with ( $p < 0.05$ ).

### **الخلاصة:**

في هذه الدراسة تم قياس مستوى متعدد الأمين (سبيرمدين ، سبيرمين) في مصل دم ٢٨ من مرضى سرطان الثدي وتم مقارنته مع مستويات هذا المتعدد الأمين في ٢٥ من الأصحاء جميعهم من الإناث. وتم حساب متعدد الأمين في مصل الدم بواسطة جهاز السائل عالي الكفاءة الكروماتوغرافي ، وقد وجد إن متوسط تركيز السبيرمدين و السبيرمين في مصل دم المرضى هي أعلى بكثير من تلك الموجودة في مصل دم مجموعة الأصحاء و إن القيم كانت معتمدة ( $p < 0.05$ ) في مصل دم مجموعة المرضى بالمقارنة مع مصل دم مجموعة الأصحاء .

### **Introduction :**

Breast cancer happens when cells in breast tissues begin to grow out of control and can then invade nearby tissues or spread throughout the body. Large collections of this out of control tissue are called tumors [1]. However, some tumors are not nearly cancer because they cannot spread or threaten someone life. These are called benign tumors. The tumors that can spread throughout the body or invade nearby tissues are considered cancer and are called malignant tumors [1,2,3].

A tumor marker is a substance present in a tumor itself or produced by the host in response to a tumor. It can be used to differentiate a tumor from normal tissue or to determine the presence of a tumor based on measurement in the blood or secretion. Such a substance can be found in cells and measured quantitatively by immunochemical or molecular biological methods to identify the presence of cancer [4]. Tumor markers included, carcino embryonic antigen (CEA), and human chorionic gonadotropin (HCG), placental lactogen, alpha-fetoprotein, pregnancy –specific beta 1-glycoprotein, lactoferrin ,secretory component, blood group iso-antigen neonon – specific enolase ,S-100, epithelerrin membrane antigen (EMA),and several anticytokeratins, including CAM 5.2 .CEA were the most frequently expressed tumor markers while HCG was the least frequent . Recently, there has been suggestion that, there may be a relationship between recurrence and survival in breast cancer patients and other tumor marker such Polyamines , Nucleosides , antioxidants , etc.....

Polyamines are low molecular weight highly charged organic cation that is ubiquitous in nature [5,6]. They are intimately involved in cell growth with possible importance in regulation of RNA-dependent protein synthesis [7]. Intracellular levels of polyamines, particularly putrescine  $[NH(CH_2)_4NH_2]$  ,spermidine  $[NH_2(CH_2)_4NH(CH_2)_3NH_2]$  and spermine  $[NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2]$ , increase early and dramatically with neoplastic cells [8]. In humans, polyamines originally come from the amino acid called ornithine. Each step of the pathway produces one of three different polyamines. In first step, a specific enzymes converts ornithine into the polyamine known as putrescine work together to change the putrescine into a

separated polyamine called spermidine, finally with the help of two enzymes, the spermidine transforms into the polyamine called spermine.

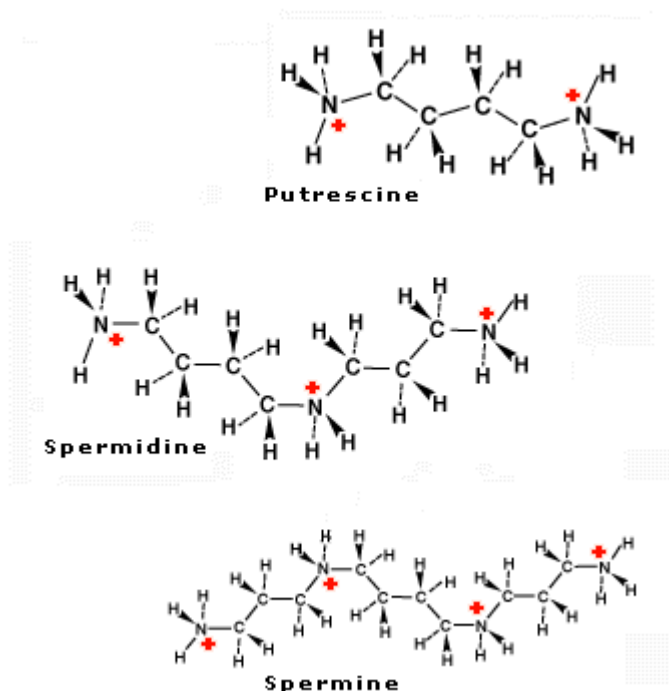


Figure (1) Structure of polyamines [9].

The most marked synthesis and accumulation of polyamines occurs in rapidly growing tissues [10]. Although the precise physiological role of polyamines is not quite clear yet. Their involvement in the regulation of cell proliferation is well established. Lowering of intracellular polyamine concentration, induced by treatment with polyamine synthesis inhibitors was shown to result in decreased rates of proliferation in a variety of experimental tumor systems [11, 12].

Humans synthesize about 0.5 mmol of spermine per day [13]. Spermidine and spermine function in diverse physiologic processes that share as a common thread in a close relationship to cell proliferation and growth. They are growth factors for cultured mammalian and bacterial cells and function in the stabilization of intact cells. Polyamines associate readily with polyanions charges such as DNA and RNA and stimulate their biosynthesis, DNA stabilization and packaging of DNA in bacteriophage [14].

This study is conducted to measure the concentration levels of the poly amine (spermidine & spermine), in breast cancer patients and compared with control subjects.

## **Materials and methods**

I-Samples

Grouping:

Blood samples from patients with breast cancer were collected. The patients were attended Kadhm'ya teaches hospital. These blood sample were obtained from fifty eight breast cancer females, their age range where form 24-73 year. A second group of blood serum were obtained from 25 healthy controls of different ages.

## **Sample Collection :**

Blood sample were drawn using 10ml syringes with steel needles. A 5ml blood sample was drawn from each patient, the blood sample was immediately transferred to a plain tubes.

The blood samples were allowed to stand at room temperature for 10 minutes for clot formation .The clots were separated from the wall of the tube using a wooden applicator stick .The tube was centrifuged for 10 minutes at 3000(r.p.m) .The serum was then transferred to a second tube using a micropipette and stored at  $-20\text{ }^{\circ}\text{C}$  until the day of analysis. All samples were analyzed at the Medical Research Center, College of Medicine.

## **II-Instruments**

### **HPLC Analysis :[15]**

Shimadzu high performance liquid chromatograph model (SCL-10AVP) was used to analyze benzoylated polyamines (spermidine and spermine) with a  $10\mu\text{L}$  sample loop, UV absorption detector (254nm), a shim-pack C18,  $5\mu\text{m}$  particles, ODS column ( $25*0.46\text{cm i.d}$ ). The mobile phase was (60:40) methanol-water run isocratically at a flow rate of  $1\text{ml/min}$ .

### **Reagents and Solvents**

Benzoyl Chloride, spermidine and spermine standards were obtained from sigma (St. Louis, Mo, USA). Methanol (HPLC grade) was obtained from fisher scientific (Ottawa, Canada).

### **Derivatization[15]**

Stock solution ( $1\text{ml}$ ) of 2% benzoyl chloride in methanol was added to  $500\mu\text{L}$  of serum in  $10\text{ ml}$  screw –capped vial.

A  $1\text{ml}$  of 2M sodium hydroxide was added and the mixture vortexed for 30 s and incubated at  $37\text{ }^{\circ}\text{C}$  for 18-20 min. The reaction was terminated by addition of  $2.0\text{ml}$  of a saturated aqueous sodium chloride solution followed by  $3.0\text{ml}$  of diethyl ether. This solution was vortexed for about 1-2min. then centrifuged at 3000 r.p.m for 10 min. to separate the aqueous and organic solvent phases .The upper ether phase containing benzoylated polyamines was transferred to another set of screw–capped tubes and evaporated to dryness.

A  $300\mu\text{L}$  of methanol added to the residual, this methanol solution was filtered through Millipore .HV filters ( $0.45\mu\text{m}$ ) to remove particulates

### **Chromatographic Separation of Polyamine Derivative**

Isocratic conditions using methanol /water mixture as the mobile phase was used for the separation of spermidine and spermine. Chromatograms of spermidine and spermine are shown in figure (2), (3) respectively. Benzoylated polyamines were separated to within 15min. the figure(3) have two peaks due to the original spermine mixed with spermidine to loss hygroscopicity of spermine to prepare the standard solution .

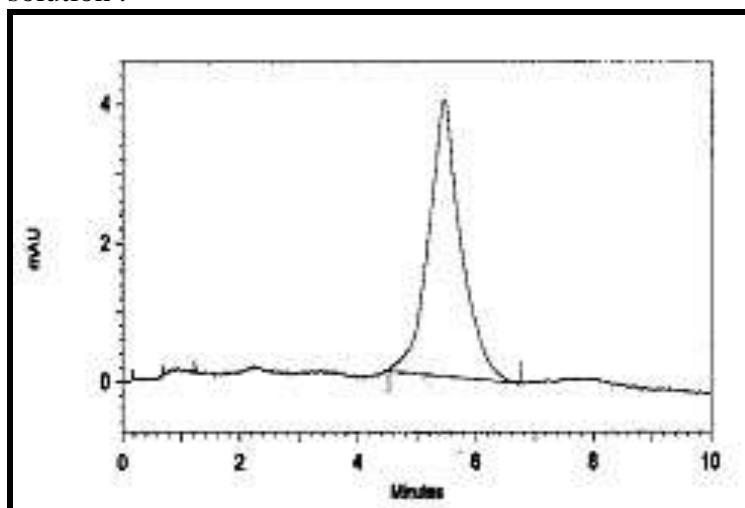


Figure (2) Chromatogram of spermidine standard on ODS column ( $25 * 0.46\text{ cm i.d}$ ), mobile phase (60:40%) methanol:water, flow rate  $1\text{ml/min}$  and detection wavelength at 254nm.

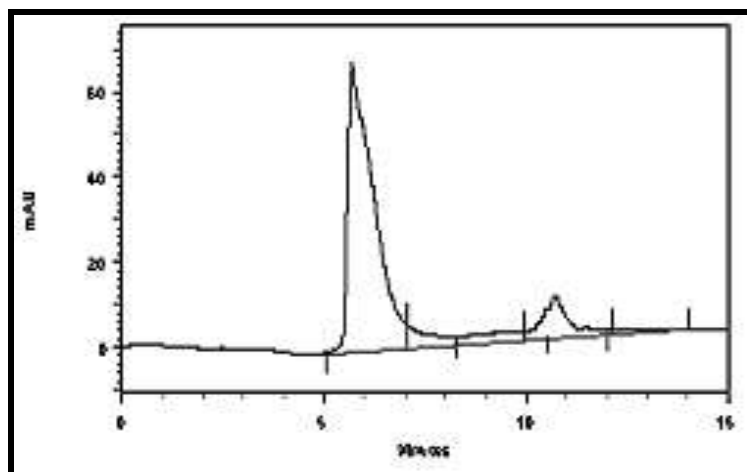


Figure (3) Chromatogram of spermine standard, condition the same as in figure (2).  
The chromatograms for the separation of normal and patient samples are shown in fig.(4) and fig.(5) respectively.

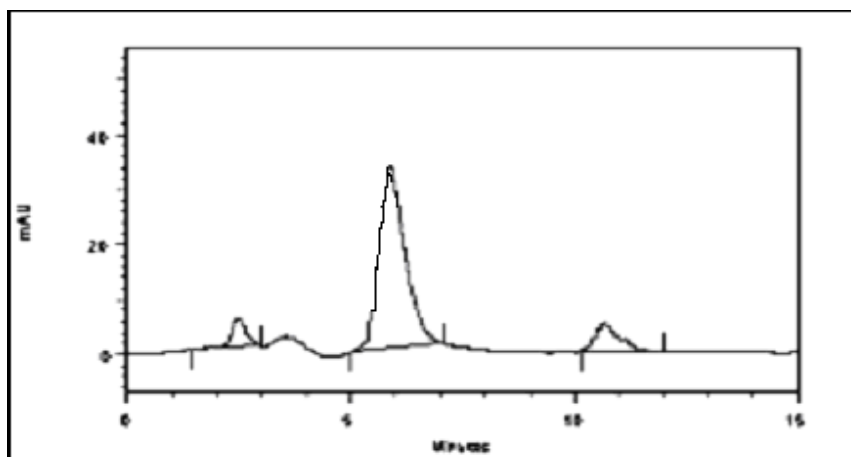


Figure (4) Chromatogram of polyamine to serum of normal, condition the same as in figure (2)

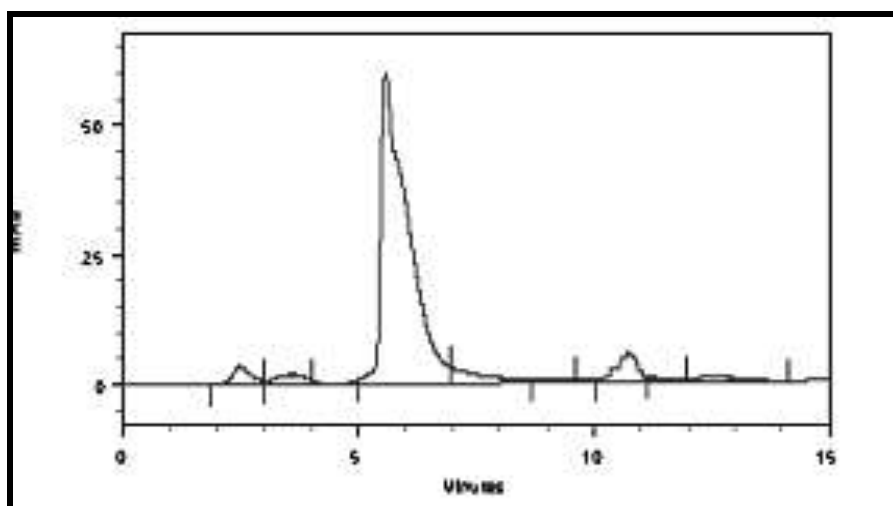


Figure (5) Chromatogram of polyamine in the serum of breast cancer patient, condition the same as in figure (2).

### **III-Quantitative Analysis**

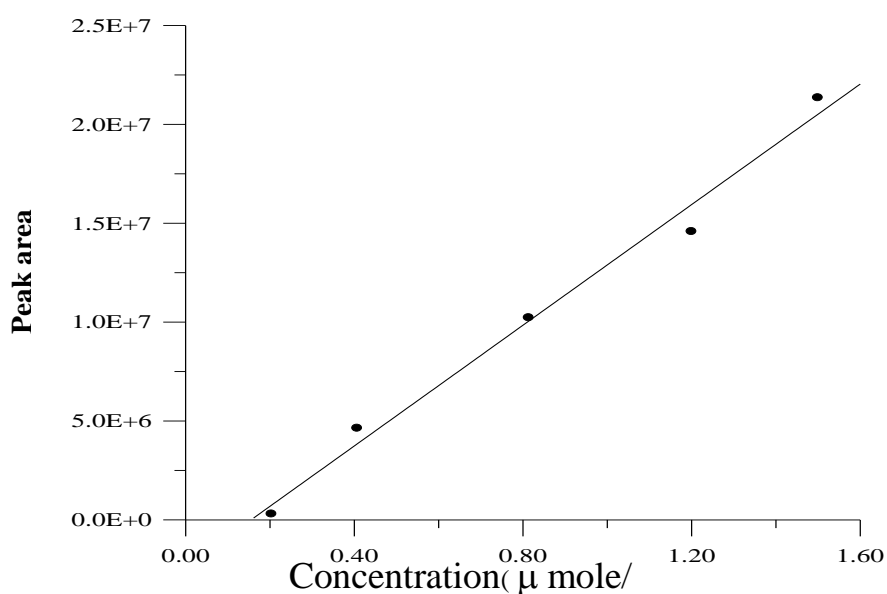
Standard solution of spermidine and spermine were prepared by subsequence dilution by deionized water .The prepared concentration ranges for polyamine (spermidine and spermine) were from (0.002-1.60)  $\mu\text{mole/ml}$  . Derivatization method above was used for standard spermidine and spermine .These calibration curve give a linear response curve for spermidine and spermine as shown in figures (6), (7) and with their linear equations. The samples were run on HPLC using the above method, the area under the peak were measured for each sample. A calibration curve were constructed to be used the peak area to measure the concentration of each sample.

Linear equation for spermidine curve.

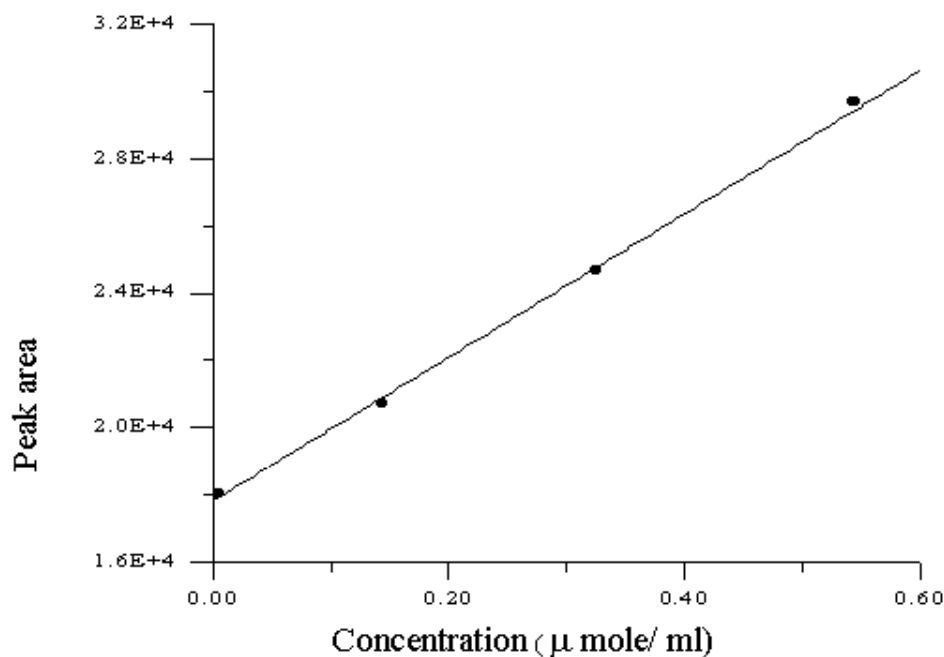
$$Y=15251200X - 2361900$$

$$R^2 = 0.98754$$

Where Y represent the peak area, and X represent the concentration



**Figure (6): Calibration curve analysis for spermidine**



**F (7):Calibration curve [PLC analysis for spermine**

Linear equations for spermine curve.

$$Y=213501X+17817.3$$

$$R^2 = 0.9971$$

Where Y represent the peak area, and X represent the concentration

### **Results and Discussion:**

Polyamines Level (spermidine, spermine)

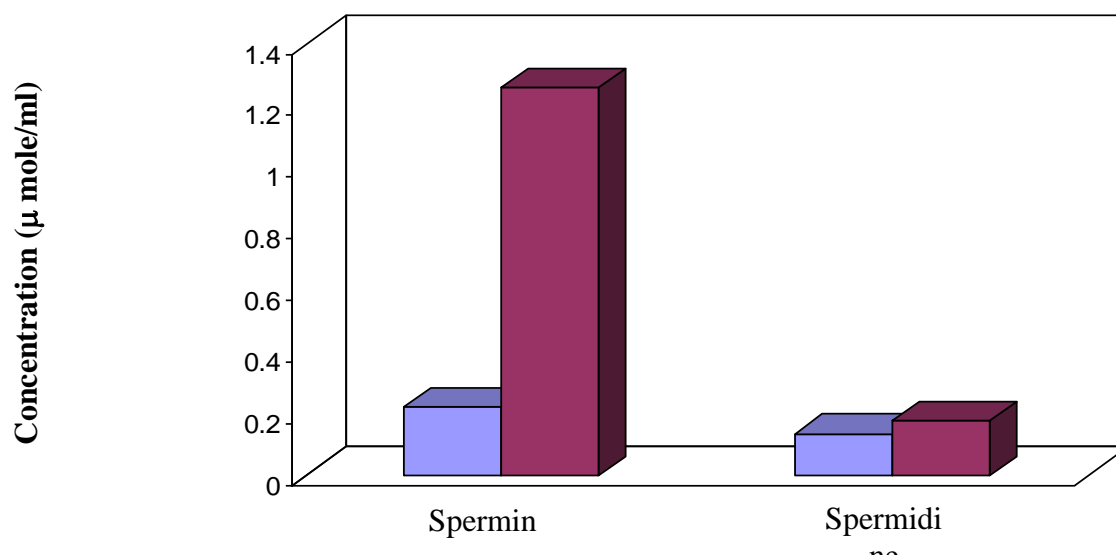
It was found that there was a significant increase ( $p < 0.05$ ) in spermidine levels in breast cancer patient ( $0.18 \pm 0.02 \mu\text{mole/ml}$ ) when compared with normal subjects ( $0.14 \pm 0.01 \mu\text{mole/ml}$ ), and a significant increase in spermine in patients ( $1.26 \pm 0.48 \mu\text{mole/ml}$ ) in comparison to that of normal ( $0.22 \pm 0.08 \mu\text{mole/ml}$ ) subjects ( $p < 0.05$ ) as noticed in table (1) and figure (8). Intercellular polyamine has an important role in proliferation of normal and malignant cells [16]. Polyamines are hypothermic and hypotensive since they bear multiple positive charge. Polyamines stimulate DNA and RNA biosynthesis [17].

The polyamine are small, charged, organic molecules that are essential for normal cell physiology and growth and death, the regulation pathways of the polyamines have been targeted as a means to elucidate the mechanisms of cancer. Much research has been done to demonstrate the important role of polyamines in breast cancer.

Many proteins involved in polyamine control, ornithine decarboxylase (ODC), the first enzyme in the production of polyamines, appears to be the most significant for alterations in polyamine levels associated with breast cancer. In addition to inhibiting synthesis of polyamines, antizyme acts to inhibit uptake of polyamine from outside the cell where antizyme is a small protein, found in mammalian cells, plays a critical role in ODC regulation by binding to, inactivating and targeting ODC for degradation [18].

Table (1) Mean concentration of polyamines (Spermidine, spermine) in serum of patient and normal

polyamine	Mean+SD( $\mu$ mole/ml)of patient	Mean+SD( $\mu$ mole/ml)of normal	p-value
Spermidine	0.18+0.02	0.14+0.01	<0.05
Spermine	1.26+0.48	0.22+0.08	<0.05



**Figure (8): Mean concentration of polyamines (spermidine and spermine) in serum of breast cancer patients and normal subject.**

In normal cells a high cellular concentration of polyamines in the cell. But in addition to regulating ODC, antizyme is also responsible for the control of the polyamine up take into the cell. For these reasons, antizyme is considered the key regulatory enzyme for polyamine levels in the cell [19]. Also, Increases of polyamines in body fluids to highest in early stage of tumor growth. Because growth and tumor cell loss are probably greatest during tumor genesis, there could at least be used to screen those pupations with known high cancer incidence that as suggested Lipton et al [20].

Further studies are needed as to the efficacy of plasma and serum vs. urinary polyamines as markers of tumor kinetic .At present it appears that plasma would be better, because there is less possibility of cellular contamination [21].

#### Conclusion

In this study it was found that polyamine (spermidine&spermine) levels in serum of breast cancer patient were significantly higher than the healthy control population to the spermidine and significantly higher than the healthy control population to the spermine.

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