بسم الله الرحمن الرحيم

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Evaluation of Parathyroid Hormone, Calcium and Phosphorus Levels Among Women with Osteoporosis in Khartoum State

تقييم هرمون الغدة الجار درقية ، مستويات الكالسيوم والفوسفور لدى النساء المصابات بهشاشة العظام في ولاية الخرطوم

A dissertation submitted in partial fulfilment for the requirement of the degree of M.Sc. in medical laboratory science (Clinical Chemistry)

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قال تعالى :

(يَا بُنَيَّ أَقِمِ الصَّلَاةَ وَأْمُرْ بِالْمَعْرُوفِ وَانْهَ عَنِ الْمُنكَرِ وَاصْبِرْ عَلَىٰ مَا أَصَابَكَ ۖ إِنَّ ذَٰلِكَ مِنْ عَزْمِ الْأُمُورِ (17) وَلَا تُصَعِّرْ خَدَّكَ لِلنَّاسِ وَلَا تَمْشِ فِي الْأَرْضِ مَرَحًا ۖ إِنَّ اللَّهَ لَا يُحِبُّ كُلَّ مُخْتَالٍ فَخُورٍ (18))

سورة لقمان ايه 17-18

Dedications

I dedicate this work to those who support me since childhood and paved the way for me to reach what I am now,

my family…

To those I find beside them all love and happiness,

my friends …

To my teachers in all academic stages …

To all people...

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Abstract

Osteoporosis is a systemic skeletal disease characterized by low bone mineral density (BMD) and microarchitecture deterioration of the bone, leading to increased fragility and a high risk of fracture. It is a silent disease until fractures occur, which causes important secondary health problems and even death. Osteoporosis is the most common bone disease in humans, representing a major public health problem. The aim of this study was determine of Parathyroid hormone, calcium and phosphorus among women with osteoporosis in compare with control group

This is case control study conducted in Khartoum state in period from February to December 2018, this study includes 90 Sudanese women 50 of them are diagnosed to have osteoporosis and 40 are healthy controls for comparison. Three ml of venous blood obtained from each participant and placed in heparin containers for estimation of calcium, phosphorus and parathyroid hormone, the samples were centrifuged to obtain plasma, then plasma transferred to Eppendorf tube and kept at -30 until time of analysis. The calcium and phosphorus were analyzed by spectrophotometer using reagent kits supplied by BioSystem company and parathyroid hormone was analyzed by ELISA.

The results show significant decrease in mean level of calcium in osteoporotic patients when compared to control group (p. value: 0.000), while parathyroid significantly increased in osteoporotic women when compared to control group (p.value 0.001) and insignificant differences in phosphorus and BMI mean levels between case and control group (p.value : 0.246 and 0.068 respectively).

Also results revealed a significant positive correlation between parathyroid hormone and phosphorus (r = 0.296, p. value 0.037), and no correlation between parathyroid hormone and calcium (r = 0.111 p. value 0.441), and no correlation between calcium and phosphorus (r = -0.071 p.value 0.623).

The study concluded that, osteoporotic patients had decreased calcium level and increased PTH level when compared to healthy control group, while there was no difference in phosphorus and BMI between case and control and there was significant positive correlation between PTH and phosphorus in osteoporotic women.

مستخلص الدراسه

هشاشة العظام هو مرض الهيكل العظمي يتميز بإنخفاض الكثافة العظميه المعدنيه وتعطل العماره المجهريه العظميه مما يؤدي الى زيادة الهشاشة وارتفاع مخاطر الكسر . هشاشة العظام مرض صامت حتى يحدث الكسر مما يسبب مشاكل صحيه ثانوية ويمكن ان يؤدي للموت . وهذا المرض يمثل مشكله صحيه عامه وكبيره . وكان الهدف من هذه الدراسه هو تحديد هرمون الغده الجار درقيه والكالسيوم والفسفور لدى النساء المصابات بهشاشة العظام بالمقارنه مع مجموعه التحكم الصحيه . هذه الدراسه اجريت في ولاية الخرطوم في الفتره من فبراير حتى ديسمبر 2018 ، تضمنت الدراسه 90 إمرأه سودانيه 50 منهن تم تشخيصهن بأنهن مصابات بهشاشه العظام و40 مجموعه تحكم صحيه للمقارنه. ثلاثه مل من الدم الوريدي اخذت من كل مشارك ووضعت في حاويات الهيبارين ثم تم عمل الطرد المركزي للحصول على البلازما ، ثم نقلت البلازما الى أنابيب إبندورف وحفظت في -30 درجه مئويه حتى وقت التحليل . تم تحليل الكالسيوم والفسفور بواسطة مقياس الطيف المرئي بإستخدام كواشف تم توفيرها عن طريق شركة بايوسيستم وتم تحليل هرمون الغدة جار الدرقيه بواسطة مقايسة الممتز المناعي المرتبط بالإنزيم.

تشير النتائج الى وجود إنخفاض معنوي في مستوى الكالسيوم في مرضى هشاشة العظام عند مقارنته بمجموعة التحكم وكانت القيمه الاحتماليه 0.000 . في حين ان هرمون الغده جار الدرقيه زاد بشكل كبير في مرضى هشاشة العظام عند مقارنته بمجموعة التحكم القيمه الإحتماليه 0.001 . ولا يوجد اختلاف ذو دلاله احصائيه في مستويات الفسفور ومؤشر كتلة الجسم بين المرضى ومجموعة التحكم وكانت القيمه الاحتماليه (0.068 و 0.246 على التوالي). كما كشفت النتائج وجود علاقه ايجابيه ذات دلالة احصائيه بين هرمون الغده جار الدرقيه والفسفور (معامل الارتباط 0.296 والقيمه الاحتماليه 0.037) ولا توجد علاقه بين هرمون الغده جار الدرقيه والكالسيوم (معامل الارتباط 0.111 والقيمه الاحتماليه 0.441) وكذلك لا توجد علاقه بين الكالسيوم والفسفور(معامل الارتباط -0.071 والقيمه الاحتماليه 0.623).
خلصت الدراسه الى ان النساء المصابات بهشاشة العظام لديهن انخفاض ملحوظ في مستوى الكالسيوم وزياده كبيره في مستوي هرمون الغده جار الدرقيه مقارنة بمجموعة التحكم و لم يكن هنالك إختلاف في الفسفور ومؤشر كتلة الجسم بين النساء المصابات ومجموعة التحكم . وأيضا وجود إرتباط إيجابي ذو دلاله احصائيه بين هرمون الغده جار الدرقيه والفسفور في النساء المصابات بهشاشة العظام.

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List of abbreviations

Symbol	Abbreviation
BMD	Bone mineral density
cAMP	Cyclic adenosine monophosphate
CaSR	Calcium sensing receptor
DEXA	Dual energy x-ray absorptiometry
DHCC	Dihydroxy cholecalciferol
DNA	Deoxyribonucleic acid
DPA	Dual photon absorptiometry
ECF	Extracellular Fluid
ELISA	Enzyme Linked Immunosorbent Assay
ER	Endoplasmic reticulum
ERs	Estrogen receptors
FGF	Fibroblast growth factor
FGF23	Fibroblast growth factor 23
GIT	Gastro intestinal tract
HRP	Horseradish peroxidase
Mab	Monoclonal antibody
PAb	Polyclonal antibody
PHPT	Primary hyperparathyroidism
QCT	Quantitative computed tomography
RNA	Ribonucleic acid
SD	Standard deviation
SHPT	Secondary hyperparathyroidism
SPSS	Statistical package of social science
VDR	Vitamin D receptor

CHAPTER ONE

1. Introduction, Rationale and Objectives

1.1 Introduction:

Osteoporosis is a disease characterized by low bone mass, deterioration of bone tissue, and disruption of bone microarchitecture: it can lead to compromised bone strength and an increase in the risk of fractures. Osteoporosis is the most common bone disease in humans, representing a major public health problem. It is more common in Caucasians, women, and older people. Osteoporosis is a risk factor for fracture and affects an enormous number of people, of both sexes and all races, and its prevalence will increase as the population ages.(Sozen, Ozisik and Calik Basaran, 2017).

Generally, the processes of bone formation and resorption are coupled so that there is no net change in the bone mass. Through childhood and early adulthood, formation exceeds resorption so that bone density increases and then plateaus until the age of 30 to 40 years. After that, resorption exceeds formation and bone density decreases through the rest of life, which in turn may lead to osteoporosis (Elmalik *et al.*, 2016)

The World Health Organization estimates that 200 million women and men suffer from osteoporosis worldwide. In the United States and the European Union, approximately 30% of all postmenopausal women have osteoporosis, and it has been predicted that more than 40% of them will suffer one or more fragility fractures during their remaining lifetime.(Zhao *et al.*, 2016)

Many environmental factors have been identified as risk of osteoporosis, including exercise and calcium intake. In addition, twin and family studies have shown that approximately 50–85% of heritability for BMD in the general population may be attributed to genetic factors. Genetic factors may also play a role in the development of osteoporosis.(Zhao *et al.*, 2016)

Calcium has definite role in bone metabolism is particularly important in elderly women because low dietary intake have been associated with reduced bone mineral density.(Mishra, M Manju, *et al.*, 2015)

Osteoporosis occurs due to imbalance in hormonal factor and change in bone formation marker (calcium and phosphorus) and bone resorption marker.(Surya Prabha *et al.*, 2015)

Phosphorus and magnesium are among minerals that have been proposed as having an important role in bone metabolisms. Phosphorus, as phosphates combine with calcium ions to form hydroxyapatite, the major inorganic molecule in teeth and bones.(Mishra, M Manju, *et al.*, 2015) PTH influences both calcium and phosphate homeostasis directly through its actions on both bone and kidney and indirectly on the intestine. PTH increases total and free plasma calcium, decreases plasma phosphate, and increases urinary excretion of inorganic phosphate (Yacoub, 2017)

1.2 Rationale:

Osteoporosis is the most common bone disease that affect numerous number of peoples, it responsible for the majority of fracture in the elderly and postmenopausal women. osteoporotic fractures are the one of major causes of morbidity and mortality. There is no enough data published in Sudan to determine parathyroid hormone, calcium and phosphorus in patients with osteoporosis. Accordingly, this study was conducted to estimate parathyroid hormone, calcium and phosphorus in Sudanese women with osteoporosis to help in good management of disease and reduce the risk of fracture.

Objectives

General objective:

- To determine parathyroid hormone, calcium and phosphorus in Sudanese women with osteoporosis.

Specific objectives:

1. To estimate parathyroid hormone, calcium and phosphorus in women with osteoporosis and control group.
2. To compare mean level of parathyroid hormone, calcium and phosphorus in both study group.
3. To correlate between different study variables in osteoporotic patients.

CHAPTER TWO

2.Literature review

2.1. Osteoporosis:

Bone metabolism is a dynamic and continuous process to maintain a balance between the resorption of old and injured bone initiated by osteoclasts and the formation of new bone under the control of osteoblasts. In general, the processes of bone formation and resorption are 'coupled', so that there is no net change in the bone mass. Through childhood and early adulthood, formation exceeds resorption so that bone density increases and then plateaus until the age of 30 to 40 years. After that, resorption exceeds formation and bone density decreases through the rest of life, which in turn may lead to osteoporosis. (Indumati, Patil and Jailkhani, 2007)

Osteoporosis is a systemic skeletal disease characterized by low bone mineral density (BMD) and microarchitecture deterioration of the bone, leading to increased fragility and a high risk of fracture(Kim *et al.*, 2015). It is a silent disease until fractures occur, which causes important secondary health problems and even death(Sozen, Ozisik and Calik Basaran, 2017).

Osteoporosis is not exclusive to older age. It is however one of diseases preventable by adequate nutrition and physical activity. Bone is a metabolically active organ. Puberty, as well as infancy is a period of life with most intensive bone growth: 90 % of bone mass will have formed by the end of adolescence. In the first 5-6 years of life, around 100 mg of calcium is retained daily for bone formation, up to 400 mg or even more during puberty. After adolescence, absorption of calcium declines, in young adults daily absorption of calcium amounts to maximally 150 mg.(Stransky and Rysava, 2009)

The social and economic burden of osteoporosis is increasing steadily because of the aging of the world population. Currently affecting more than 10 million people in the United States, osteoporosis is projected to impact approximately 14 million adults over the age of 50 by the year 2020. Worldwide, approximately 200 million women have osteoporosis. Although the likelihood of developing osteoporosis currently is greatest in North America and Europe, it will increase in developing countries as population longevity in these countries continues to increase (Lane, 2006).

Women are more susceptible to osteoporosis than men. They present approximately 80% of all patients with osteoporosis. Generally, women have smaller and thinner bones than men and there

is a sharp decline in production of estrogen which increases the bone formation after menopause (Al-Daghri *et al.*, 2014)

Bone undergoes a continuous remodeling process (i.e., replacement) involving resorption of old bone by osteoclasts and The activity of osteoclasts formation of new bone by osteoblasts. and osteoblasts ordinarily is balanced and regulated by physical factors and hormonal influences. Osteoporosis is characterized by an imbalance between osteoclast and osteoblast activity and a rate of bone resorption that exceeds the rate of bone formation, resulting in bone loss and skeletal fragility. Osteoporosis may be primary or secondary to an identifiable cause (i.e., a drug, disease, or condition) (Kamel, 2006).

Osteoporosis is classified into primary and secondary ones. Primary osteoporosis is manifested by deterioration of bone mass that is unassociated with other chronic illness and is related to aging and decreased gonadal function. Therefore, early menopause or perimenopause estrogen deficiency states may hasten the development of primary osteoporosis. Prolonged periods of inadequate calcium intake, sedentary lifestyle and tobacco and alcohol abuse also contribute to this condition. Secondary osteoporosis results from chronic conditions that contribute significantly to accelerated bone loss. These chronic conditions include endogenous and exogenous thyroxine excess, hyperparathyroidism, malignancies, gastrointestinal diseases, medications, renal failure and connective tissue diseases (Mahmoud, 2008).

2.1.1 Risk Factors for Osteoporosis:

Major risk factors for osteoporosis include low body weight, history of fracture, family history of osteoporosis, and smoking. Established risk factors for osteoporosis and associated fractures are increasing age, female sex, white race, removal of the ovaries at an early age, prolonged immobility, and prolonged use of corticosteroids. Furthermore, factors that probably or possibly increase risk in postmenopausal white women include a low calcium intake, cigarette smoking, and, at least for hip fractures, use of long half-life psychotropic drugs and heavy alcohol consumption (Abdelrahman, Mohammed and Abdelsalam, 2010).

2.1.2 Diagnosis of Osteoporosis:

The diagnosis of osteoporosis is based on the presence of a fracture after minimal trauma or by detecting low bone mineral density measured by Dual Energy X-ray Absorptiometry(DEXA) (Baccaro *et al.*, 2015).

Dual Energy X-ray Absorptiometry (DEXA), peripheral dual energy X-ray absorptiometry (P-Dexa), Dual Photon Absorptiometry (DPA), Ultrasound and Quantitative Computed Tomography (QCT) are different methods for the measurement of bone mineral density. Dual energy x- ray is the most accurate way and DEXA results are described in terms of T-score (Shakoor *et al.*, 2014).

The T-score is a comparison of the patient's bone density with healthy, young individuals of the same sex. A negative T-score of –2.5 or less at the femoral neck defines osteoporosis. The Z-score is a comparison with the bone density of people of the same age and sex as the patient. A negative Z-score of –2.5 or less should raise suspicion of a secondary cause of osteoporosis (Sheu and Diamond, 2018).

DEXA is considered the gold standard of methods used to diagnose osteoporosis. This test is capable of measuring bone mineral content at any site in the body but usually is used at central sites (the lumbar spine and the proximal femur) and peripheral sites, including the distal forearm. This is accomplished by passing 2 beams of different energies through the bone at the site being measured (Lane, 2006).

DEXA of the lumbar spine and proximal femur (hip) provides accurate and reproducible BMD measurements at important osteoporosis-associated fracture sites. Optimally, both hips should be initially measured to prevent misclassification and to have a baseline for both hips in case a fracture or replacement occurs in 1 hip. These axial sites are preferred over peripheral sites for both baseline and serial measurements. The most reliable comparative results are obtained when the same instrument and, ideally, the same technologist are used for serial measurements. Diagnostic criteria, therapeutic studies, and cost effectiveness data have been primarily based on DEXA measurements of the total hip, femoral neck, and/or lumbar spine (L1-L4), and are the preferred measurement sites (Camacho *et al.*, 2016).

DEXA scan fallouts are reported as T-scores:

• Normal bone: T-score beyond -1

• Osteopenia: T-score sandwiched between -1 and -2.5

• Osteoporosis: T-score further down -2.5 (Naveed and Hameed, 2016)

2.1.3 Premenopausal Osteoporosis:

Osteoporosis in younger women results from either a low peak bone mass, increased bone loss prior to menopause or both. In the case of premenopausal osteoporosis, secondary causes are responsible for at least half of cases (Table 1).(McLendon and Woodis, 2014)

Table 2.1 : Secondary Causes of Premenopausal Osteoporosis

Cause	Examples
Medications	Glucocorticoids, anticonvulsants, aromatase inhibitors, heparin, alcohol, LHRH agonists, medroxyprogesterone acetate, high-dose levothyroxine, cytotoxic chemotherapy
Endocrine disease	Hypogonadism, hyperthyroidism, Cushing's disease, growth hormone deficiency, hypopituitarism, hyperparathyroidism, Type 1 diabetes
Malnutrition or malabsorption	Anorexia nervosa, inflammatory intestinal disease, celiac disease, intestinal resection Inflammatory
Inflammatory disease	Rheumatoid arthritis, systemic lupus erythematous
Transplant patients	Solid organ and bone marrow transplants
Other causes	Liver disease, osteogenesis imperfecta, HIV infection, hemochromatosis, idiopathic osteoporosis, pregnancy, systemic mastocytosis, chronic kidney disease, malignancies, hyperprolactinemia, multiple myeloma, depression

Risk factors for low BMD in premenopausal women include low body weight, amenorrhea, lack of physical activity, smoking, low dietary calcium or vitamin D, personal or family history of fracture, pregnancy and Caucasian or Asian race (McLendon and Woodis, 2014).

2.1.4 Postmenopausal Osteoporosis:

Menopause is defined as the permanent cessation of menses resulting from reduced ovarian hormone secretion that occurs naturally or is induced by surgery, chemotherapy, or radiation. Natural menopause is recognized after 12 months of amenorrhea that is not associated with a pathologic cause. The average age of menopause in the United States is 51 years, and can vary normally between 40 and 58 years (Danby *et al.*, 2005).

Due to menopause ovarian follicles loss its function, which results in decreased production of estradiol and other hormones. Decreased level of estrogen leads to increased osteoclast formation and enhanced bone resorption, which intern leads to loss of bone density and destruction of local architecture resulting in osteoporosis (Mishra, M Manju, *et al.*, 2015).

Estrogen hormone inhibits the production of inflammatory maker IL 6 which in turn inhibits the osteoclast apoptosis and leads to decrease bone resorption resulting in remodeling of bones in females. Therefore, deficiency of estrogens may cause longer life span of osteoclast. In the age group of 40 to 50years females menstrual cycle becomes irregular, failure of ovulation in menstrual cycle and cessation of menstrual cycle occur ultimately which is known as menopause. Bone turnover becomes higher in female as soon as menopause occur. Moreover, deficiency of estrogens hormones leads to calcium loss as it has an indirect effect on calcium homeostasis of bones. Decrease of calcium leads to osteoporosis (Kalia and Deep, 2017).

Estrogens are known to play an important role in regulating bone homeostasis and preventing postmenopausal bone loss. They act through binding to two different estrogen receptors (ERs), ERα and ERβ, which are members of the nuclear receptor superfamily of ligand-activated transcription factors. Both ER kinds are expressed in osteoblasts, osteoclasts, and bone marrow stromal cells. And also ESRα has a prominent role in regulating bone turnover and the maintenance of bone mass (Mohammadi *et al.*, 2014).

A large number of etiological factors may lead to a decrease in bone density, but the most common form of osteoporosis is postmenopausal osteoporosis in which the estrogen deficiency is a major

risk factor in early and the late phase of bone loss in women, since estrogens have a very favorable, antiresorptive and a discreet anabolic effect on bone tissue (Milivojac[1] *et al.*, 2015).

2.1.5 Treatment of Osteoporosis:

The aim of treatment of osteoporosis is the avoidance of bone fractures by overcoming bone loss or by increasing bone density. Drugs which are used in treatment of osteoporosis include:

• Bisphosphonates drugs which responsible for decrease bone loss hence reduce risk of fracture.

• Estrogen antagonists which act as inhibitor for spine fractures.

• Calcitonin which responsible for prevention spinal fracture in postmenopausal women.

• Parathyroid hormone which stimulates formation of bones.

• Calcium supplements.

• Vitamin D supplements .

As well as, prevention of osteoporosis is most important than treatment.

Osteoporosis can be prevented by:

• Lifestyle changes, including take off smoking, excessive alcohol intake, take sufficient calcium and vitamin D.

• Take drugs that stop bone loss and increase bone strength, alendronate, risedronate, calcitonin.

• Take medications which increase bone formation such as teriparatide (Elbossaty, 2017).

2.2 Calcium

Calcium is the most abundant mineral in the human body. Approximately 99% of the total calcium stores are contained in the skeleton. The remaining stores are in the cells of soft tissue (0.9%) and in the bloodstream and extracellular fluid (0.1%), where they exert effects on the cardiovascular, nervous, and muscular systems. Calcium requirements for skeletal maintenance fluctuate throughout a woman's life. During the teen years, calcium requirements are high because of the demands of a rapidly growing skeleton. During woman's 20s, less calcium is required for bone health as bone turnover stabilizes (bone formation and resorption rates become balanced) and peak

adult bone mass is achieved. Calcium requirements remain stable until menopause, when the bone resorption rate increases in association with the decrease in ovarian estrogen production. Calcium needs rise at that time because of decreased efficiency in the utilization of dietary calcium, which is due, in large part, to estrogen related shifts in intestinal calcium absorption and renal conservation (Bilezikian *et al.*, 2006).

Through interacting with numerous proteins distributed in different cellular compartments, calcium is involved in a large amount of aspects of life, such as muscle contraction, enzyme activation, cell differentiation, immune response, programmed cell death and neuronal activity. Such broad functions are maintained by tightly controlled calcium concentration in extracellular fluid and cellular compartments.

The concentrations of calcium in blood and extracellular fluid are usually maintained at 1–2 mmol/L, while the concentration of intracellular calcium at resting state is maintained at 100 nmol/L or less by calcium ATPase, channels, and exchangers located in plasma membrane and endoplasmic reticulum (ER) membrane. During the signaling process of calcium, the concentration of intracellular calcium is increased to approximately 100 μM, which triggers calcium signaling through the activation or deactivation of an array of calcium-binding proteins (Pu, Chen and Xue, 2016).

2.2.1 Dietary Source of Calcium

Calcium is present in both plant and animal foods. The richest source of calcium amongst animal foods are dairy products (milk, yoghurt and cheese) and amongst the plant foods are green leafy vegetables like amaranth, fenugreek leaves and broccoli. Cereals like Ragi, nuts and seeds like almonds , pistachios and sesame seeds, fishes like salmon, sardines etc. are good sources of calcium (Trailokya *et al.*, 2017).

2.2.2 Calcium Absorption

Calcium absorption from gut depends upon number of factors. Absorption is greatest when the intake of calcium is low and the need is high. Vitamin D levels, an acidic environment, age, estrogen levels, and dietary fiber intake play a role in calcium absorption. Calcium absorption declines with age, low vitamin D levels, hypochlorhydria , low estrogen levels, and a high-fiber diet (Trailokya *et al.*, 2017).

The active vitamin D metabolite, 1,25-dihydroxy vitamin D (1,25(OH) 2 D) stimulates the active calcium transport through the intestinal wall. The active metabolite binds to the vitamin D receptor in the intestinal epithelial cell, subsequently the calcium binding protein CaBP-9K is synthesized and the calcium channels TRPV6 and TRPV5 are activated. Calcium can enter the cell from the intestinal lumen and is transported through the cell by the calcium binding protein and transferred to the interstitium by an ATP mechanism. This active transport mechanism has a maximum. Besides the active transport, paracellular transport takes place by diffusion. The paracellular transport depends on the calcium gradient, i.e. on calcium intake. In case of vitamin D deficiency, the active transport is lower and diffusion becomes more important especially when calcium intake is high (Lips, 2012).

2.2.3 Regulation of Calcium

Calcium homeostasis is largely regulated through an integrated hormonal system that controls calcium transport in the gut, kidney, and bone. It involves two major calcium regulating hormones and their receptors -PTH and the PTH receptor (PTHR) and 1,25(OH)2D and the vitamin D receptor (VDR) as well as serum ionized calcium and the calcium-sensing receptor (CaSR) (Munro Peacock, 2010).

Calcium concentration within a narrow range (8.5-10.5 mg/dL) is very important for calcium homeostasis. Approximately 40% of plasma calcium is protein-bound and 10% of calcium is in a complex with anions like phosphate, citrate, and sulfate etc. Only half of plasma calcium is in its free form (ionized form, iCa^{2+}) and physiologically important. The ionized calcium is tightly regulated by hormones like parathyroid hormone (PTH), 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), calcitonin, and calcium itself. The kidney, intestine, and bone are the main target organs of these regulators, and the kidney plays a key role in the fine regulation of calcium excretion (Jeon, 2008).

Parathyroid hormone is secreted by the parathyroid glands in response to a decrease in the calcium plasma concentration from the set point. PTH acts mainly on the bone and kidney. Upon the increase in PTH concentration, a process known as osteocytic osteolysis takes place, in which PTH causes the removal of bone salts from the bone matrix by lacunar osteocytes. This occurs within minutes and proceeds without actual resorption of bone matrix. More short-term needs are met through osteocytic osteolysis. If high concentrations of PTH persist, a delayed response (hours to

days), known as osteoclastic bone resorption, takes place due to the activation of the bone osteoclasts. This process involves resorbing the bone matrix itself and allows the response to PTH to continue beyond what can be handled by osteocytic osteolysis (El-Samad, *etal.*, 2002).

Thus, the need for maintaining plasma calcium concentrations is deemed more important than maintaining the integrity of the bone. The effect of PTH on the kidney is to increase tubular reabsorption of calcium thus reducing calcium loss through urine. Therefore, the impact of PTH is to increase immediate calcium transfer into the blood plasma. On the other hand, the main role of 1,25-dihydroxy cholecalciferol (DHCC) is to stimulate intestinal calcium absorption through increasing formation of a calcium-binding protein in the intestinal epithelial cell. In fact, 1,25-DHCC is considered to be the most potent stimulator of calcium absorption from the intestine. It is well known that 1,25-DHCC is produced from cholecalciferol, a biologically inactive form of vitamin D after it undergoes several hydroxylation steps in the liver and kidney. The last hydroxylation step in the kidney takes place only under stimulation by PTH. Calcitonin, the third hormone involved in calcium homeostasis, has relatively little relevance during hypocalcemia and therefore will not be considered. In fact, calcitonin is not secreted until plasma calcium levels exceed 9.5 mg/dl. Above this calcium level, plasma calcitonin is directly proportional to plasma calcium (El-Samad,*etal.*, 2002).

2.2.4 Hypercalcemia and Hypocalcemia

Hypocalcemia and hypercalcemia are terms used clinically to refer to abnormally low and high serum calcium concentrations. It should be noted that, because about one half of serum calcium is protein bound, abnormal serum calcium, as measured by total serum calcium, may occur secondary to disorders of serum proteins rather than as a consequence of changes in ionized calcium (Peacock, 2010).

Disturbances in both serum and whole body Ca2+ levels can cause severe pathological conditions, the etiology of which is both complex and variable. Hypercalcemia can result from Ca2+ hyper-absorption from the gastrointestinal tract (GIT), decreased urinary excretion, or an increased resorption from bone. Elevated serum PTH levels, secondary to hyperparathyroidism or a hypophosphatemic state, will cause increased Ca2+ absorption from the GI tract. Increased Ca2+ loss from bone is caused by elevated PTH and/or 1,25(OH)2D3 levels or skeletal metastasis, while severe dehydration will increase serum Ca2+ concentration without altering the total amount in

blood. Symptoms and findings of hypercalcemia include fatigue, electrocardiogram abnormalities, nausea, vomiting, constipation, anorexia, abdominal pain, hypercalciuria, and consequently kidney stone formation. Treatment of hypercalcemia depends on the severity of the abnormality and ranges from dietary adaptation to the administration of calcimimetic compounds that activate the CaSR in the parathyroid glands, reducing blood PTH levels. Hypocalcemia can result in muscle cramping, depression, psychosis, and seizures. Causes include decreased Ca2+ absorption due to a poor intake, 1,25(OH)2D3 deficiency or resistance, lack of sunlight, decreased bone resorption, a complication of thyroid surgery (i.e. parathyroidectomy), or renal Ca2+ wasting. Oral Ca2+ and 1,25(OH)2D3 supplementation and ultraviolet light exposure are the current treatments for hypocalcemia (Civitelli and Ziambaras, 2011).

2.3 Phosphorus

Phosphorus is an essential mineral component of the human body, and is essential for energy metabolism (ATP formation), maintenance of intracellular pH, and cell signaling; it is also an important element of cell membranes, nucleic acids (i.e. DNA and RNA molecules), and the skeletal system. Phosphorous has a very strong electronegative attraction with calcium, having electronegative values on the Pauling scale of 2.1 for phosphorus and 1.0 for calcium. At physiologic pH (7.4), extracellular phosphate is mostly present as Na2HPO4 and NaH2PO4. Once absorbed, phosphate combines with calcium and accumulates mostly in bone and teeth to form the structural basis and rigidity of these organs (Mahdi *etal*., 2015).

The total body phosphorus is around 700 grams (23,000 mmol) and is distributed mainly in the bones (80%), viscera (10.9%), skeletal muscle (9%), and only 0.1% is in the extracellular space. The average diet usually provided 800- 1400 mg of phosphorus daily, of which 60-80% is absorbed in the gut mainly by passive transport though there is an active transport of phosphorus via the action of 1, 25- dihydroxy vitamin d3 (1, 25[OH]2D3). Parathyroid hormone (PTH) and low phosphorus diet also stimulate absorption phosphorous. (Tucker and Thornley-Brown, 2013)

Inorganic phosphate in serum exists in three forms: ionized (about 55%), complexed to cations (about 35%) and protein bound (about 10%). Approximately 90% of plasma inorganic phosphate is ultrafiltrable, i.e. non-protein bound fraction. The relative proportion of HPO_4 2- and H_2PO_4- depends on the pH, and at normal pH the ratio is 4:1. This ratio decreases in acidic conditions and increases in alkaline states (Manghat *etal*., 2014).

Phosphorus and magnesium are among minerals that have been proposed as having an important role in bone metabolisms. Phosphorus, as phosphates combine with calcium ions to form hydroxyapatite, the major inorganic molecule in teeth and bones (Mishra, M. Manju, *et al.*, 2015a).

2.3.1 Dietary Source and Absorption of Phosphorus

Dietary phosphate is found in many foods, with dairy, meats, and cereals being the richest sources. Intestinal phosphate absorption occurs primarily in the duodenum and the jejunum via active cellular and passive para-cellular pathways, and up to75% of dietary phosphate is absorbed in the small intestine. The active cellular absorptive pathway is dependent on the luminal sodium/phosphate (Na/Pi) co-transporter type 2b. In addition, 1,25-(OH)2 vitamin D is involved in a feedback loop with serum phosphate levels in that decreased serum phosphate levels lead to an increase in 1,25-(OH)2 vitamin D levels. Increased 1,25- (OH)2 vitamin D consequently leads to increased intestinal absorption of phosphate. (OH)2 vitamin D consequently leads to increased intestinal absorption of phosphate (Day,*etal.*, 2018).

The normal plasma phosphorus level (expressed as phosphates) is usually between 0.84 - 1.44 mm/l (2.8 – 4.5 mg/dL). The plasma concentration of phosphorus is determined by dietary intake, intestinal absorption, renal tubular reabsorption and transfer between intra and extracellular fluid compartment (Tucker and Thornley-Brown, 2013).

Red cells are richer in phosphorus compared to plasma mainly because they contain more ester phosphates (Eldin and Hussein, 2015).

2.3.2 Regulation of Phosphorus

Maintenance of phosphate homeostasis based on the regulation of phosphate handling by bone, intestine, and kidney. Diseases affecting these organs frequently are accompanied by significant hyper- or hypophosphatemia. The major identifiable regulatory substances are parathyroid hormone (PTH), vitamin D, and fibroblast growth factor 23 (FGF23) with its required cofactor, the glucuronidase klotho. Disorders of these three circulating hormones result in defined syndromes of hyper- or hypo-phosphataemia and phosphate overload or phosphate deficiency (Lederer, 2014).

1,25-Dihydroxycholecalciferol raises calcium and phosphate levels by increasing gut absorption. Here, the type IIb sodium phosphate co-transporter appears to be regulated at the membrane level by 1,25-dihydroxcholecalciferol, but not at a transcriptional level. 1,25-Dihydroxycholecalciferol also increases activity of the osteoblasts, laying down calcium into the bone matrix and increases calcium and phosphate reabsorption by the epithelial cells of the renal tubules. Parathyroid hormone is released in response to changes in calcium concentration but also acts on phosphate. As parathyroid hormone levels increase, the bone releases calcium and phosphate- containing minerals into the circulation. Parathyroid hormone also has a direct effect on the kidney to increase tubular reabsorption of calcium, but also to decrease the phosphate reabsorption by acting on the proximal tubules. The net effect is that phosphate levels decrease in response to parathyroid hormone. Parathyroid hormone also promotes formation in the kidneys of 1,25-dihydroxycholecalciferol, therefore enhancing the absorption of calcium and phosphate from the gastrointestinal (GI) tract. Phosphate is completely reabsorbed by the kidney when the plasma concentration is below 1 mmol/liter with none lost to the urine. Above this critical concentration, phosphate loss is proportional to the concentration present (Wadsworth and Siddiqui, 2016).

FGF23 suppresses the expression of type 2a and 2c sodium–phosphate cotransporters and thereby inhibits proximal tubular phosphate reabsorption. FGF23 also reduces the expression of CYP27B1 that encodes 25-hydoxy-vitamin D[25(OH)D]-1a-hydroxylase and enhances CYP24 expression that produces 25(OH)D-24-hydroxylase. By these actions, FGF23 reduces circulatory level of 1,25(OH)2D. Several studies indicated that 1,25(OH)2D enhances FGF23 production. In addition, oral phosphate administration increases FGF23 level in both humans and animals. However, it has not been shown that phosphate directly modulates FGF23 expression so far. It was reported that increase or decrease of serum phosphate did not alter FGF23 levels during several hours, indicating that there is no acute regulation of FGF23 by phosphate. On the other hand, FGF23 levels are low in patients with chronic hypophosphatemia such as Fanconi syndrome, and it was shown that serum phosphate can regulate FGF23 level. These results suggest that phosphate chronically regulates FGF23 production. In addition, FGF23 was shown to inhibit both production and secretion of PTH (Fukumoto, 2014).

2.3.3 Hypophosphatemia

Hypophosphatemia is defined as a serum- or plasma- measured phosphate level below the reference interval, usually 2.5 to 4.5 mg/dL (0.81-1.45 mmol/L) in adults. The underlying cause of a decrease in phosphate concentration below the reference interval can be decreased intestinal absorption, increased urinary excretion, or a shift of extracellular phosphate into the cells.(Bazydlo,*etal*., 2014)

2.3.3.1 Common Causes of Hypophosphatemia

2.3.3.1.1 Decreased Urinary Phosphate

-Intracellular shift:

- Increased insulin levels: diabetic ketoacidosis, insulin-dependent diabetes, refeeding syndrome
- Hungry bone syndrome
- Acute respiratory alkalosis
- Tumor consumption: lymphoma, leukemia blast crisis
- Sepsis

-Decreased intestinal absorption Vitamin D deficiency

- Malabsorption: bowel surgery, pancreatitis, Crohn's, celiac disease, chronic diarrhea
- Chronic kidney/liver disease
- Phosphate absorption inhibitors: niacin, phosphate binders, antacids
- Nutritional deficiency: anorexia, alcoholism, marasmus

2.3.3.1.2 Increased Urinary Phosphate

-Increased urinary losses:

- Primary/secondary hyperparathyroidism
- Medications: tenofovir, acetazolamide, bicarbonate, IV iron
- Tumor-induced osteomalacia/mutations
- Fanconi syndrome. (Day, Morgan and Saag, 2018)

2.3.4 Hyperphosphatemia

hyperphosphatemia occurs when phosphate concentrations in the serum and/or the plasma exceed the reference interval. There are 3 main circumstances in which this occurs: a decrease in renal phosphate excretion, increased phosphate load, or a shift of phosphate into the extracellular fluid. The most common cause is decreased renal phosphate excretion, which can occur due to renal failure, hypoparathyroidism, or PTH resistance.(Bazydlo, *etal.*, 2014)

2.3.4.1 Causes of Hyperphosphatemia

-Increased phosphate intake

- Oral (laxatives)
- Enema
- Intravascular fluids with phosphate supplementation
- Vitamin D intoxication (increased absorption)

-Transcelluar shift

- Tumour lysis syndrome
- Rhabdomyolysis
- Malignant hyperpyrexia
- Massive haemolysis Acidosis: diabetic ketoacidosis and lactic acidosis

-Reduced excretion:

- Renal failure – acute and chronic
- Hypoparathyroidism

-Genetic causes (Manghat, Sodi and Swaminathan, 2014)

Renal failure is the most common cause of hyperphosphatemia in clinical practice. In mild to moderate chronic kidney disease retention of phosphorus results in increase in parathyroid hormone (PTH) and increase in renal phosphate excretion.(Tucker and Thornley-Brown, 2013)

2.4. Parathyroid Hormone

Human parathyroid hormone (PTH) is an 84-amino acid polypeptide secreted from the parathyroid gland. acting predominantly on the skeletal system and renal tubules, the hormone modulates serum calcium and phosphate (Osagie-Clouard *et al.*, 2017). PTH is first translated in the endoplasmic reticulum of the parathyroid chief cell as a 115-amino acid pre pro-PTH molecule. Twenty-five amino acids at the amino-terminal end of the peptide are cleaved as it leaves the endoplasmic reticulum forming pro-PTH. The next 6 amino-terminal amino acids are removed as pro-PTH leaves the cell cytoplasm. This final product consists of 84 amino acids and is a functionally intact PTH molecule abbreviated as (1–84) PTH.(Carter and Howanitz, 2003).PTH secretion results in raised serum calcium through its release from the bones, reduced renal excretion, and increased small intestine absorption (Zobel *et al.*, 2017).

PTH has receptors widely expressed in bone on cells of osteoblast lineage and in kidney proximal and distal tubule cells. While the major physiological regulator of PTH secretion is the ionized calcium level through interaction with the calcium sensing receptor, PTH secretion is also modulated by serum phosphate, FGF23, and 1,25-dihydroxyvitamin D3 (Lederer, 2014). Even minor changes in the extracellular ionized calcium concentration cause large changes in PTH release, and the serum level of PTH may be considered a marker of the body's calcium status (Sneve *et al.*, 2008).

The normal physiological role of PTH on skeletal homeostasis, when secreted endogenously, is more complex, but probably serves to regulate bone remodeling rather than overall skeletal mass. Within the physiological range of concentrations, PTH stimulates the bone forming activity of osteoblasts at the same level as the bone-losing activity of osteoclasts (Misiorowski, 2011).

2.4.1 Action and Regulation of Parathyroid Hormone

PTH is a hormone essential in the process of bone remodeling in the regulation of calcium levels in the blood. Its effect is exerted by binding to receptors in the membrane of osteoblasts and tubular cells of the kidneys. It stimulates mature osteoblasts, which are unable to proliferate and to produce growth factors such as FGF-2 and IGF-1, which stimulates proliferation osteoprogenitor cells that have the receptor for PTH. FGF (fibroblast growth factor) is the primary mediator of action of

PTH on bone. PTH leads to the accumulation of a multilayer of osteoblasts at sites of bone formation (Milivojac[1] *et al.*, 2015).

Osteoblasts, bone lining cells, and bone marrow stromal cells have PTH receptors, and intermittent PTH stimulates these cells through the modulation of cAMP concentrations and cAMP-dependent protein kinase A. The PTH receptor also activates the calcium protein kinase C pathway, stimulating proliferation of cells in the osteoblastic lineage (Borba and Mañas, 2010).

Regulation of syntheses and secretion of PTH with Ca ions includes the negative feedback mechanism: the reduction of calcium levels leading to increased secretion and activity of PTH, which stimulates osteoclasts (indirectly via osteoblasts) to produce cytokines which are stimulating osteoblast and osteoclast precursors (IL-1, PG E2, TNF, etc.), leading to bone resorption and release of calcium into the blood. PTH increases Ca reabsorption in the kidney, GIT absorption of Ca through stimulation of the synthesis of the D hormones, it increases re- sorption of phosphate from bones and increases excretion of phosphates (Milivojac[1] *et al.*, 2015). Additional mechanisms of PTH signal propagation and control include internalization of the PTH receptor its association with importins and its nuclear translocation, where it may regulate gene transcription. The exact signaling pathway responsible for the anabolic effect is not known, but the various pathways used by PTH may determine whether it has anabolic or catabolic actions (Borba and Mañas, 2010).

2.4.2 Hypoparathyroidism

Hypoparathyroidism is a rare disorder characterized by the presence of decreased serum calcium and absent or inappropriately decreased serum parathyroid hormone (PTH). This condition is most often acquired, but may also be inherited. The acquired form is most often due to removal of, or damage to, the parathyroid glands or their blood supply at the time of neck surgery for thyroid disease, head and neck cancer, or parathyroid disease. Postsurgical hypoparathyroidism is responsible for about 75% of acquired cases. The next most common cause in adults is thought to be autoimmune disease, affecting either only the parathyroid glands, or the parathyroid glands in addition to multiple other endocrine organs. Remaining cases are due to a variety of rare infiltrative disorders, metastatic disease, iron or copper overload, ionizing radiation exposure, or rare genetic disorders (Clarke, 2014).

2.4.3 Hyperparathyroidism

Hyperparathyroidism occurs when serum PTH levels are elevated relative to serum Ca levels. Although hyperparathyroidism is associated with abnormal Ca metabolism, 2%-5% of patients are asymptomatic. The diagnosis may be made upon incidental finding of hypercalcemia during routine blood tests or when routine BMD testing is abnormal (Velez *et al.*, 2016).

2.4.3.1 Primary Hyperparathyroidism

Primary hyperparathyroidism as a condition with a high bone turnover is caused by an increased secretion of parathyroid hormone (PTH). It is usually originated from a solitary parathyroid adenoma. PTH through its complex actions on the kidneys, gastrointestinal tract, and the bones is involved in the hemostasis of vitamin D, calcium, and phosphate. Primary hyperparathyroidism occurs with no symptoms in up to 80% of patients and increased levels of PTH are characterized during work up of hypercalcemia. Long-standing elevated serum calcium level can lead to renal insufficiency which is dependent to duration and degree of hypercalcemia. Generally, hyperparathyroidism is diagnosed through parathyroid immunoassay. When high PTH has been confirmed, the high serum level of calcium is confirmatory factor for primary hyperparathyroidism (Einollahi *et al.*, 2014).

Primary hyperparathyroidism (PHPT) is a disease caused by overactive parathyroid glands with consequent hypercalcemia. The main cause in 85%-90% of cases, is the presence of a solitary parathyroid adenoma. In the other affected patients, hyperplasia or multiple adenomas occur, the latter common in familial forms. PHPT occurs most commonly in individuals over 50 years of age and in postmenopausal women, showing a prevalence of about 0.78% in patients evaluated in reference services (Francisco Bandeira *et al.*, 2013).

PHPT can have significant, insidious effects on the body, most commonly increasing bone turnover and decreasing bone mineral density (BMD). Failure to correct bone abnormalities has a negative impact on fracture risk (Velez *et al.*, 2016).

2.4.3.2 Secondary Hyperparathyroidism

Secondary hyperparathyroidism (SHPT) is an adaptive and in many cases ultimately maladaptive process that develops in response to declining kidney function, impaired phosphate excretion, and

failure to bio activate vitamin D. Dysregulation of calcium and phosphorous homeostasis leads to decreased renal phosphate excretion, increased serum phosphorous, elevated levels of the phosphatonin fibroblast growth factor 23 (FGF-23), and reduced synthesis of calcitriol, the active form of vitamin D. These changes result in increased synthesis and secretion of parathyroid hormone (PTH) and parathyroid hyperplasia, contributing to the development of a vicious cycle (Cunningham *etal.*, 2011).

In some patients with advanced renal failure, hypercalcemia is due to progression from appropriate parathyroid hyperplasia to autonomous overproduction of PTH, a disorder termed tertiary hyperparathyroidism. Patients with normocalcemic hyperparathyroidism may present with low bone density, osteoporosis, or a fragility fracture. Many of these patients will probably evolve into having hyperparathyroidism, although the exact natural history is not known. It is important to exclude vitamin D deficiency and chronic kidney disease before making this diagnosis (Kelly *et al.*, 2013).

2.5 PTH, Calcium and Phosphorus Status in Osteoporosis

Decrease in serum calcium level and increase in phosphorus and PTH levels in osteoporosis patient when compared to control group was reported by,(Nadu *et al.*, 2016, Surya Prabha *et al.*, 2015 and Al-Daghri *et al.*, 2014). Also (Khatake and Jadhav, 2014) reported significant decrease in serum calcium in postmenopausal women (08.32 ± 0.43 mg/dl), compared to control group (09.05 ± 0.50 mg/dl) p. value <0.0001. other study showed significant increase in serum calcium but no significant change in serum phosphorus in postmenopausal women compared to control p. value >0.05 ,(Mishra, *et al.*, 2015b) . other study stated that there was no significant difference in mean level of calcium, phosphorus and parathyroid hormone and BMI in osteoporosis patient (8.7±0.38, 3.76±0.40, 65.57±35.91, 28.4±4.5 respectively) and control group (9.09±0.39, 3.95±0.59, 69.43±24.55, 27.7±4.3 respectively) (Razmandeh *et al.*, 2014). other previous study show significant decrease in mean of BMI in osteoporotic patients (30.4 ± 5.29) compared to control group (33.1 ± 5.28) and insignificant deference in serum calcium and parathyroid hormone between osteoporotic patients and control group (Kharroubi *et al.*, 2017).

CHAPTER THREE

3. Materials and Methods

3.1 Materials:

3.1.1 Study Design

This was analytical case control study

3.1.2 Study Area and Duration

This study was conducted in Khartoum state in period between May and December 2018.

3.1.3 Study Population

A total of 90 Sudanese women selected by simple random sampling technique were included in this study, 50 women were attended to Dr. Mawahib Aledrisi clinic at Alryan laboratory and diagnosed to have osteoporosis by DEXA scan (BMD T-score less than -2.5) as case group, the remaining 40 women were apparently healthy as control group.

- **Inclusion Criteria**

  All women diagnosed to have osteoporosis were included in this study.

- **Exclusion criteria**

  Women with other bone disease, renal disease and parathyroid gland problem were excluded.

3.1.4 Ethical Consideration

The study was approved by scientific committee of Clinical Chemistry Department, college of Medical Laboratory Science, Sudan University of Science and Technology. And verbal approval was taken from each participant and demographic data collected using questionnaire (appendix I).

3.1.5 Specimen Collection

three ml of venous blood was collected from each subject using standard vein puncture technique directly on lithium heparin container and then centrifuged at 3500 r/m for 10 minute to obtain plasma. After centrifugation the plasma separated from mixture using automatic pipette and placed in Eppendorf tube and stored at -30° until time of analysis.

3.2. Methods:

3.2.1. Method of Calcium Estimation

Estimation of total plasma calcium was done by Methyl Thymol Blue (MTB) method using reagents supplied by BioSystems company.

3.2.1.1 Principle of Calcium Estimation

Calcium in the sample react with methylthymol blue in alkaline medium forming colored complex that can be measured by spectrophotometry. Hydroxyquinoline is included in the reagent to avoid magnesium interference. (appendix II)

3.2.2 Method of Phosphorus Estimation

Estimation of plasma phosphorus was done by phosphomolybdate method using reagents supplied by BioSystems company.

3.2.2.1 Principle of Phosphorus Estimation

Inorganic phosphorus in the sample reacts with molybdate in acid medium forming a phosphomolybdate complex that can be measured by spectrophotometry. (appendix III)

3.2.3 Method of Parathyroid Hormone Estimation

Parathyroid hormone was estimated by using DIASource hPTH ELISA kit.

3.2.3.1 Principles of The Method

The DIAsource hPTH-ELISA is a solid phase Enzyme Amplified Sensitivity Immunoassay performed on breakable microtiterplates. Calibrators and samples react with the capture polyclonal antibodies (PAb, goat anti 1-34 PTH fragment) coated on microtiter well. After incubation, the excess of antigen is removed by washing. Then monoclonal antibodies (MAb, mouse anti 44-68 PTH fragment) labelled with horseradish peroxidase (HRP) are added. After an incubation period allowing the formation of a sandwich: coated PAbs – human PTH – Mab – HRP, the microtiterplate is washed to remove unbound enzyme labelled antibody. Bound enzyme-labelled antibody is measured through a chromogenic reaction. The chromogenic solution (TMB) is added and incubated. The reaction is stopped with the addition of Stop Solution and the microtiterplate is then read at the appropriate wavelength. The amount of substrate turnover is determined

colourimetrically by measuring the absorbance, which is proportional to the PTH concentration. A calibration curve is plotted and PTH concentration in samples is determined by interpolation from the calibration curve. The use of the ELISA reader (linearity up to 3 OD units) and a sophisticated data reduction method (polychromatic data reduction) result in a high sensitivity in the low range and in an extended calibration range. (appendix 4)

3.3 Quality Control:

To ensure the accuracy of the results the normal and pathological control sera and calibrators, calibrated pipettes and instruments and clean wares were used.

3.4 Statistical Analysis:

The data obtained was analyzed using Statistical Package of Social Science (SPSS) software program, the students' T test was used to compare between means and person's correlation test used for correlations between variables. the level of significance was set at $p < 0.05$. Results were expressed as Mean ± Standard Deviation (SD).

CHAPTER FOUR

4. Results

The present study includes 90 Sudanese women 50 are osteoporotic patients as case group and 40 women are healthy control group.

Table 4.1 show descriptive statistics of study variables represent, minimum and maximum value for different study variables for both osteoporotic patients and control group.

For osteoporotic patients the minimum age was 24 and maximum age 90 years, the minimum weight was 42.5 kg and maximum weight was 93.7 kg, the minimum high was 126 cm and maximum high was 178 cm, the minimum level of calcium was 1.2mg/dl and maximum level was 10.3 mg/dl, the minimum level of phosphorus was 2.0 mg/dl and maximum level was 6.5 mg/dl, the minimum level of parathyroid hormone 14.2 pg/ml and maximum level was 183 pg/ml, the minimum level of BMI was 15.3 kg/m^2 and maximum level was 45.3 kg/m^2.

For control group the minimum age was 26 years and maximum age was 81 years, the minimum weight 36.5 kg and maximum weight was 108.2 kg, the minimum high was 146 cm and maximum high was 179 cm, the minimum level of calcium was 6.0 mg/dl and maximum level was 10.9 mg/dl, the minimum level of phosphorus was 2.0 mg/dl and maximum level 6.5 mg/dl, the minimum level of parathyroid hormone was 5.1 pg/ml and maximum level was 175.5 pg/ml, the minimum level of BMI was 15.1 kg/m^2 and maximum level was 41.4 kg/m^2.

Table 4.3 shows comparisons of deferent variable means between case and control. shows a significant decrease in mean level of calcium in osteoporotic patients (7.30±2.28mg/dl) when compare to control group (8.82±1.15mg/dl), p.value: 0.000, Also significant increase in parathyroid hormone in cases (65.8±49.5pg/ml) when compared to control group (36.9±30.5 pg/ml) p. value 0.000, and no differences in phosphorus and BMI mean levels between case (4.42±1.12mg/dl, 29.7±5.9.kg/m^2 respectively) and control group (3.98±1.12 mg/dl, 28.1±6.7 kg/m^2), (p.value : 0.068 , 0.246, respectively).

Figure 4.1 shows no correlation between parathyroid hormone and calcium in osteoporotic women (r = 0.111 , p. value 0.441).

Figure 4.2 shows a significant positive correlation between parathyroid hormone and phosphorus in osteoporotic women (r = 0.296 , p. value 0.037).

Figure 4.3 shows no correlation between calcium and phosphorus in osteoporotic women (r = -0.71, p. value 0.623).

Figure 4.4. shows PTH mean level according to age group in osteoporotic women and control group.

Table 4.1: descriptive statistics of study variables.

Variable	sample	N	minimum	maximum
Age(year)	Case	50	24	90
	Control	40	26	81
Weight (kg)	case	50	42.5	93.7
	control	40	36.5	108.2
Height (cm)	case	50	126	178
	control	40	146	179
body mass index (kg/m^2)	Case	50	19.3	45.3
	Control	40	15.1	41.4
Calcium (mg/dl)	Case	50	1.2	10.3
	Control	40	6.0	10.9
Phosphorus (mg/dl)	Case	50	2.0	6.5
	Control	40	2.0	6.5
parathyroid (pg/ml) hormone	Case	50	14.2	183
	Control	40	5.1	175.5

Table 4.2: comparison of study variables between case and control group

Parameter	Case	control	p.value
BMI (kg/m^2)	29.7±5.9	28.1±6.7	0.246
Calcium (mg/dl)	7.30±2.28	8.82±1.15	0.000
Phosphorus (mg/dl)	4.42±1.12	3.98±1.12	0.068
Parathyroid hormone(pg/ml)	65.8±49.5	36.9±30.5	0.001

-Independent sample t-test was used to compare between means

-p. value of <0.05 considered significant

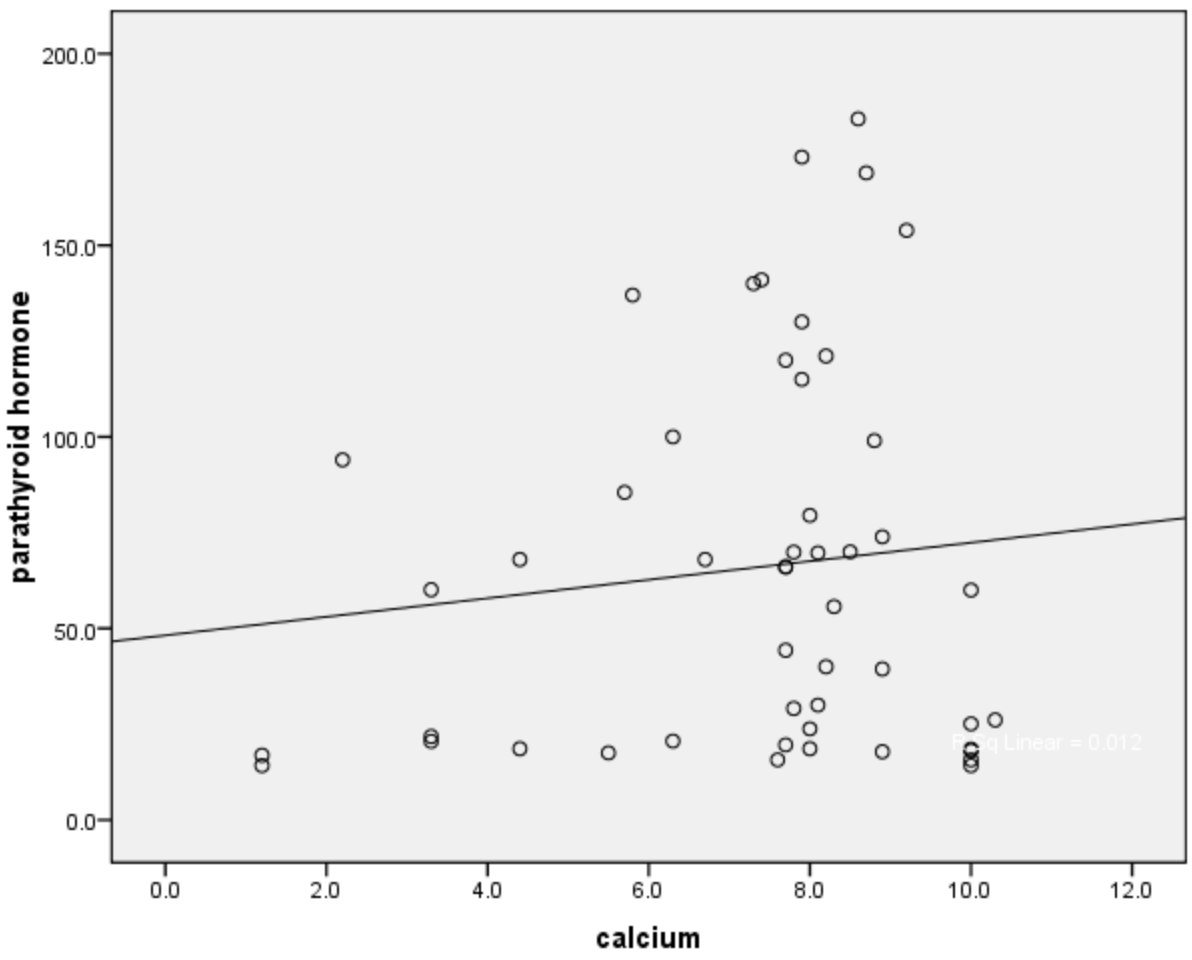


Figure 4.1: Correlation between PTH and calcium among osteoporotic women

r = 0.111 p. value 0.441

Y axis: Parathyroid hormone (pg/ml)

X axis: Calcium (mg/dl)

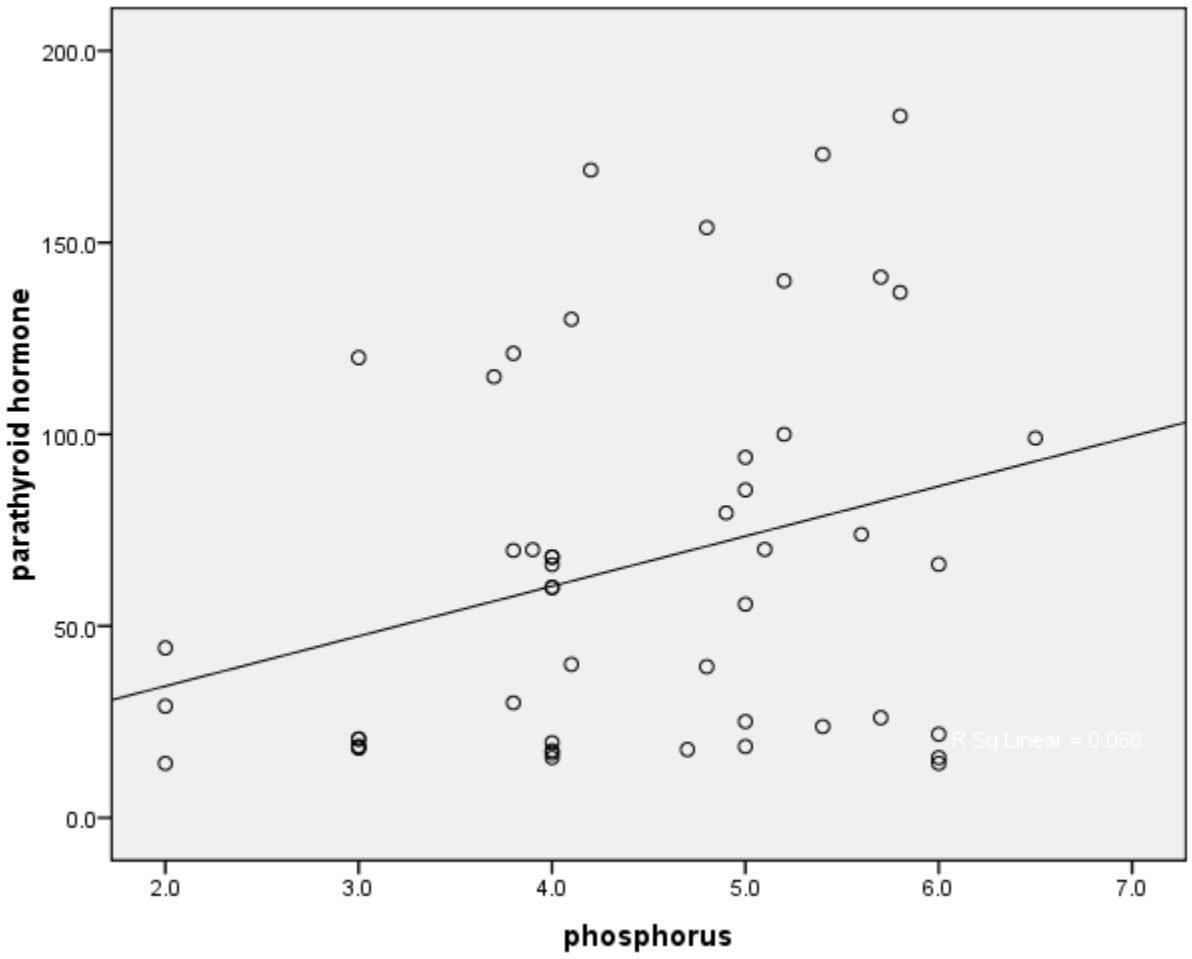


Figure 4.2: correlation between PTH and phosphorus among osteoporotic women

r = 0.296 p. value 0.037

Y axis: Parathyroid hormone (pg/ml)

X axis: phosphorus (mg/dl)

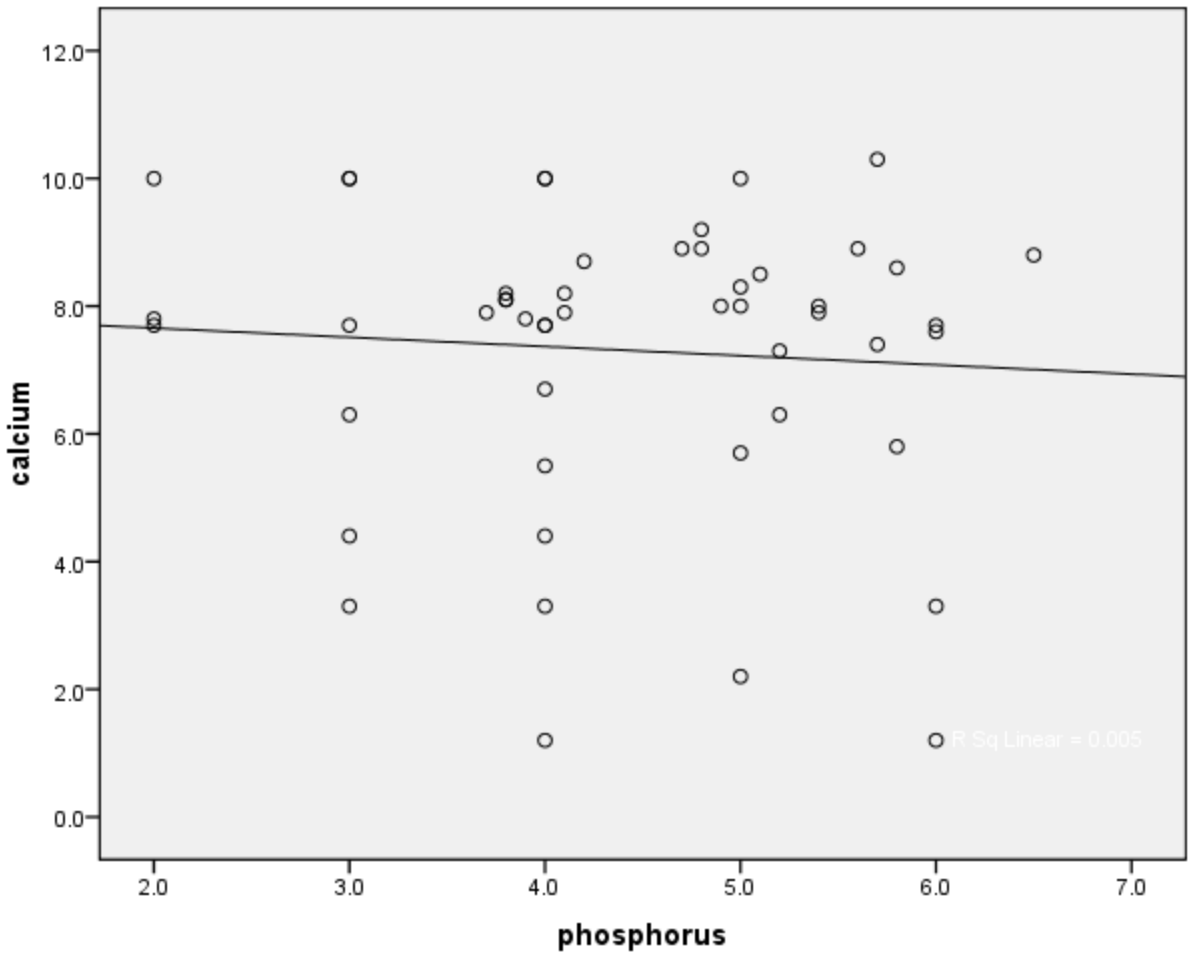
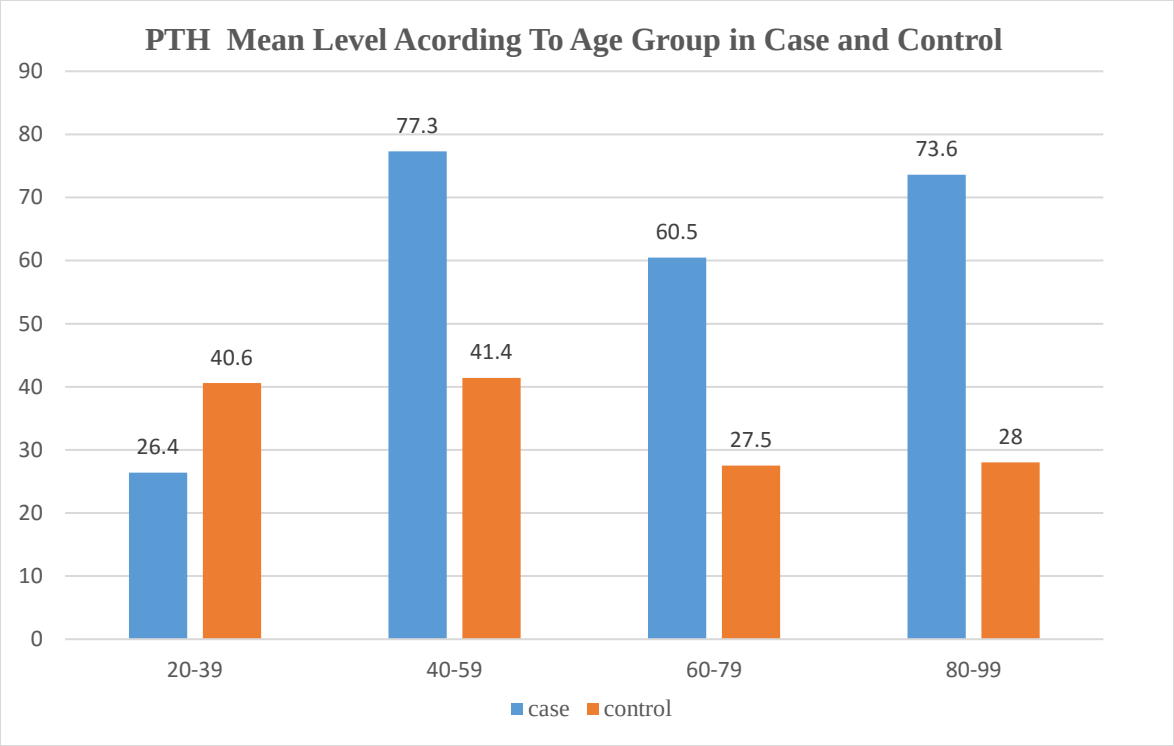


Figure 4.3: correlation between calcium and phosphorus among osteoporotic women

r = -0.071 p. value 0.623

Y axis: Calcium (mg/dl)

X axis: Phosphorus (mg/dl)


PTH Mean Level Acording To Age Group in Case and Control
90
80
70
60
50
40
30
20
10
0
26.4
40.6
77.3
41.4
60.5
27.5
73.6
28
20-39
40-59
60-79
80-99
case
control

CHAPTER FIVE

5. discussion, conclusion and recommendations

5.1 Discussion

Osteoporosis is a metabolic disorder of bone characterized by low bone mineral density and deterioration micro architecture of the bone tissue leading to increased skeletal fragility and increased fracture risk (V,sujatha *et al.*, 2015) . It is a degenerative bone disorder where there is thinning and weakening of the bone, and a general decrease in bone mass and density. So susceptible to fractures. Fractures related to osteoporosis are estimated to affect around 30% of women in developed countries and are a major health problem.(Surya Prabha *et al.*, 2015).

In this study the minimum age among cases was 24 years and maximum age was 90 years. The prevalence of osteoporosis increases with age and by World Health Organization (WHO) definition, up to 70% of women over the age 80 years have osteoporosis. (Mishra, M. Manju, *et al.*, 2015b).

In our study we found that serum calcium level was decreased in cases when compared to control group ‘p’ value <0.000, which is statistically significant and significant increase in mean level of parathyroid hormone when compare to control group p. value 0.001.The values in the present study are in consistent with the study of (Nadu *et al.*, 2016) (Surya Prabha *et al.*, 2015), (Khatake and Jadhav, 2014).but this finding was not agreed with results obtained by (Kharroubi *et al.*, 2017) done in Palestine and study done by (Razmandeh *et al.*, 2014) in Iran which report insignificant difference in calcium and parathyroid hormone level between case and control group.

Bone formation is a process in which deposition of inorganic mineral is controlled by an organic matrix. The mineral phase is composed of calcium and phosphorus. The concentrations of these ions in the plasma and extracellular fluid (ECF) influences the rate at which mineral is formed. When bone is resorbed calcium and phosphorus ions are released into the ECF and the organic matrix is resorbed. Ageing and loss of estrogen leads to a significant increase in osteoclastic activity. In addition, a decrease in calcium intake or impaired absorption of calcium from the gut lowers the serum level of ionized calcium. Decrease could be due to decline in either the active calcium transport or diffusion component of the calcium absorption system, probably a result of loss of direct effect of estrogen on calcium transport in gastro intestinal tract. Deficiency of calcium and malabsorption due to hormonal imbalance may lead to disorder of calcium absorption system,

probably a result of loss of direct effect of estrogen on calcium transport in gastro intestinal tract(Surya Prabha *et al.,* 2015). An inadequate intake of either calcium, vitamin D, or both will influence calcium-regulating hormones. It is also known that intestinal absorption of calcium decreases with age, this may be due to an age related decrease in serum levels of 1,25-dihydroxycholecalciferol. (Khatake and Jadhav, 2014).

A deficiency of either calcium or vitamin D will result in reduced calcium absorption and a lower concentration of circulating ionized calcium. When this occurs, parathyroid hormone (PTH) secretion is stimulated and there is a resulting increase in PTH levels. The cumulative effect of higher PTH levels, secondary to poor calcium and vitamin D nutrition (secondary hyperparathyroidism), is an increase in bone remodeling leading to significant loss of bone and an increased fracture risk (Mishra, M. Manju, *et al.*, 2015b).

In our study also, there was insignificant differences in phosphorus mean level between case and control group (p. value 0.068), this finding agreed with study done by (Mishra, M. Manju, *et al.*, 2015b), which reported no significant difference in phosphorus between osteoporotic women and control group p. value >0.05.and not agreed with studies done by (Al-Daghri *et al.*, 2014),(Surya Prabha *et al.,* 2015),(Nadu *et al.*, 2016) which report there were statistically significant increase in mean level of phosphorus and between osteoporosis patients and healthy control group.

Our study also showed statistically insignificant difference in mean level of BMI between case and control (p. value 0.246). the finding of this study agreed with study done by (Razmandeh *et al.*, 2014) which report that there were no significant differences in mean level BMI between osteoporotic group and control group.

In our study, there was insignificant positive correlation between PTH and calcium and significant positive correlation between parathyroid hormone and phosphorus, also insignificant negative correlation of calcium with phosphorus among osteoporosis patients.

5.2 Conclusion

The study concluded that, osteoporotic patients had decrease level of calcium and increase in PTH level when compared to healthy control group, while there was no difference in phosphorus and BMI between case and control and PTH is positively correlate with phosphorus in osteoporotic women.

5.3 Recommendations

1. Investigation of bone minerals should be done in postmenopausal women as routine for any change can lead to osteoporosis.
2. We recommend that women should be screened for osteoporotic fracture risks which might be an important strategy in the management of postmenopausal osteoporotic fracture risk.
3. Also we recommended that women should consume a diet rich in calcium and with sun light exposure for vitamin D absorption. Osteoporosis is associated with high morbidity and mortality, so preventive measures can be instituted with early diagnosis.
4. Further studies should be done to estimate ionized calcium, phosphorus, active parathyroid hormone, vitamin D, estrogen and ALP in women especially after menopause.

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APPEDICES

Appendix I

Questionnaire NO ◯

بسم الله الرحمن الرحيم

Sudan University of Science and Technology

Faculty of Graduate Studies

Estimation of Parathyroid Hormone, Calcium and Phosphorus Among Women with Osteoporosis in Khartoum State

(October, 2018)

Participant:

Name _____

Address _____

Phone number _____

Age _____

Marital status:

Single ☐ Married ☐ Divorced ☐ Widowed ☐

Do you have renal disease?

Yes ☐ No ☐

Laboratory investigation:

Biochemical Investigation	Results	Comment
Parathyroid Hormone		
Calcium		
Phosphorus		

COD 11527 4 x 50 mL	COD 11507 2 x 250 mL
STORE AT 15-30ºC	
Reagents for measurement of calcium concentration Only for *in vitro* use in the clinical laboratory	

CALCIUM - MTB

BioSystems
REAGENTS & INSTRUMENTS

CE

CALCIUM - MTB
METHYLTHYMOL BLUE

PRINCIPLE OF THE METHOD

Calcium in the sample reacts with methylthymol blue in alkaline medium forming a coloured complex that can be measured by spectrophotometry. Hydroxyquinoline is included in the reagent to avoid magnesium interference[1,2].

CONTENTS

	COD 11527	COD 11507
A. Reagent	2 x 50 mL	1 x 250 mL
B. Reagent	2 x 50 mL	1 x 250 mL
S. Standard	1 x 5 mL	1 x 5 mL

COMPOSITION

A. Reagent. Potassium cyanide 7.7 mmol/L, ethanolamine 1.5 mol/L.

B. Reagent. Methylthymol blue 0.1 mmol/L, hydrochloric acid 10 mmol/L, hydroxyquinoline 17 mmol/L.

S. Calcium/Magnesium Standard. Calcium 10 mg/dL (2.5 mmol/L), magnesium 2 mg/dL. Aqueous primary standard.

STORAGE

Store at 15-30ºC.

Reagents and Standard are stable until the expiry date shown on the label when stored tightly closed and if contaminations are prevented during their use.

Indications of deterioration:

– Reagents: Presence of particulate material, turbidity, absorbance of the blank over 0.800 at 610 nm.

– Standard: Presence of particulate material, turbidity.

REAGENT PREPARATION

Standard (S) is provided ready to use.

Working Reagent: Mix equal volumes of Reagent A and Reagent B (Note 1). Mix gently. Stable for 2 days at 2-8ºC.

ADDITIONAL EQUIPMENT

– Analyzer, spectrophotometer or photometer able to read at 610 ± 20 nm.

SAMPLES

Serum, heparinized plasma or urine collected by standard procedures.

Calcium in serum or plasma is stable for 10 days at 2-8ºC. Anticoagulants other than heparin should not be used.

Collect a 24-hour urine specimen in a bottle containing 10 mL of 50 % (v/v) nitric acid. Stable for 10 days at 2-8ºC. Centrifuge or filter and dilute ½ with distilled water before testing.

PROCEDURE

1. Pipette into labelled test tubes: (Notes 1, 2)

	Blank	Standard	Sample
Calcium Standard (S)	—	10 µL	—
Sample	—	—	10 µL
Working Reagent	1.0 mL	1.0 mL	1.0 mL

2. Mix thoroughly and let stand the tubes for 2 minutes at room temperature.
3. Read the absorbance (A) of the Standard and the Sample at 610 nm against the Blank. The colour is stable for at least 1 hour.

CALCULATIONS

The calcium concentration in the sample is calculated using the following general formula:

$$\frac{A_{Sample}}{A_{Standard}} \times C_{Standard} \times \text{Sample dilution factor} = C_{Sample}$$

If the Calcium Standard provided has been used to calibrate (Note 3):

	Serum and plasma	Urine
$\frac{A_{Sample}}{A_{Standard}}$	x 10 = mg/dL calcium x 2.5 = mmol/L calcium	x 20 = mg/dL calcium x 5 = mmol/L calcium

REFERENCE VALUES

Serum and plasma[3]: 8.6-10.3 mg/dL = 2.15-2.58 mmol/L

Urine[3]: 100-300 mg/24-h = 2.5-7.5 mmol/24-h

These ranges are given for orientation only; each laboratory should establish its own reference ranges.

QUALITY CONTROL

It is recommended to use the Biochemistry Control Serum level I (cod. 18005, 18009 and 18042) and II (cod. 18007, 18010 and 18043) to verify the performance of the measurement procedure.

Each laboratory should establish its own internal Quality Control scheme and procedures for corrective action if controls do not recover within the acceptable tolerances.

METROLOGICAL CHARACTERISTICS

– Detection limit: 0.6 mg/dL calcium = 0.15 mmol/L calcium.

– Linearity limit: 15 mg/dL calcium = 3.75 mmol/L calcium. For higher values dilute sample 1/2 with distilled water and repeat measurement.

– Repeatibility (within run):

Mean calcium concentration	CV	n
11 mg/dL = 2.75 mmol/L	0.7 %	20
13 mg/dL = 3.25 mmol/L	2.6 %	20

– Reproducibility (run to run):

Mean calcium concentration	CV	n
11 mg/dL = 2.75 mmol/L	3.9 %	25
13 mg/dL = 3.25 mmol/L	4.7 %	25

– Sensitivity: 30 mA·dL/mg = 120 mA·L/mmol

– Trueness: Results obtained with this reagent did not show systematic differences when compared with reference reagents (Note 3). Details of the comparison experiments are available on request.

– Interferences: Hemolysis (hemoglobin < 10 g/L) and bilirubin (< 20 mg/dL) do not interfere. Lipemia (triglycerides > 1.25 g/L) interfere. Other drugs and substances may interfere[4].

These metrological characteristics have been obtained using an analyzer. Results may vary if a different instrument or a manual procedure are used.

DIAGNOSTIC CHARACTERISTICS

Calcium is the most prevalent cation found in the body, distributed in bone (99%), soft tissues and extracellular fluid. Its concentration in plasma is regulated by parathyroid hormone, vitamin D and calcitonin.

Calcium ion is important in the transmission of nerve impulses, in the maintenance of normal muscle contractility, as a cofactor in certain enzyme reactions, and in the coagulation of the blood.

Hypercalcemia can be due to vitamin D intoxication, enhanced renal retention, osteoporosis, sarcosidosis, thyrotoxicosis, hyperparathyroidsm, multiple mieloma, idiopathic hypercalcemia of infancy, and carcinoma metastasic to bone[3,5].

Elevated calcium concentration in urine is found in nephrolithiasis and metabolic acidosis[3,5].

Hypocalcemia may be caused by primary and secondary hypoparathyroidism, pseudohypoparathyroidism, vitamin D deficiency, malnutrition and intestinal malabsorption[3,5].

Clinical diagnosis should not be made on the findings of a single test result, but should integrate both clinical and laboratory data.

NOTES

1. Contamination of glassware with calcium will affect the test. Use acid-washed glassware or plastic tubes.
2. These reagents may be used in several automatic analysers. Instructions for many of them are available on request.
3. Calibration with the provided aqueous standard may cause a matrix related bias, specially in some analyzers. In these cases, it is recommended to calibrate using a serum based standard (Biochemistry Calibrator, cod. 18011 and 18044).

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Quality System certified according to EN ISO 13485 and EN ISO 9001 standards

04/2011

COD 11508 170 mL
STORE AT 2-30ºC
Reagents for measurement of phosphorus concentration Only for *in vitro* use in the clinical laboratory

Appendix III
PHOSPHORUS

CE

PHOSPHORUS
PHOSPHOMOLYBDATE/UV

PRINCIPLE OF THE METHOD

Inorganic phosphorus in the sample reacts with molybdate in acid medium forming a phosphomolybdate complex that can be measured by spectrophotometry[1,2].

CONTENTS AND COMPOSITION

A. Reagent: 3 x 40 mL. Sulfuric acid 0.36 mol/L, sodium chloride 154 mmol/L.

DANGER: H314: Causes severe skin burns and eye damage. P280: Wear protective gloves/protective clothing/eye protection/face protection. P303+P361+P353: IF ON SKIN (or hair): Remove/Take off immediately all contaminated clothing. Rinse skin with water/shower.

B. Reagent: 1 x 50 mL. Sulfuric acid 0.36 mol/L, sodium chloride 154 mmol/L, ammonium molybdate 3.5 mmol/L.

DANGER: H314: Causes severe skin burns and eye damage. P280: Wear protective gloves/protective clothing/eye protection/face protection. P303+P361+P353: IF ON SKIN (or hair): Remove/Take off immediately all contaminated clothing. Rinse skin with water/shower.

S. Phosphorus Standard. 1 x 5 mL. Phosphorus 5 mg/dL (1.61 mmol/L). Aqueous primary standard.

For further warnings and precautions, see the product safety data sheet (SDS).

STORAGE

Store at 2-30ºC.

Reagents and Standard are stable until the expiry date shown on the label when stored tightly closed and if contaminations are prevented during their use.

Indications of deterioration:

– Reagents: Presence of particulate material, turbidity, absorbance of the blank over 0.500 at 340 nm.

– Standard: Presence of particulate material, turbidity.

REAGENT PREPARATION

Standard (S) is provided ready to use.

Working Reagent: Mix thoroughly in the proportion: 7 mL Reagent A + 3 mL Reagent B. Stable for 12 months at 15-30ºC.

ADDITIONAL EQUIPMENT

– Analyzer, spectrophotometer or photometer able to read at 340 ± 20 nm.

SAMPLES

Serum, heparinized plasma or urine collected by standard procedures.

Phosphorus in serum or plasma is stable for 7 days at 2-8ºC.

Collect 24-hour urine in a bottle containing 10 mL of 10% (v/v) hydrochloric acid. Stable for 10 days at 2-8ºC. Centrifuge or filter the sample and dilute 1/10 with distilled water before measurement.

PROCEDURE

1. Pipette into labelled test tubes: (Note 1)

	Reag.Blank	Sample Blank	Sample	Standard
Distilled Water	10 µL	—	—	—
Sample	—	10 µL	10 µL	—
Phos. Standard (S)	—	—	—	10 µL
Reagent (A)	—	1.0 mL	—	—
Working Reagent	1.0 mL	—	1.0 mL	1.0 mL

2. Mix thoroughly and let stand the tubes for 5 minutes at room temperature.
3. Read the absorbance (A) of the Sample Blanks at 340 nm against distilled water.
4. Read the absorbance (A) of the Samples and of the Standard at 340 nm against the Reagent Blank.

CALCULATIONS

The phosphorus concentration in the sample is calculated using the following general formula:

$$\frac{A_{Sample} - A_{Sample\ Blank}}{A_{Standard}} \times C_{Standard} \times \text{Sample dilution factor} = C_{Sample}$$

If the Phosphorus Standard provided has been used to calibrate (Note 2):

	Serum and plasma	Urine
$\frac{A_{Sample} - A_{Sample\ Blank}}{A_{Standard}}$	x 5 = mg/dL x 1.61 = mmol/L	x 50 = mg/dL x 16.1 = mmol/L

REFERENCE VALUES

Serum[3]: Adults: 2.5-4.5 mg/dL = 0.81-1.45 mmol/L
Children: 4.0-7.0 mg/dL = 1.29-2.26 mmol/L

Urine[3]: 0.4-1.3 g/24-h = 12.9-42 mmol/24-h

Concentrations in plasma are about 0.25 mg/dL (0.08 mmol/L) lower than in serum. These ranges are given for orientation only; each laboratory should establish its own reference ranges.

QUALITY CONTROL

It is recommended to use the Biochemistry Control Serum level I (cod. 18005, cod. 18009 and cod. 18042), level II (cod. 18007, cod. 18010 and cod. 18043) and the Biochemistry Control Urine (cod. 18054 and cod. 18066) to verify the performance of the measurement procedure.

Each laboratory should establish its own internal Quality Control scheme and procedures for corrective action if controls do not recover within the acceptable tolerances.

METROLOGICAL CHARACTERISTICS

– Detection limit: 0.13 mg/dL phosphorus = 0.042 mmol/L phosphorus.

– Linearity limit: 20 mg/dL phosphorus = 6.46 mmol/L phosphorus. For higher values dilute sample 1/2 with distilled water and repeat measurement.

– Repeatibility (within run):

Mean concentration	CV	n
4.34 mg/dL = 1.40 mmol/L	1.3 %	20
8.20 mg/dL = 2.65 mmol/L	0.7 %	20

– Reproducibility (run to run):

Mean concentration	CV	n
4.34 mg/dL = 1.40 mmol/L	2.9 %	25
8.20 mg/dL = 2.65 mmol/L	2.5 %	25

– Sensitivity: 48 mA·dL/mg = 149 mA·L/mmol

– Trueness: Results obtained with this reagent did not show systematic differences when compared with reference reagents (Note 2). Details of the comparison experiments are available on request.

– Interferences: hemoglobin (10 g/L), lipemia (triglycerides 10 g/L) and bilirubin (20 mg/dL) do not interfere. Other drugs and substances may interfere[4].

These metrological characteristics have been obtained using an analyzer. Results may vary if a different instrument or a manual procedure are used.

DIAGNOSTIC CHARACTERISTICS

Approximately 80% of the phosphorus in the human body is found in the calcium phosphate salts, which make up the inorganic substance of bone. The remainder is involved in the esterification of carbohydrate metabolism intermediaries and is also found as a component of phospholipids, phosphoproteins, nucleic acids and nucleotides.

Hypophosphatemia can be caused by shift of phosphate from extracellular to intracellular spaces, increased renal loss (renal tubular defects, hyperparathyroidism) or gastrointestinal loss (diarrhea, vomiting), and decreased intestinal absorption[3,5].

Hyperphosphatemia is usually secondary to inhability of the kidneys to excrete phosphate due to renal failure or hypoparathyroidism[3,5].

Clinical diagnosis should not be made on the findings of a single test result, but should integrate both clinical and laboratory data.

NOTES

1. These reagents may be used in several automatic analysers. Instructions for many of them are available on request.
2. Calibration with the provided aqueous standard may cause a matrix related bias, specially in some analyzers. In these cases, it is recommended to calibrate using a serum based standard (Biochemistry Calibrator, cod. 18011 and 18044).

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Appendix IV

Read entire protocol before use.

hPTH-ELISA

I. INTENDED USE

Immunoenzymetric assay for the *in vitro* quantitative measurement of human Intact Parathyroid Hormone (PTH) in serum and plasma.

II. GENERAL INFORMATION

A. Proprietary name : DIAsource hPTH-ELISA Kit

B. Catalogue number : KAP1481 : 96 tests

C. Manufactured by : DIAsource ImmunoAssays S.A.
Rue du Bosquet, 2, B-1348 Louvain-la-Neuve, Belgium.

For technical assistance or ordering information contact :
Tel : +32 (0)10 84.99.11 Fax : +32 (0)10 84.99.91

III. CLINICAL BACKGROUND

A. Biological activities

Human parathyroid hormone (hPTH) is a major physiological regulator of phosphocalcic metabolism. hPTH increases serum calcium concentrations by its actions on kidney (enhancing tubular Ca^{++} reabsorption and phosphate excretion) and bone (stimulating osteoclastic activity and bone resorption). It indirectly affects intestinal absorption of Ca^{++} by stimulating renal 1α-hydroxylation of 25 hydroxyvitamin D. The release of PTH is controlled in a negative feedback loop by the serum concentration of Ca^{++}.

PTH is synthesized in the chief cells of the parathyroid glands and secreted as an 84 amino acid molecule called "intact PTH", which is the main bioactive product. This molecule is degraded by proteolytic cleavage between amino acids 33-37 at peripheral sites to form biologically active amino-terminal fragments and biologically inactive carboxyl-terminal fragments. The carboxyl-terminal fragments are cleared only by glomerular filtration, while the bioactive intact PTH and amino-terminal fragments are also metabolically degraded in the liver and other tissues. The half-life of the carboxyl-terminal fragments increases dramatically in patients with renal failure. Thus, the measurement of intact PTH correlates best with the hormone production and biological activity.

B. Clinical application

The measurement of intact hPTH is used to establish the diagnosis of primary hyperparathyroidism by demonstrating elevated serum levels of bioactive PTH. It allows documenting the occurrence of secondary hyperparathyroidism in patients with Vit.D deficiency, intestinal malabsorption, or renal failure. In this last situation, the absence of interference with the inactive carboxyl-terminal fragments is especially valuable. The specificity and high sensitivity of the assay also allows differentiating clearly low serum PTH levels in hypoparathyroidism or in tumor-induced hypercalcaemia.

IV. PRINCIPLES OF THE METHOD

The DIAsource hPTH-ELISA is a solid phase Enzyme Amplified Sensitivity Immunoassay performed on breakable microtiterplates. Calibrators and samples react with the capture polyclonal antibodies (PAb, goat anti 1-34 PTH fragment) coated on microtiter well. After incubation, the excess of antigen is removed by washing. Then monoclonal antibodies (MAb, mouse anti 44-68 PTH fragment) labelled with horseradish peroxidase (HRP) are added. After an incubation period allowing the formation of a sandwich: coated PAbs – human PTH – MAb – HRP, the microtiterplate is washed to remove unbound enzyme labelled antibody. Bound enzyme-labelled antibody is measured through a chromogenic reaction. The chromogenic solution (TMB) is added and incubated. The reaction is stopped with the addition of Stop Solution and the microtiterplate is then read at the appropriate wavelength. The amount of substrate turnover is determined colourimetrically by measuring the absorbance, which is proportional to the PTH concentration.
A calibration curve is plotted and PTH concentration in samples is determined by interpolation from the calibration curve. The use of the ELISA reader (linearity up to 3 OD units) and a sophisticated data reduction method (polychromatic data reduction) result in a high sensitivity in the low range and in an extended calibration range.

V. REAGENTS PROVIDED

Reagents	96 tests Kit	Color Code	Reconstitution
Microtiterplate with 96 anti PTH (polyclonal antibodies) coated breakable wells	96 wells	blue	**Ready** for use
Ab **HRP** Conjugate: HRP labelled anti-PTH (monoclonal antibodies) in TRIS-maleate buffer with bovine serum albumin, thymol and sheep serum	1 vial 11 ml	red	**Ready** for use
CAL **0** Zero calibrator in human plasma and thymol	1 vial lyophilized	yellow	**Add** 3.0 ml distilled water
CAL **N** Calibrator N = 1 to 5 (see exact values on vial labels) in human plasma and thymol	5 vials lyophilized	yellow	**Add** 1.0 ml distilled water
WASH **SOLN** **CONC** Wash Solution (NaCl-Tween20)	1 vials 25 ml	brown	**Dilute** 28 x with distilled water (use a magnetic stirrer).
CONTROL **N** Controls - N = 1 or 2 in human plasma with thymol	2 vials lyophilized	silver	**Add** 1.0 ml distilled water
INC **BUF** Incubation Buffer with EDTA, Benzamidin and azide (< 0.1%)	1 vial 6 ml	blue	**Ready** for use
CHROM **TMB** Chromogenic Solution (TMB : Tetramethylbenzydine)	1 vial 25 ml	white	**Ready** for use
STOP **SOLN** Stopping reagent: HCl 1.0N	1 vial 25 ml	white	**Ready** for use

Note: 1. Use the zero calibrator for sample dilutions.
2. 1 pg of the calibrator preparation is equivalent to 1 pg of a synthetic PTH (1-84) from the Japanese Peptide Institute.

VI. SUPPLIES NOT PROVIDED

The following material is required but not provided in the kit:
1. High quality distilled water
2. Pipettes for delivery of: 50 µl, 100 µl, 200 µl, 1 ml, 2 ml and 3 ml (the use of accurate pipettes with disposable plastic tips is recommended)
3. Vortex mixer
4. Magnetic stirrer
5. Horizontal microtiterplate shaker capable of 700 rpm ± 100 rpm
6. Washer for microtiterplates
7. Microtiterplate reader capable of reading at 450 nm, 490 nm and 650 nm (in case of polychromatic reading) or capable of reading at 450 nm and 650 nm (monochromatic reading)
8. Optional equipment: The ELISA-AID™ necessary to read the plate according to polychromatic reading (see paragraph XI.A.) can be purchased from Robert Maciels Associates, Inc. Mass. 0.2174 USA.

VII. REAGENT PREPARATION

A. Calibrators : Reconstitute the zero calibrator with 3.0 ml distilled water and other calibrators with 1 ml distilled water.
B. Controls : Reconstitute the controls with 1 ml distilled water.
C. Working Wash solution : Prepare an adequate volume of Working Wash solution by adding 27 volumes of distilled water to 1 volume of Wash Solution (28x). Use a magnetic stirrer to homogenize. Discard unused Working Wash solution at the end of the day.

VIII. STORAGE AND EXPIRATION DATING OF REAGENTS

- Before opening or reconstitution, all kits components are stable until the expiry date, indicated on the vial label, if kept at 2 to 8°C.
- Unused strips must be stored, at 2-8°C, in a sealed bag containing a desiccant until expiration date.
- After reconstitution, calibrators and controls should be frozen immediately after use and kept at –20°C for 3 months. Avoid successive freeze thaw cycles.
- The concentrated Wash Solution is stable at room temperature until expiration date.
- Freshly prepared Working Wash solution should be used on the same day.
- After its first use, the conjugate is stable until expiry date, if kept in the original well-closed vial at 2 to 8°C.
- Alterations in physical appearance of kit reagents may indicate instability or deterioration.

IX. SPECIMEN COLLECTION AND PREPARATION

- Blood samples should be promptly separated from the blood cells.
- Serum and plasma must be kept at 2 - 8°C.
- If the test is not run within 8 hours, storage in aliquots at -20°C is recommended. Avoid subsequent freeze thaw cycles.
- Prior to use, all samples should be at room temperature. It is recommended to vortex the samples before use.
- It is advisable to assay serum samples.
- Do not use haemolysed samples.

X. PROCEDURE

A. Handling notes

Do not use the kit or components beyond expiry date.
Do not mix materials from different kit lots.
Bring all the reagents to room temperature prior to use.
Thoroughly mix all reagents and samples by gentle agitation or swirling.
Perform calibrators, controls and samples in duplicate. Vertical alignment is recommended.
Use a clean plastic container to prepare the Wash Solution.
In order to avoid cross-contamination, use a clean disposable pipette tip for the addition of each reagent and sample.
For the dispensing of the Chromogenic Solution and the Stop Solution avoid pipettes with metal parts.
High precision pipettes or automated pipetting equipment will improve the precision.

Respect the incubation times.
To avoid drift, the time between pipetting of the first calibrator and the last sample must be limited to the time mentioned in section XIII paragraph E (Time delay).
Prepare a calibration curve for each run, do not use data from previous runs.
Dispense the Chromogenic Solution within 15 minutes following the washing of the microtiterplate.
During incubation with Chromogenic Solution, avoid direct sunlight on the microtiterplate.

B. Procedure

1. Select the required number of strips for the run. The unused strips should be resealed in the bag with a desiccant and stored at 2-8°C.
2. Secure the strips into the holding frame.
3. Pipette 50 µl of Incubation Buffer into all wells.
4. Pipette 200 µl of each Calibrator, Control and Sample into the appropriate wells.
5. Incubate for 2 hours at room temperature on a horizontal shaker set at 700 rpm ± 100 rpm.
6. Aspirate the liquid from each well.
7. Wash the plate 4 times by:
   - Dispensing 0.4 ml of Wash Solution into each well
   - Aspirating the content of each well
8. Pipette 100 µl of anti-PTH-HRP conjugate into all the wells.
9. Incubate for 1 hour at room temperature on a horizontal shaker set at 700 rpm ± 100 rpm.
10. Aspirate the liquid from each well.
11. Wash the plate 4 times by:
    - Dispensing 0.4 ml of Wash Solution into each well
    - Aspirating the content of each well
12. Pipette 100 µl of the Chromogenic Solution into each well within 15 minutes following the washing step.
13. Incubate the microtiterplate for 30 minutes at room temperature on a horizontal shaker set at 700 rpm ± 100 rpm, avoid direct sunlight.
14. Pipette 200 µl of Stop Solution into each well.
15. Read the absorbancies at 450 nm and 490 nm (reference filter 630 nm or 650 nm) within 1 hour and calculate the results as described in section XI.

XI. CALCULATION OF RESULTS

A. Polychromatic Reading:

1. In this case, the ELISA-AID™ software will do the data processing.
2. The plate is first read at 450 nm against a reference filter set at 650 nm (or 630 nm).
3. A second reading is performed at 490 nm against the same reference filter.
4. The ELISA-AID™ Software will drive the reader automatically and will integrate both readings into a polychromatic model. This technique can generate OD's up to 10.
5. The principle of polychromatic data processing is as follows:
   - Xi = OD at 450 nm
   - Yi = OD at 490 nm
   - Using a standard unweighted linear regression, the parameters A & B are calculated : Y = A*X – B
   - If Xi < 3 OD units, then X calculated = Xi
   - If Xi > 3 OD units, then X calculated = (Yi-B)/A
   - A 4 parameter logistic curve fitting is used to build up the calibration curve.
   - The PTH concentration in samples is determined by interpolation on the calibration curve.

B. Bichromatic Reading

1. Read the plate at 450 nm against a reference filter set at 650 nm (or 630 nm).
2. Calculate the mean of duplicate determinations.
3. On semi-logarithmic or linear graph paper plot the OD values (ordinate) for each calibrator against the corresponding concentration of PTH (abscissa) and draw a calibration curve through the calibrator points by connecting the plotted points with straight lines.
4. Read the concentration for each control and sample by interpolation on the calibration curve.
5. Computer assisted data reduction will simplify these calculations. If automatic result processing is used, a 4 parameter logistic function curve fitting is recommended.

XII. TYPICAL DATA

The following data are for illustration only and should never be used instead of the real time calibration curve.

hPTH-ELISA		**OD units Polychromatic model**
Calibrator	0 pg/ml	0.050
	22 pg/ml	0.149
	70 pg/ml	0.344
	224 pg/ml	0.999
	666 pg/ml	2.721
	1400 pg/ml	4.483

XIII. PERFORMANCE AND LIMITATIONS

A. Detection Limit

Twenty zero calibrators were assayed along with a set of other calibrators. The detection limit, defined as the apparent concentration two standard deviations above the average OD at zero binding, was 2 pg/ml.

B. Specificity

Cross-reactive hormones or fragments were added to the zero calibrator, a high value calibrator (900 pg/ml) and a low value calibrator (100 pg/ml). The apparent PTH response was measured.

Cross-reactant	**No significant interference up to**
PTH 1-34 synthetic fragment, human	1000 pg/ml
PTH 44-68 synthetic fragment, human	20000 pg/ml
PTH 53-84 synthetic fragment, human	20000 pg/ml
PTH 73-84 synthetic fragment, human	100000 pg/ml
PTH-related protein 1-34 synthetic fragment, human	100000 pg/ml

C. Precision

INTRA ASSAY				INTER ASSAY			
Serum	**N**	**<X> ± SD (pg/ml)**	**CV (%)**	**Serum**	**N**	**<X> ± SD (pg/ml)**	**CV (%)**
A	10	41.0 ± 0.5	1.1	A	20	45.7 ± 3.3	7.1
B	10	594 ± 12	2.0	B	20	381 ± 11.1	2.9

SD : Standard Deviation; CV: Coefficient of variation

D. Accuracy

RECOVERY TEST

Sample	**Added PTH (pg/ml)**	**Recovered PTH (pg/ml)**	**Recovery (%)**
Serum	371	333	90
Heparin plasma	371	347	93
EDTA plasma	371	350	94

DILUTION TEST

Sample	**Dilution**	**Theoretical Concent. (pg/ml)**	**Measured Concent. (pg/ml)**
Serum	1/1	-	955
	1/2	477	506
	1/4	239	229
	1/8	119	124
	1/16	60	63
	1/32	34	34

Samples were diluted with zero calibrator.

E. Time delay between last calibrator and sample dispensing

As shown hereafter, assay results remain accurate even when a sample is dispensed 60 minutes after the calibrators have been added to the coated wells.

TIME DELAY					
	T0	15 min	30 min	45 min	60 min
S1	158	146	166	144	137
S2	77	72	70	67	72
S3	328	320	323	342	357
S4	260	250	250	258	251

XIV. INTERNAL QUALITY CONTROL

- If the results obtained for Control 1 and/or Control 2 are not within the range specified on the vial label, the results cannot be used unless a satisfactory explanation for the discrepancy has been given.
- If desirable, each laboratory can make its own pools of control samples, which should be kept frozen in aliquots. Controls which contain azide will interfere with the enzymatic reaction and cannot be used.
- Acceptance criteria for the difference between the duplicate results of the samples should rely on Good Laboratory Practises
- It is recommended that Controls be routinely assayed as unknown samples to measure assay variability. The performance of the assay should be monitored with quality control charts of the controls.
- It is good practise to check visually the curve fit selected by the computer.

XV. REFERENCE INTERVALS

These values are given only for guidance; each laboratory should establish its own normal range of values.

	N	Median (pg/ml)	Range (pg/ml)
Normal patients	156	29	16 - 46
Hyperparathyroidism	64	291	106 - > 1000
Hypoparathyroidism	11	0	0 – 6.4

The range is based on 5% to 95% percentiles.

XVI. PRECAUTIONS AND WARNINGS

Safety

For *in vitro* diagnostic use only.

The human blood components included in this kit have been tested by European approved and/or FDA approved methods and found negative for HBsAg, anti-HCV, anti-HIV-1 and 2. No known method can offer complete assurance that human blood derivatives will not transmit hepatitis, AIDS or other infections. Therefore, handling of reagents, serum or plasma specimens should be in accordance with local safety procedures.

All animal products and derivatives have been collected from healthy animals. Bovine components originate from countries where BSE has not been reported. Nevertheless, components containing animal substances should be treated as potentially infectious.

Avoid any skin contact with all reagents, Stop Solution contains HCl, the chromogen contains TMB. In case of contact, wash thoroughly with water.

Do not smoke, drink, eat or apply cosmetics in the working area. Do not pipette by mouth. Use protective clothing and disposable gloves.

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XVIII. SUMMARY OF THE PROTOCOL

	CALIBRATORS (µl)	**SAMPLE(S) CONTROLS (µl)**
Incubation buffer Calibrators (0-5) Samples, Controls	50 200 -	50 - 200
Incubate for 2 hours at room temperature with continuous shaking at 700 rpm. Aspirate the contents of each well. Wash 4 times with 400 µl of Wash Solution and aspirate.		
Anti-PTH-HRP	100	100
Incubate for 1 hour at room temperature with continuous shaking at 700 rpm. Aspirate the contents of each well. Wash 4 times with 400 µl of Wash Solution and aspirate.		
Chromogenic Solution	100	100
Incubate for 30 min at room temperature with continuous shaking at 700 rpm.		
Stop Solution	200	200
Read on a microtiterplate reader and record the absorbance of each well at 450 nm (versus 630 or 650 nm) and 490 nm (versus 630 or 650 nm)		

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