The Effect of Growth Hormone Release on Plasma and Erythrocyte Selenium and Zinc Levels in Children

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THE EFFECT OF GROWTH HORMONE RELEASE ON PLASMA AND ERYTHROCYTE SELENIUM AND ZINC LEVELS IN CHILDREN

by

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A Thesis submitted to the Faculty of
Old Dominion University in Partial Fulfillment of the Requirement for the Degree of

MASTER OF SCIENCE

CHEMISTRY

OLD DOMINION UNIVERSITY
August 1997

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ABSTRACT

THE EFFECT OF GROWTH HORMONE RELEASE ON PLASMA AND ERYTHROCYTE SELENIUM AND ZINC LEVELS IN CHILDREN

Maria Estela Legal de Reid
Old Dominion University, 1997
Director: Dr. Patricia Pleban

Research studies have suggested that selenium may be important in the release of growth hormone, GH. A selenium-containing amino-acid residue, selenocysteine, has been identified as part of the active site of the three isoenzymes (Type I, II and III iodothyronine 5’-deiodinase) responsible for the synthesis of active thyroid hormone, T₃, which is reported to stimulate GH secretion. The importance of zinc in growth is well-documented because of its involvement in transcription and expression of GH and its intermediary, insulin growth factor I or IGF-I. Thus, selenium and zinc levels in plasma may be altered in response to GH release such as occurs during GH evaluation testing.

Plasma and erythrocyte levels of selenium and zinc were measured in 43 short-stature children undergoing GH stimulation using clonidine, insulin-arginine and glucagon as the pharmacologic agents to provoke GH release. Children were classified as GH-deficient when they failed to respond to two of these agents. Baseline and post-administration specimens were collected and the elements assayed using Zeeman-effect atomic absorption spectroscopy. In addition, the long-term effects of the GH therapy on plasma and erythrocyte levels of these elements were investigated by collecting specimens at 2 and 3 months after initiation of therapy in five of the GH-deficient children.

We found no significant differences between baseline and post-administration levels of the elements in either GH-deficient or normal-responding children as evaluated by Student’s
t-test. However, we observed lowered selenium and zinc (p < 0.01) in plasma and erythrocytes from children undergoing GH therapy when we evaluated the data using analysis of variance.
To my father
ALFREDO STROESSNER

my mother
MARIA ESTELA

To my husband Frank and our daughters

STELI AND MELISSA
ACKNOWLEDGMENTS

I would like to extend my appreciation to my advisor Dr. Pleban for her constant encouragement and guidance during this program. Also I would like to thank the members of the committee, Dr. Laura Moen, Dr. John Donat, and Dr. Reuben Rohn, for their time in reviewing this thesis. I especially would like to thank Dr. Reuben Rohn for his collaboration. I would like to take this opportunity to thank Mrs. Rana Morris and everyone in the chemistry department who were always so helpful and kind to me.

I need to mention Mrs. Bonnie Reid for her loving care of my daughters while I was in this program and I must thank my dear husband Frank for giving his continued enthusiasm and support as I diligently work to accomplish my goals.
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CHAPTER I

INTRODUCTION

Human growth is stimulated by interaction of several hormones. Thyroid and steroid hormones play a relevant role; but growth hormone (GH), also known as somatotrophic hormone, is considered to be the most important /1/. In addition, normal development results from other factors such as genetics, a healthy environment and balanced nutrition /1/.

It has been established that trace elements and other micronutrients are important as part of our daily diet. The importance of zinc for growth and other actions has already been determined /2/. Several studies have demonstrated the direct “hormonal-like” effects of zinc on growth in children /3/ and it has been established that Zn stabilizes the hGH by promoting dimerization of two hGH monomers /4/.

Selenium has been identified as a cofactor of the antioxidant enzyme gluthatione peroxidase (GSH px) /5/ and also as a component of the active site of three isoenzymes, Type I, II, III iodothyronine 5’-deiodinases, which catalyze deiodination of thyroid prohormone, thyroxine (T₄), and active hormone, triiodothyronine (T₃), in rat and human liver /6,7,8/. Little is known about the effects of selenium on growth other than studies which report that toxic levels damage the liver and impair release of insulin-like growth factor (IGF I) /9/ which is an intermediary of the GH.

Deficiency also results in stunted growth. When selenium stores are low, the concentration of T₃ decreases in plasma because the deiodination of T₄ is impaired by decrease in production of the selenoprotein type I 5’-iodothyronine deiodinase /10/. Since

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The journal model used is Clinical Chemistry.
"T'3 is involved in growth hormone expression" depletion of selenium may result in poor growth /10/.

The present study was focused on the effect of growth hormone release on selenium and zinc levels in plasma and erythrocytes of children being assessed for growth hormone disorders using a growth hormone stimulation test. We hypothesized that plasma concentrations of selenium and zinc would increase several hours after the initiation of the growth hormone stimulation test in children with normal response to stimulation testing, due to mobilization of these trace elements for use in the tissues. In addition, we hypothesized that 2-3 months after the initiation of GH therapy in GH-deficient children (and possibly erythrocytes levels) of selenium and zinc would decrease due to the depletion of body stores of these trace elements during rapid growth.
CHAPTER II

BACKGROUND

Nutrients that are found in low concentrations (on the order of parts per million or less) in tissues of the body are designated micronutrients or trace elements. These micronutrients are consumed in the diet and are important for the well-being of the individual due to their participation in diverse biochemical reactions [11].

Selenium

Selenium (Se) is a metalloid located in group VI A of the periodic table and in its ionic forms has oxidation states of -2, +4, +6 [12]. In 1917, Jacob Berzelius, a Swedish chemist discovered selenium which he called Selene. But, in 1860, long before its discovery the symptoms of a gastrointestinal toxicity were described and designated "alkali disease" which were later attributed to selenium toxicity [12, 13].

In 1972, Rotruck et al. [5] demonstrated in laboratory rats that selenium plays an important role in maintaining the activity of the antioxidant enzyme, glutathione peroxidase (GSHpx). The identification of this selenoprotein and its antioxidant mechanism could be one of the reasons why tissue damage by free radicals is observed in Se deficiency [14]. By 1989 the role of Se as an antioxidant was established [14].

In 1952 it was reported that "the production of T₃ not only was produced directly from the thyroidal synthesis, but also from enzymatic deiodination of the prohormone T₄" [7]. In 1970 two publications confirmed this theory [15, 16]. It is now known that all the iodothyronine deiodinases are selenocysteine-containing enzymes [8]. Types I and II-iodothyronine deiodinase catalyzes the monodeiodination of the inner tyrosyl ring [7]. These deiodination reactions result in the production of both active and inactive thyroid
hormones, T₃ and reverse T₃, [7] as shown in Figure 1.

Selenoprotein P is another recently-identified extracellular glycoprotein that contains selenocysteine in its structure and has a molecular weight of 57-kDa. Its role has been partially elucidated and some studies suggest that it acts as a transport protein for Se or that it may be an antioxidant whose role is to “trap free radicals” [17,18]. In rats, selenoprotein P (about 60%) is the major form of Se of plasma selenium [17]. After injection of 25 μg/kg Se into selenium deficient rats, this selenoprotein appears within hours and has a half-life of 3 to 4 hours [18] in the plasma. The main sources of selenium in the animal diet are the selenoacids (selenite and selenate), selenocysteine and, in lower relative percentage, selenomethionine [19,20]. When selenite enters the blood stream it is rapidly taken up by red blood cells and approximately 70% is reduced by thiols to hydrogen selenide [20]. It then is released into the plasma where it is bound to the alpha and beta globulins as a selenosulfide and transported to the different tissues [20]. Selenoaminoacids are actively transported across the cell membrane [20]. There is now a large amount of evidence that a specific codon, UGA, exists in the m-RNA of prokaryotes [21,22] and eukaryotes for synthesis of selenoproteins [22]. This codon can be transduced to a specific selenocysteyl-tRNA (sec t-RNA) when the appropriate downstream signal is present. Selenocysteine can be synthetized by a conversion of ser-tRNA to sec-tRNA [22].

Glutathione peroxidase acts as an antioxidant as in Equation 1 shown below.

\[
2 \text{GSH} + \text{ROOH} \xrightleftharpoons{\text{GSHpx}} \text{R} \text{OH} + \text{H}_2\text{O} + \text{GSSG} \tag{1}
\]

The activities of some enzymes such as heme oxidases which promote oxidative reactions are increased [14]. Other selenium-dependent enzymes such as iodothyronine deiodinases are also affected by Se deficiency.
Fig. 1. Diagram showing the metabolism of thyroxine to active and inactive triiodothyronine.
In plasma, greater than 80% of the selenium is contained in proteins with half-lives ranging from a few hours to several days, making plasma Se levels an indicator of short-term Se nutritional status /23/. In erythrocytes, Se is primarily found in hemoglobin (as selenomethionine) or GSHpx /23/. In general, erythrocyte levels respond more slowly to Se supplementation due to their 120 day half-life in blood and are considered to be long term indicators of Se nutritional status /23/. Nutritional deficiency of selenium is thought to be involved in the pathogenesis of diseases such as Keshan disease which is observed in children and young people of certain rural areas of Keshan Province in China /12/. These individuals shared a common cardiomyopathy, skin rashes and other symptoms such as susceptibility to infections due to the suppression of the immune system as well as growth retardation. This disease could be attributed to the lack of Se in the soil and, consequently, in their food /12,14/, and was eliminated by giving selenium supplements. Se deficiency can also be seen in patients on prolonged total parenteral nutrition without Se supplementation. These patients developed “muscular dystrophy, cardiac affections and rheumatic arthritis” /12/. The symptoms could be prevented by Se supplementation /14/. In 1986 Levander concluded that the severity of the deficiency depends on the “duration of the deficiency, age and species” /24/.

The understanding of these mechanisms of selenoprotein action has confirmed the important biochemical role that selenium plays in the metabolism and, therefore, can be considered as a essential micronutrient /14/.

Zinc

Zinc is part of a large number of metalloenzymes (about 200) including the carbonic anhydrase isoenzymes which catalyze the reaction between carbon dioxide and water to
form carbonic acid \cite{25,26} and alkaline phosphatase found in mammalian cells specifically in the “intestinal endothelium and cells along the lining of the newly calcified bone”, which is important for the calcification of the bone \cite{26}. Other enzymes such as lactic and alcohol dehydrogenases are zinc metalloenzymes. Additionally zinc is a cofactor for catalytic activity of the RNA polymerases which makes it essential for gene expression \cite{26,27}. Zn\(^{2+}\) binds to the two cysteine and two histidine residues forming the characteristic tetrahedral structure found in “zinc finger proteins” \cite{28}, which are necessary for DNA transcription.

Because of its importance in metabolism, the recommended daily allowance (RDA) has been established. For adults of all ages and gender the RDA is about 15 mg \cite{11}. The important role that zinc plays in growth and in the prevention of certain pathological conditions has been stated by Todd et al. in 1934 \cite{25}. Further studies in different species of animals by other researchers confirmed Todd et al.’s findings \cite{25}. In 1963 evidence of growth retardation and infertility were observed in a nutritional zinc-deficient male \cite{25}. Some signs of zinc deficiency are anorexia, irritability, loss of hair, skin lesions in specific areas of the body, infections due to a depressed immunity, impaired blood coagulation due to reduced platelet aggregation, growth retardation and sexual hypofunction \cite{14}.

In addition to the condition of malnutrition and malabsorption, zinc deficiency is also found in alcoholism, pregnancy, severe burns, gastrointestinal disorders and chronic renal failure \cite{29}. The probability of finding zinc toxicity in humans appears to be very low in contrast with selenium which is a very toxic trace element \cite{11,14}. It has been reported that in all animal species, the signs of zinc deficiency were similar \cite{25}.

Growth Hormone

The genes for human growth hormone (hGH) is found on the long arm of the
chromosome 17 in a group of five loci: GH1 (normal growth hormone), hCS A and B or chorionic somatomammatropin, one pseudogene and another type of growth hormone, GH2. Further splicing of GH1 results in two different proteins one with MW of 20 kD that represents 15% of the circulating hGH and the second with MW of 22 kD /1,30/. The 22 kD protein consist of 191 amino acid chain and is mainly responsible for hGH action. The hGH is secreted by the acidophilic cells of the adenohypophysis or anterior lobe of the pituitary gland /4,30/. In addition, synthesis of hGH occurs in the secretory granules of the somatotroph cells where the new hGH molecule can also be stored by forming a complex in which Zn$^{2+}$ is chelated by the intermolecular disulfide bonds of the hGH resulting in a more stable dimeric structure. The release of hGH is accomplished by exocytosis and is Ca$^{2+}$ dependent /30/.

The three dimensional structure of the hGH contains four antiparallel alpha-regions (helices1,2,3,4) /30/ consisting of two peptide loops containing four cysteine residues bound together by disulfide bonds /4,30/. The structure is shown in Figure 2.

GH is released into the bloodstream where it binds to two specific growth hormone binding proteins (GHBP); one of low affinity and one of high affinity /31/. The high affinity GHBP primarily binds the major 22 kD form of hGH. These binding proteins where initially identified by Bauman et al. in 1986 /31,32/. Baumann and colleagues also identified the high-affinity binding protein as part of the extracellular hepatic hGH receptor /31,32/. Each binding protein can bind to one molecule of hGH and results in complexes of 85 kD (high affinity BP) or 125 kD (low affinity BP) with the low affinity protein /31/. This study suggested that the concentration of binding protein could serve to predict the receptor status /31/ and may be important in the proper action of the hGH /33/. 
Fig. 2. Molecular model of dimerized GH showing Zn$^{+2}$ in structure.
Growth Hormone Secretion

It is reported that the secretion of this hormone is in intermittent bursts and occurs after about ninety minutes of deep sleep. After birth, plasma levels of GH decrease and begin increasing again during puberty until finally decreasing in adulthood to maintenance levels. The stimulation or inhibition of the growth hormone secretion, like other hormones of the adenohypophysis is modulated by a hypothalamic neuropeptide growth hormone releasing hormone (GHRH) or growth hormone inhibiting hormone (GHIH) also called somatostatin. Growth hormone releasing hormone (GHRH) and somatostatin are secreted by the hypothalamus in response to signals (influenced by external and internal factors) that the central nervous system sends via the neurotransmitters /1, 30/.

Physical and emotional stress, hypoglycemia, hormones (e.g. thyroxine, sex steroids) and amino acids (e.g. arginine and leucine) all stimulate GH release /1, 30/. Therefore, growth hormone deficiency can be diagnosed in the laboratory by determination of the GH concentrations in blood from fasting patients (hypoglycemia), after physical stress, during the onset of sleep and after stimulation of the growth hormone secretion using pharmacological agents.

The pharmacological agents utilized in stimulating testing are based on the observation that growth hormone release is highly influenced by the level of glycemia. These agents, such as insulin act on the metabolism of carbohydrates producing hypoglycemia by different mechanisms resulting in growth hormone release. Arginine stimulates growth hormone release by suppressing GHIH and also promoting insulin release.

Clonidine and glucagon are alpha 2 adrenergic agonists which stimulate release of GHRH and, therefore, release of GH /34/. Insulin is also used as an stimulating agent because it
induces hypoglycemia. Generally, the pharmacologic stimulants used were combinations of insulin + arginine or glucagon + arginine which are given intravenously and provide optimal stimulation /35/.

In order to achieve many of GH actions, the release of an intermediary protein is necessary. This intermediary protein was initially called somatomedin C. Somatomedin C today is known as Insulin-like Growth factor I (IGF I) due to its analogy to the proinsulin hormone structure and its insulin-like effects which play a key role during prenatal development stage and after birth. This hormone, as with many other peptide hormones is secreted in a inactive “pro” form which is later transformed intracellularly to an active hormone /36/.

It has been suggested that a serine protease may be involved in the conversion of pro IGF I to the mature form IGF I by cleavage at the carboxyl terminus of an arginine residue /36/. The IGF I is produced and mainly in the liver and, in lower quantities, in almost all other tissues of the body /37/. Plasma IGF I contains 70 amino acid residues and is bound noncovalently to specific binding proteins insulin growth factor binding proteins (IGFBPs) /36,37/. The levels of the complex IGF I to its IGF BP depends on the GH secreted /32/. The administration of IGF I to humans stimulates lipid oxidation, inhibits protein catabolism and enhances sensitivity to insulin /37/. Although IGF I is an intermediary for GH activity, protein synthesis is an action only produced by growth hormone itself /37/.

Mechanism of Growth Hormone Action

Growth hormone not only promotes the enlargement of cells but also increases the number of cells /30/. The sequence of GH action starts with the binding of the hormone to its receptor. Wells et al. /38/ described a possible mechanism for growth hormone
binding to its receptor. The model suggests that growth hormone is released in a dimeric form from the pituitary gland. Once in the blood, it dissociates and one site of the monomeric form binds to site I on the receptor forming a membrane bound complex. The site II of the hGH in the primarily complex then binds to other receptor (dimerization of two receptors) [4, 36]. This mechanism is shown the Figure 3. The GH-dimerized receptors on the cell surface interact with transmembrane proteins called guanine nucleotide-binding proteins or G proteins. The signal is transferred to the interior of the cell by activation of the enzyme adenylate cyclase which catalyzes the conversion of ATP to cyclic AMP (c-AMP). The c-AMP is a second messenger which triggers a chain of reactions [28,30].

Studies have shown that there are two different mechanisms of growth hormone action. The direct action occurs when growth hormone produces anti-insulin effects. It enhances lypolysis in adipocytes liberating free fatty acids and gycerol, stimulates glycogenolysis and gluconeogenesis and decreases the uptake of glucose by cells. Protein synthesis also increases [4,30].

The indirect actions of the growth hormone are exerted through the growth factors. Growth hormone stimulates the production and release of IGF I from the liver. IGF I produces cell proliferation, especially of the chondrocytes which induces formation of cartilage and stimulates bone growth by “deposition of chondroitin sulfate and collagen” [1,32,37]. It allows the bone to grow in length and width. When the epiphyses are closed The indirect actions of the growth hormone are exerted through the growth factors. Growth hormone stimulates the production and release of IGF I from the liver. IGF I produces cell proliferation, especially of the chondrocytes which induces formation of cartilage and stimulates bone growth by “deposition of chondroitin sulfate and collagen” [1,32,37]. It
Fig. 3. Binding mechanism of GH to its membrane receptor.

II II Inactive receptor

III III Active receptor

- Growth hormone binding site I
- Growth hormone binding site II
allows the bone to grow in length and width. When the epiphyses are closed and linear growth is no longer possible, only growth in diameter occurs. As mentioned above the IGF I shows similar effects to insulin. A study by Hussain et al. [37] reported that the intravenous administration of IGF I in humans resulted in hypoglycemia lowering circulating free fatty acids and amino acids. The analogous effect of the IGF I with the insulin could be explained by a possible cross reactivity with the insulin receptor in some tissues [37]. The difference between insulin and IGF I is in the different distribution of their receptors and the fact that IGF I can act inhibiting release of GHRH and insulin secretion [1, 37, 39].

The feedback mechanisms involved in GH release are shown in Figure 4.

Selenium and Growth Hormone Secretion

Many selenoproteins have been identified but only a few have been well characterized. It is known that Se is important in regulation of the thyroid hormones. The first identified seleno-containing enzyme important in thyroid hormone action was type I 5’ - iodothyronine deiodinase (IDI). Later it was shown that the enzymes type II 5’- iodothyronine deiodinase(IDII) and type III 5 (IDIII) were also seleno enzymes [8].

Research, both in vivo and in vitro, has demonstrated that IDI and IDII are important for the deiodination of thyroxine, T₄, to its active form 3,5,3’ triiodothyronine, T₃ [6, 7]. Cells in the cortex of the thyroid gland produce T₄ which, upon entry tissue cells, is deiodinated by the one of these isoenzymes depending on the tissue site. It has been reported that IDI is located mainly in the liver and kidney. The enzyme, IDII, is found mainly in the brain, brown adipose tissue and in the pituitary and represents the major form of the isoenzymes in the tissue. Deiodinations in placenta, skin and brain are due to the activity of IDIII [40] to form inactive rT₃. Reactions are shown in Figure 5. All three of these enzymes are
EXTERNAL FACTORS → CENTRAL NERVOUS SYSTEM → NEUROTRANSMITTERS → HYPOTHALAMUS → GHRH (+) (-) SRIF → PITUITARY GLAND → GH

**Direct action**
- Decreases metabolism of carbohydrates
- Increases lypolysis
- Stimulates protein synthesis

**Indirect action**
- Production of IGF I
- Cartilage and bones

**Fig. 4.** Mechanism of GH Action.
Fig. 5. Deiodination mechanism by 5' I, II and 5 III iodothyronine deiodinases.
in the degradation of T4 to diodothyronine, T3. Studies have demonstrated that Se-deficient rats have a decrease in the concentration of circulatory triiodothyronine \[10\] and retardation of growth. T3 stimulates GH expression \[1, 9, 41\]. There is evidence that, in the rat, the thyroid receptor can induce growth hormone transcription of the growth hormone transcription of the growth hormone genes in the pituitary by binding to a specific site of the growth hormone promoter sequence on the gene \[41\]. Overall, thyroid hormones are important for skeletal maturation \[1\].

Although there is evidence of the importance that selenium may play in the process of growth, negative effects on growth of this ambiguous trace element have been demonstrated. Thorlacius-Ussing et al. \[13, 42\] observed a decrease in growth when toxic amount of NaSeO3, was administrated to rats for a short period of time. Selenium produced reduction in the release of growth hormone. The observed accumulate on the secretory granules of the somatotroph cells of the anterior pituitary and, in lesser amounts, in other cells such as thyrotrophs, corticotrophs, gonadotrophs. In addition, selenium was thought to replace the sulfur of the disulfide bridges of the growth hormone producing a change in the molecular form of the growth hormone \[13, 40\]. A previous study by Thorlacius-Ussing et al. \[9, 42, 43\] showed that oral and intraperitoneal doses of selenium in the form of sodium selenite accumulated in the pituitary bound to the zinc and, thus, impaired release. IGF I synthesis is also affected by the toxic levels of selenium \[13, 43\]. Exposed rats showed a decrease in IGF I due to decrease in growth hormone release; but mainly due to liver damage by accumulation of selenium in the tissues of that organ \[13\]. The net action is growth retardation \[9, 13, 42\].

Another possible relationship between GH and selenium is the role, as an antioxidant.
Se is present in the active site of glutathione peroxidase (GSH px). In vitro, the intake of Se decreased hemolysis and hemoglobin oxidation in erythrocytes of rats [5]. GSHpx is part of the enzymatic “antioxidant defense system” of the body [12]. The metabolism of oxygen results in the production of free radicals species and hydrogen peroxide in the various biological reaction that take place in anaerobic organism [12,44]. This selenoenzyme exerts its antioxidant activity in the cytoplasmic and in the mitochondrial matrix of the cells [12].

The role of the glutathione peroxidase is to catalyze the reaction between reduced glutathione (GSH), and H_2O_2 or lipid peroxide. During the reaction, glutathione is oxidized to form a disulfide linkage and the H_2O_2 or lipid peroxide is reduced to water or lipid alcohol. The net reaction is shown page 4. The selenium in this enzyme is easily converted to selenolate molecule because the pKa for -SeH is approximately 5.0. In this form the enzyme can act to reduce the peroxide substrate by transfer of an electron and producing an alcohol, and the selenolate ( -Se^-) residue of the enzyme is oxidized to selenic acid ( - Se OH). The sulfur of the glutathione binds to the Se of the enzyme residue and releases the hydroxyl group of the selenic acid forming a selenosulphide molecule. A second molecule of glutathione reacts with the selenosulphide and the oxidized form of the glutathione is produced [45]. This mechanism is shown in Figure 6. Glutathione peroxidase synthesis from dietary selenite takes several hours and the transport of selenium for synthesis of other selenoproteins appears to have priority. Researchers have suggested that cellular GSH px is the primary storage site of Se in the organism [23].

Zinc and Growth Hormone Secretion

The importance of zinc for growth has been extensively documented. This metal is important in the regulation of the transcription by being part of the zinc finger proteins [46].
Fig. 6. Catalytic antioxidant mechanism for GSH px activity.
The identification of nucleotide polymerase as a zinc metalloenzyme elucidated the importance of zinc in RNA and DNA synthesis and thus on growth [25, 26, 46, 47]. Zinc can be also found in the pancreas where it is stored by forming a complex with insulin [30]. Cunningham et al demonstrated that zinc is necessary for the binding of hGH to its human prolacting receptor [29] but not to the hGH receptor. Zinc diminishes binding of hGH to its receptor and enhances binding to the hprolacting receptor.

IGF I levels are reduced in zinc deficiency. This deficiency probably produced by a disruption in the growth hormone receptor signaling [29]. In addition, growth hormone binding protein mRNA is decreased in zinc deficiency. In plasma, zinc is bound to albumin and globulins, transported bound preferentially to transferrin rather than albumin [28].

A study of zinc deficiency children (and rats) showed impairment of growth; and when the children were subsequently supplemented with zinc, resulted in improvement of growth and an increase of IGF I levels in the plasma [48, 49]. Zinc also acts on other hormones. There is evidence that in zinc deficiency the levels of TSH, thyroxine and androgens all decrease [3,50]. Zinc is found in equimolar amounts levels with growth hormone in secretory granules of somatotroph of the adenohypophysis [4]. Zinc is involved in the dimerization of the growth hormone. See Figure 2. In vitro, the dimeric form of GH shows more resistance to denaturation with guanidine-HCl suggesting that the zinc form the most stable structure. Zinc gives stability to the hormone by chelation to histidine 17, histidine 21, glutamine 174 and disulphide asparagine [4]. Due to its greater stability the complex

\[ \text{Zn}^2+ (\text{hGH})_2 \] is the primary (about 70 %) storage form of GH in the secretory granules of the somatotoph of the anterior lobe of the pituitary gland [4]. GH1, once in the blood, dissociates and one molecule binds to its receptor in the cell membrane. The monomeric
growth hormone-receptor complex then binds to another receptor. The two receptors dimerize and this triggers a cascade of reactions [38]. Because the important role that plays zinc in the metabolism of thyroid hormones and in growth hormone secretion, an adequate amount of zinc will be necessary for development and maturation [3].
CHAPTER III

MATERIALS AND METHODS

METHODS

Equipment. The instrumentation used for determination of trace element selenium and zinc in blood was a polarized Zeeman-effect atomic absorption spectrophotometer (Hitachi Instruments, Inc. Model Z 8100) equipped with graphite furnace (for Se analyses) and flame attachments (for Zn analyses). An optical pyrometer was used to control furnace temperature and analyses were done using an autosampling system (model SSC 200). Pyrolytic graphite cuvets (Hitachi Instruments, Inc.) were used for the graphite furnace analyses. To determine hemoglobin concentration a UV-Visible spectrophotometer (Milton Roy Spectronic 1201) was used. Blood was collected using microtainers and butterfly syringes with multiple-draw needles (Beckton Dickinson). All plasticware was stored overnight in 5% HNO₃ solution and thoroughly rinsed with Type I water (<10 megohms cm⁻¹ in line), ≤ .05 mg Silicate/L passed through activated carbon, Millipore Systems, Inc.) before using. Metal- free pipette tips (Biorad, Inc.) and automatic pipettes (Brinkman Industries, Inc.) were used for liquid transfers.

Reagents. Selenium working standard solution were prepared using a H₂SeO₃ atomic absorption standard (1.000 g/L from Fisher Scientific). The matrix modifier for the selenium assay was prepared by dissolving 170 mg of PdCl₂ (Analytical Reagent Grade, Aldrich Chemical, Co.) and 500 μL of concentrated HCl (Analytical Reagent Grade, Fisher Scientific, Co.) into a 100 mL volumetric flask containing 75 mL of deionized water. The solution was stirred to dissolve the PdCl₂ and diluted to the mark with deionized water. The final concentrations were 9.4 mM PdCl₂ and 65 mM HCl. Prior to assay, 10 μL each of
Triton X-100 and Antifoam B (Sigma Chemical, Co.) were added to 10 mL of matrix modifier to minimize sample foaming in the graphite cuvet.

Zinc working standard solutions (0, 50, 100, 150, 200 ppm) were prepared from a 1000 ppm Zn atomic absorption standard in dilute HCl solution (Fischer Scientific Co.).

For the analyses low and high concentration metal control were included in each analytical run of plasma selenium and zinc plasma determination. A pooled red blood cell control was used for erythrocyte selenium and zinc assays.

Hemoglobin was assayed using a standard method for cyanmethemoglobin (see Appendix A). In addition, low, normal and high hemoglobin controls were run.

A 30 mM disodium ethylenediaminetetraacetate (Na₂EDTA) solution was prepared using crystallized commercial Na₂ EDTA (Primary Standard Grade, Sigma Chemical Co.) and deionized water.

Study Subjects. This study was approved by the IRB of the Medical College of Hampton Roads and was funded in part by an NIH grant. The 43 patients enrolled in this study were short stature children, one to sixteen years old, that were being evaluated for growth hormone status at Children’s Hospital of the King’s Daughter, Norfolk, VA. A child was classified as growth-hormone deficient when he/she failed to respond normally to two different pharmacological stimuli.

Subjects were grouped according to the following criteria:

a. Children with short stature but normal growth hormone response to stimulation testing served as the control group (N=25). These children did not have any other apparent illnesses.

b. Children with short stature and abnormal GH response to stimulation (all GH levels < 5
ng/mL during response period) (N=15).

c. Children with short stature, abnormal response due to panhypopituitarism (N=3)

**Specimen Collection and Handling.** Growth Hormone stimulation testing was done as an outpatient procedure at Children’s Hospital of the King’s Daughter (CHKD), Norfolk, VA.

A baseline blood specimen was collected between 7 and 8 am, followed by infusion of the GH stimulant. Blood was then collected at half-hour intervals for measurement of GH, T₄, FSH, LH, prolactin and glucose levels. Zinc and selenium were assayed in the baseline and in post infusion specimens collected at 60-120 minutes (clonidine) or 180 minutes (other pharmacological stimuli) specimens. The pharmacological stimulants used to provoke growth hormone release were clonidine, insulin+arginine, glucagon+arginine. Growth hormone concentration determination were performed by Nichols Institute Diagnostics, San Juan Capistrano, CA, USA using immunoassay techniques.

For trace element analyses approximately 1 mL of blood was immediately transferred to a microtainer containing 30 μL of 30 mM Na₂EDTA solution as anticoagulant. Plasma and red blood cells were separated by centrifugation at 1000 g for 10 minutes within 4 hours of collection. The plasma and erythrocytes were stored at -20 °C until assay.

**Hemoglobin Determination.** Specimens were diluted 4-fold and the hemoglobin level in these hemolysates was determined using the standard cyanmethemoglobin methodology.

**Selenium Analyses.** The graphite furnace determination of Se in plasma was performed on a 4-fold and 10-fold dilution of the samples. For erythrocytes on 4-fold dilution of specimen hemolyzates by following a modified method of Fan [51] shown in Appendix A. Concentrations of selenium in the specimens were determined by comparison of the specimen absorbance to the standard curve followed by multiplication by the appropriate
dilution factor. In the case of the erythrocyte selenium assays, the selenium concentration in the hemolyzate is divided by the Hb concentration in the hemolyzate to give \( \mu g \text{ Se/g Hb} \).

A typical standard curve for selenium analyses is shown in Figure 7.

**Zinc Analyses.** The flame atomic absorption analyses of plasma and erythrocyte zinc levels in specimens were performed using 10-fold and 20-fold diluted plasma and RBC zinc assay were done using a 25-fold dilution of the hemolyzates. The zinc absorbance for each specimen was determined by comparison to a standard curve and multiplied by the appropriate dilution factor. A complete description of zinc analyses is shown in Appendix A. As with the erythrocyte selenium analyses, zinc levels were normalized on the hemolyzate hemoglobin levels by dividing the specimen zinc concentration in the hemolyzate by the hemolyzate Hb level to give \( \mu g \text{ Zn/g Hb} \). A typical standard curve for zinc analyses is shown in Figure 8.

**Statistical Methods.** Results were calculated using the parametric Student’s t-test and multiple analysis of variance MANOVA as well as nonparametric Mann-Whitney and Kruskal-Wallis tests found in the Statistical Package for the Social Sciences (SPSS) computer program, version 2.0 using the Old Dominion University mainframe Unix computer.
Fig. 7. Typical standard curve for the selenium analyses ($Y = 0.0114 \text{ ppb} \cdot X + 0.0008$). Correlation coefficient, $r$: 0.9997.
Fig. 8. Typical standard curve for zinc analyses ($Y=0.0182 \text{ ppb} \times 0.0006$)
Correlation coefficient, $r$: 0.9999.
CHAPTER IV

RESULTS

Performance Parameters for the Analyses. Data on the analytical performance of the serum assays were obtained using commercial control specimens, over the data collection period beginning June 15, 1993 through October 12, 1995. During this period a pooled erythrocyte control was prepared and used to assess precision since no commercial controls were available. Precision studies and accuracy of trace element assays are shown in Tables 1 and 2. Between-run CV’s for the both selenium and zinc analyses ranged between 4 and 5% (N=20). Assay values for the low and high serum controls all fell within the acceptable ranges as shown in Tables 1 and 2. Mean values for the respective serum selenium and zinc were close to the reported commercial means (also shown in Tables 1 and 2).

Parameters of Study Population. The study population consisted of children undergoing growth evaluation at Children’s Hospital of the King’s Daughter, as in Tables 3 to 5. The control group was composed of children with short stature and normal response to GH stimulation testing. This consisted of 19 males and 6 females between ages 3 and 15 years. The short stature, growth hormone deficient children consisted of 12 males and 3 females with ages between 1 to 16 years and a second group of children that were growth hormone deficient due to panhypopituitarism consisting of 2 females and one male.

Subjects were also grouped according to other variables such as blood collection time (baseline, post administration of stimulating agent, time since start of the GH therapy).

In addition, all patients of our study population had normal concentration levels in thyroxine and TSH except the children with panhypopituitarism. Subjects in the panhypopituitary group with the exception of one had selenium and zinc levels within the reference range.
Table 1. Between-run precision studies for the selenium assay.

<table>
<thead>
<tr>
<th>Expected Mean value (Range)</th>
<th>Assay Mean value</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selenium assay N=20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UTAK Serum controls</td>
<td>96 (86 - 106)</td>
<td>102 (5)</td>
</tr>
<tr>
<td>normal range, µg/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UTAK Serum controls</td>
<td>320 (290 - 350)</td>
<td>308 (13)</td>
</tr>
<tr>
<td>high range, µg/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erythrocyte control</td>
<td>0.70 (0.03)</td>
<td></td>
</tr>
<tr>
<td>µg/g Hb</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The serum controls used were purchased commercially and reconstituted in the lab following the manufacturers recommended procedure. RBC control is an "in house" prepared from pooled human RBC blood specimens and diluted four-fold with Type I water.
Table 2. Between-run precision studies for the zinc assay.

<table>
<thead>
<tr>
<th></th>
<th>Expected Mean value (range)</th>
<th>Assay Mean value</th>
<th>CV,%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Zinc assay N=20</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UTAK Serum controls</td>
<td>122 ( 110 - 134 )</td>
<td>119 (6)</td>
<td>5</td>
</tr>
<tr>
<td>normal range, μg/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UTAK Serum controls</td>
<td>330 ( 300 - 360 )</td>
<td>328 (15)</td>
<td>5</td>
</tr>
<tr>
<td>high range, μg/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erythrocyte control</td>
<td>39 (2)</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>μg/g Hb</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The serum controls used were purchased commercially and reconstituted in the lab following the manufacturers recommended procedure. RBC control is an “in house” prepared from pooled human RBC blood specimens and diluted four-fold with Type I water.
### Table 3. Study population: Short stature children, normal GH response (N=25).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean</th>
<th>Std. Dev.</th>
<th>Reference Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>10</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Plasma Se, µg/L</td>
<td>125</td>
<td>52</td>
<td>* 99 - 205</td>
</tr>
<tr>
<td>RBC Se, µg/gHb</td>
<td>0.71</td>
<td>0.26</td>
<td>* 0.40 - 1.2</td>
</tr>
<tr>
<td>Plasma Zn, µg/dL</td>
<td>81</td>
<td>20</td>
<td>** 60 - 140</td>
</tr>
<tr>
<td>RBC Zn, µg/gHb</td>
<td>34</td>
<td>10</td>
<td>** 26 - 49</td>
</tr>
</tbody>
</table>

**From Pleban PA, Numerof BS, Wirth FH. Clinics in endocrinology and metabolism, 1985;14(3):545-566.
Table 4. Study Population: Short stature children, abnormal GH response due only to growth hormone deficiency (N=15).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean</th>
<th>Std. Dev.</th>
<th>Reference range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>9</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Plasma Se, μg/L</td>
<td>124</td>
<td>42</td>
<td>* 99 - 205</td>
</tr>
<tr>
<td>RBC Se, μg/gHb</td>
<td>0.71</td>
<td>0.23</td>
<td>* 0.40 - 1.2</td>
</tr>
<tr>
<td>Plasma Zn, μg/dL</td>
<td>86</td>
<td>20</td>
<td>** 60 - 140</td>
</tr>
<tr>
<td>RBC Zn, μg/gHb</td>
<td>36</td>
<td>9</td>
<td>** 26 - 49</td>
</tr>
</tbody>
</table>

** From Pleban PA, Numerof BS, Wirth FH. Clinics in endocrinology and metabolism, 1985;14(3):545-566.
Table 5. Study Population: Short stature children, abnormal GH response due to panhypopituitarism (N=3).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean</th>
<th>Std. Dev.</th>
<th>Reference range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>7</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Plasma Se, μg/L</td>
<td>134</td>
<td>59</td>
<td>* 99 - 205</td>
</tr>
<tr>
<td>RBC Se, μg/gHb</td>
<td>0.70</td>
<td>0.22</td>
<td>* 0.40 - 1.2</td>
</tr>
<tr>
<td>Plasma Zn, μg/dL</td>
<td>75</td>
<td>53***</td>
<td>** 60 - 140</td>
</tr>
<tr>
<td>RBC Zn, μg/gHb</td>
<td>41</td>
<td>12</td>
<td>** 26 - 49</td>
</tr>
</tbody>
</table>

** From Pleban PA, Numerof BS, Wirth FH. Clinics in endocrinology and metabolism, 1985;14(3):545-566.
*** The plasma Zn concentration for one of the patients included in the calculations is low resulting in a high standard deviation.
Comparison Between Groups. In order to establish the suitability of using parametric tests we evaluated each group using the Kolmogorov-Smirnoff Goodness of Fit test available in the SPSS software package. All selenium plasma and erythrocytes levels were normally distributed (p > 0.05) except for erythrocyte zinc concentrations in the control population (p= 0.031). Therefore, we used parametric statistical tests for comparisons for all parameters except erythrocyte zinc levels. When the baseline values for the study groups (shown in Tables 3 - 5) were compared using Student’s t-test (or Mann-Whitney U-test in the case of erythrocyte zinc levels), we found no significant differences between these groups.

Short Term Effect of GH Stimulation Tests on Plasma and RBC Selenium and Zinc Concentrations. We compared baseline and post-administration concentrations in both the control group and the GH - deficient populations using the Student’s t-test (Mann-Whitney U -test for erythrocyte zinc concentration) and observed no significant differences as a function of the stimulation test used or the collection time. Because there was no significant difference between the type of stimulation testing used, we pooled zinc and selenium results obtained for baseline and post administration collection periods for each group (trace element assays were done for only one set of stimulation test specimens). Data are shown in Table 6.

Long Term Effects of GH Therapy on Plasma and RBC Selenium and Zinc Concentrations. To investigate the effects of GH therapy we assayed blood specimens of five GH- deficient and panhypopituitary patients collected at 2 and 3 month after the start of the GH therapy to determine in plasma and erythrocytes if the levels of these elements dropped as growth began. Due to the small sample number in this group (GH-deficient, N=2 and panhypopituitary N=3) we pooled data from these groups.
Table 6. Response of plasma and erythrocyte selenium and zinc concentrations in the study population (N=43).

<table>
<thead>
<tr>
<th>Collection Time</th>
<th>Control</th>
<th>GH-deficient</th>
<th>Panhypopituitary</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (SD)</td>
<td>t-test **</td>
<td>Mean (SD)</td>
</tr>
<tr>
<td></td>
<td>pooled variance</td>
<td>2-tailed</td>
<td>pooled variance</td>
</tr>
<tr>
<td><strong>Plasma selenium</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>125 (52)</td>
<td></td>
<td>124 (42)</td>
</tr>
<tr>
<td>Post adm.</td>
<td>121 (42)</td>
<td>n.s.</td>
<td>128 (39)</td>
</tr>
<tr>
<td><strong>Plasma zinc</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>80 (20)</td>
<td></td>
<td>86 (20)</td>
</tr>
<tr>
<td>Post adm.</td>
<td>82 (15)</td>
<td>n.s.</td>
<td>91 (22)</td>
</tr>
<tr>
<td><strong>RBC selenium</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>0.71 (0.26)</td>
<td></td>
<td>0.70 (0.22)</td>
</tr>
<tr>
<td>Post adm</td>
<td>0.69 (018)</td>
<td>n.s.</td>
<td>0.73 (0.19)</td>
</tr>
<tr>
<td><strong>RBC zinc</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>34 (10)</td>
<td></td>
<td>35 (9)</td>
</tr>
<tr>
<td>Post adm</td>
<td>35 (10)</td>
<td>n.s.</td>
<td>34 (9)</td>
</tr>
</tbody>
</table>

All baseline and post administration values were not significantly different.

n.s. = not significant  * Mann-Whitney U-test
We found significantly lowered plasma Zn levels ($p = 0.01$). Plasma Se levels were lower, though not significantly, after initiation of GH therapy. RBC levels of both metals did not appear to be affected. See Table 7 for results of the Multiple Analysis of Variance performed using SPSS software program.
Table 7. Long term effect of GH stimulation on plasma and RBC selenium and zinc concentrations.

<table>
<thead>
<tr>
<th>Collection Time</th>
<th>Plasma Zn μg/dL Mean (SD)</th>
<th>RBC Zn μg/dL Mean (SD)</th>
<th>Plasma Se μg/L Mean (SD)</th>
<th>RBCSe μgSe/gHb Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>97 (15)</td>
<td>33 (8)</td>
<td>149 (55)</td>
<td>0.86 (0.14)</td>
</tr>
<tr>
<td>2 month</td>
<td>52 (12)</td>
<td>42 (17)</td>
<td>128 (56)</td>
<td>0.85 (0.31)</td>
</tr>
<tr>
<td>3 month</td>
<td>63 (27)</td>
<td>37 (17)</td>
<td>94 (33)</td>
<td>0.57 (0.43)</td>
</tr>
</tbody>
</table>
Fig. 9. Longitudinal changes in plasma selenium concentration of GH-deficient children after the start of the GH therapy.
\[ \text{Fig. 10. Longitudinal changes in plasma zinc concentration of GH-deficient children after the start of the GH therapy.} \]
CHAPTER V
DISCUSSION

Precision for the assay was sufficient to see changes due to biological variation. Individual variations or biological variation for each parameter (plasma and RBC selenium and zinc) were calculated using equation 2;

\[ SD^2_{\text{individual}} = SD^2_{\text{overall}} - SD^2_{\text{analytical}} \]  

(2)

The biological and analytical SD values for our study population are shown in Table 8 for both the short stature, control group and the short stature, growth hormone-deficient population. Using 5 % as CV for the analytical variation we found that biological components (due to the effects of nutrition, posture, age, metabolic differences, gender, race) accounted for the most of the variation seen in our results. Therefore, we concluded that these analytical methods for trace element analyses were suitable for use in this study.

We tested for a Gaussian distribution of our data results which will allow us to use parametric statistical tests. The nonparametric Kolmogorov-Smirnov Goodness of Fit Test showed a normal distribution of our sample population for all parameters studied with the exception of RBC zinc concentration in the short stature, normal children.

For this group we used the corresponding nonparametric, in this case the Mann-Whitney U-test in place of parametric T-test and the Kruskall-Wallis One Way ANOVA (for MANOVA).

We observed that after stimulation of the GH release there was no significant change in the levels of either trace elements selenium or zinc in plasma and erythrocytes. This may be
Table 8. Comparison of the biological and analytical components of the overall standard deviation for the analyses.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean</th>
<th>Sd_{overall}</th>
<th>* Sd_{analytical}</th>
<th>** SD_{biological}</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma Se, ( \mu g/L )</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>125</td>
<td>52</td>
<td>6.25</td>
<td>52</td>
</tr>
<tr>
<td>GH-deficient +</td>
<td>129</td>
<td>51</td>
<td>6.45</td>
<td>51</td>
</tr>
<tr>
<td><strong>RBC Se, ( \mu gSe/gHb )</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>0.71</td>
<td>0.26</td>
<td>0.03</td>
<td>0.24</td>
</tr>
<tr>
<td>GH-deficient +</td>
<td>0.70</td>
<td>0.22</td>
<td>0.03</td>
<td>0.21</td>
</tr>
<tr>
<td><strong>Plasma Zn, ( \mu g/dL )</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>81</td>
<td>21</td>
<td>4.05</td>
<td>21</td>
</tr>
<tr>
<td>GH-deficient +</td>
<td>80</td>
<td>36</td>
<td>4.00</td>
<td>36</td>
</tr>
<tr>
<td><strong>RBC Zn, ( \mu gZn/gHb )</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>34</td>
<td>10</td>
<td>1.1</td>
<td>9.9</td>
</tr>
<tr>
<td>GH-deficient +</td>
<td>38</td>
<td>10</td>
<td>1.9</td>
<td>9.8</td>
</tr>
</tbody>
</table>

\[
* \text{SD}_{\text{analytical}} = \frac{5 \% \text{CV}}{100} \times \text{mean} \\
** \text{SD}_{\text{biological}} = \sqrt{\text{SD}_{\text{overall}}^2 - \text{SD}_{\text{analytical}}^2}
\]
due to the longer time required for the hGH to exert its metabolic action in the organism. There is no apparent mobilization of selenium or zinc detectable up to 180 min after the initiation of GH stimulation testing. However, GH release did not occur until 60 to 90 min later for the clonidine test and 45 to 90 min later for the other stimulation testing. The short term mobilization of selenium and zinc has been noted; this may be due to the short duration between the administration of the pharmacological stimulant, the GH release and the sample collection time.

In order to check for the long term effects of the hGH administration, we used analysis of variance which enabled us to compare the growth hormone deficient children at 2 months and 3 months after initiation of the therapy. We concluded that plasma zinc dropped significantly with respect to time; this drop was due probably to the utilization of zinc during initiation of growth. This decrease was apparent after 2 months of therapy as shown in Figure 9. Zinc concentrations in plasma of the five patients in figure 9 showed a significant decrease as indicated in our results, shown in Table 7.

The importance of zinc for the process of growth has been well documented. The changes of zinc during the hGH release found in our study agreed with the conclusions of a previous researcher [55], suggesting that the method selected is adequate.

Collipp et al. [52] studied the importance of zinc on growth. He stated that in hypopituitary children the growth hormone therapy increased urinary zinc excretion and lowered serum concentration. In his study he concluded that "there are some children with growth hormone deficiency who are zinc deficiencies and some children with zinc deficiencies who are also growth hormone deficient". Nakamura et al./53/ showed that zinc supplementation induces growth in children with mild zinc deficiency and short stature.
without endocrinology abnormalities.

Another study by Cheruvanky et al.\textsuperscript{54} demonstrated that zinc supplementation improved growth rates in growth hormone-deficient children (detected by insulin and arginine stimulation test) who had been treated with hGH for at least three months. Our study indicates that earlier supplementation with zinc may be beneficial. All the studies above indicated an interrelationship between hGH and zinc.

Aihara et al.\textsuperscript{55} evaluated zinc, selenium and other trace element metabolism in fourteen growth hormone-deficient children ages 6 - 15 years and six patient ages 15 - 48 years suffering from acromegaly (high levels of hGH). The control group consisted of 40 healthy children ages 6 - 12 years old and forty healthy adults ages 20 - 56 years old. These researchers determined selenium and zinc concentrations by flameless atomic absorption spectroscopy in plasma and RBC of growth hormone deficient children before and after four to twelve months of hGH therapy. Their finding that the concentration levels of Zn in plasma were significantly reduced 4 - 12 months after the start of treatment. Their observations agree with our results. They also reported that RBC zinc concentrations in these growth hormone-deficient children were higher than in the “age matched” control group and became “normal” after 4 - 12 month after starting therapy. Their control group did not undergo GH stimulation testing and may have had undetected abnormalities due to the fact that they did not actually assay other endocrinological abnormalities. Additionally, since we only followed RBC zinc levels over a period of 3 months, we could not have detected the drop in zinc levels reported by Aihara et al.\textsuperscript{55}. In the study of Aihara et al.\textsuperscript{55}, the same analytical method was used to determined the effects of hGH on plasma and erythrocyte selenium concentrations. They observed a significant decrease of selenium after
4 to 12 months of growth hormone therapy. Also plasma selenium concentrations in acromegalic patients increased after treatment. These findings by Aihara et al.55/ demonstrate that hGH release has an influence or “affects” selenium metabolism “directly and / or indirectly”. We also observed a decrease in plasma Se over a three month period as shown in Figure 10. This decrease was not observed in RBC concentrations.

Research has established the importance of zinc for growth and suggested a possible relationship between growth hormone and selenium. We also conclude that growth hormone release can have a long term influence on plasma zinc concentrations as early as two months after the initiation of GH therapy. The interrelationship of growth hormone action and selenium is possible but more research needs to be done in order to elucidate this mechanism.

Although we only had 43 patients in our study, we determined trace element levels in a very large number of specimens in order to obtain this study group. We noted that many individuals did not return for growth hormone testing, limiting the number that could be used in our longitudinal study of the effects of GH therapy. Some of these individuals may have experienced so much improvement that they felt no further testing was necessary and this would bias our longitudinal study toward identifying zinc- and selenium-deficient patients.

We also noted that a higher percentage of males were tested relative to female patients. This was also true of the study done by Aihara et al. /55/ mentioned in this discussion. This gender bias was probably introduced by sociological factors. It is important to mention that the decrease in Se observed by MANOVA after a period of time would likely be significant if a larger number of subjects had been included in the study.
As mentioned earlier, the collection time for the post administration specimen may have been too soon after the administration of the GH stimulant in the investigation of the short term effects of GH release on the plasma levels of selenium and zinc. In addition, it may be that the speciation of the metals is altered rather than the total metal level during GH release. For example, it may be that the form of selenium which is taken up by the cells (selenoprotein P) is increased rather than the total selenium content of the plasma. Measurement of this species may be more meaningful. Likewise, much of the plasma zinc is bound to albumin, and determination of the unbound (to albumin) or small molecular weight species (which is thought to be the form available to the cells) may give more insight into the effects of GH release on short term zinc metabolism.
REFERENCES


41. Koenig RJ, Brent GA, WarneRL. Thyroid hormone binds to a site in the rat GH promoter Proc Natl Acad Sci 1987; 84: 5670-74.


APPENDIX A

Zinc in Serum / Plasma and Erythrocytes by Flame Atomic Absorption Spectroscopy

Principle

Zinc is determined using Zeeman-effect flame atomic absorption spectroscopy by direct aspiration of diluted plasma or erythrocyte hemolysate specimens. The zinc concentration is determined by comparison of absorbances to an aqueous standard curve.

Equipment

- Atomic absorption spectrophotometer (Hitachi Model Z 8100) equipped with flame attachments with Zeeman correction capabilities.
- Collection equipment consisting of syringe (Sarstedt, Inc.) and Trace Element vacutainers tubes without additive or with heparin or EDTA as preservative (Becton-Dickinson, Inc.).
- Polypropylene microcentrifuge tubes, (Fisher Scientific Co., Inc.); polypropylene test tubes and caps, conical bottom, 13x75mm(Baxter Scientific Co., Inc) and polypropylene test tubes and caps, 17x121mm (Fisher Scientific Co., Inc.).
- Automatic pipettes with fixed and variable volume (Eppendorf, Brinkmann Industries, Inc.) and metal-free tips (Bio-Rad Industries, Inc.).
- 100 mL Nalgene volumetric flask (Nalge Corporation, Inc.)

All plasticware are stored overnight in a 5% HNO₃ solution to remove of metal ion contamination on the surface. Rinse thoroughly with deionized water before using.

Reagents and Controls

- Concentrated nitric acid (Ultrapure Grade, Fisher Scientific Co., Inc. for assays, and Analytical Reagent Grade for acid washing procedures).
- Deionized water, Type I.
• Calibrators.
  a. 1.000 g/L Zinc atomic absorption standard solutions dilute HCL solution (Fisher Scientific Co.).
  b. Working standard containing 0, 50, 100, 150, 200 mg Zn/L solution and 50 mL of concentrated nitric acid.

• Controls.
  a. Serum Trace Elements Toxicology normal and high controls (UTAK, Lab.Inc.)
     Reconstitute with 5 mL Type I water.
  b. Hemolyzate control (for erythrocyte assay only). Prepared from pooled human red blood cell specimens and diluted fourfold with Type I water.

All reagents must be checked for expiration dates.

**Specimen Collection and Handling**

Venous blood or capillary blood is collected using a metal-free polypropylene syringe (Sarstedt, Inc.) or Trace Element vacutainer tubes (Becton Dickinson, Inc.) with heparin or EDTA as the anticoagulant for erythrocyte and plasma measurements or with no additive when serum is assayed. Separation of plasma and erythrocytes is done by centrifugation at 1000 g for 10 minutes within 4 hours of collection and stored frozen at -20 C until assay. Minimum amount of blood for the assay is 200 mL.

Hemolyzed specimens must be avoided; erythrocytes contain a high level of zinc and, therefore, hemolyzed specimens are not suitable for serum or plasma zinc analyses.

**Instrumental Analytical Conditions**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lamp current</td>
<td>5.0 mA</td>
</tr>
<tr>
<td>Wavelength</td>
<td>213.8 nm</td>
</tr>
<tr>
<td>Slit with</td>
<td>1.3 nm</td>
</tr>
<tr>
<td>Atomizer</td>
<td>Std Burner</td>
</tr>
<tr>
<td>Flame</td>
<td>C₂H₂-Air</td>
</tr>
<tr>
<td>Fuel Flow</td>
<td>1.5 l/min</td>
</tr>
<tr>
<td>Oxidant Press</td>
<td>160 kPa</td>
</tr>
<tr>
<td>Flow</td>
<td>15.0 l/min</td>
</tr>
<tr>
<td>Burner Height</td>
<td>7.5 nm</td>
</tr>
</tbody>
</table>
Assay procedure

1. Remove the specimens, normal, high and hemolysate controls from freezer.
2. Allow the samples to thaw at room temperature.
3. Write out the manifest sheet describing order of assay of standards, blanks, controls and specimens.
4. Rinse thoroughly with deionized water Type I five 13 mL (17 x 121mm) plastic test tubes with top for the working standard solutions and three (13 x 75mm) conical bottom plastic tubes for controls (for each set of 10 specimens) and one tube for each of the samples. Allow them to dry.
5. Turn on the instrument by pushing “on” of the power bottom.
6. The Zn program is automatically called if the program has been already loaded. If not, select the Zn flame program by pushing “memory recall” at bottom of the keyboard, go to edit mode and select Zn plasma /RBC Zn program from menu. Press “enter” and exit edit mode. Press the “program” button on the left, top side of the keyboard. The program menu will appear on the screen and analysis name should be zinc. Be sure that the parameter for atomization are flame and the autosampler mode is “off”.
7. Select analysis condition bottom. Zn parameter should appear on the screen.
8. Press “condition set” to warm the lamp. The warm up of the lamp takes about 15 minutes. Meanwhile proceed to prepare the Zn reference standard solution as describe in step 9.
9. Prepare Zn stock standard 1.000 mg/L or 1,000 ppm, concentration in dilute nitric acid by:
   a. Fill a clean, 100 mL Nalgene volumetric flask half ways with deionized water type I.
   b. Add 0,500 mL. of concentrated nitric acid (Ultra Pure Reagent Grade) to small amount of the acid to a tube first and transfer it from tube).
   c. Pipette 100 mL of the Zn reference standard solution (1,000 ppm) into the flask containing half filled deionized water and 0,500 mL of concentrated nitric acid.
   d. Fill the volumetric flask with deionized water out to its mark and mix gently by inversion at least 14 times.
   e. Label the volumetric flask. It is important to prepare a new working standard solution daily.
10. Check the lamp emission profile and by depressing the “profile” key and make sure that the lamp emission peak is sharp and that no interfering peaks appear in the band pass.

11. Organize the dry plastic test tubes for the analysis from step 4 in a rack, and label them starting with the five working standard solutions, the blank, the normal and high serum control and hemolyzate control followed by number of samples and additional controls. Run normal and high controls every ten samples. Cap the tubes and mix the samples by gently inverting the tubes.

12. Dilute plasma and controls as follows:

- **Zn normal control**: 10-fold dilution
- **Zn high control**: 20-fold dilution
- **Hemolyzate control**: 10-fold dilution

**Samples**

- **PLASMA**: Depending on the availability dilute 10-fold or 20-fold.
- **ERYTHROCYTES**: 25-fold dilution.

**10-fold dilution**: Transfer 100 mL of serum or normal control in a tube containing 900 mL of deionized water type I. (Hint: pipette 1 mL of water into the tube then take out 100 mL of water and add 100 mL of serum or normal control into the tube)

**20-fold dilution**: Transfer 100 mL of serum or high control in a tube containing 1,900 mL of deionized water type I. (Hint: pipette 2 mL of deionized water into the tube and then pipette 100 mL of water out and add 100 mL of serum or high control into the tube).

**25-fold dilution**: Transfer 40 mL of erythrocyte in a tube containing 960 mL of deionized water type I. (Hint: pipette 1 mL of deionized water into the tube then 40 mL of water out and add 40 mL of the erythrocyte sample and hemolysate control into the tube).

13. Prepare standard working solution by adding Zn atomic absorption standard solution HNO₃ washed tubes containing deionized water and concentrated nitric acid as shown in the following table.
<table>
<thead>
<tr>
<th>Number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I, H₂O, mL</td>
<td>10</td>
<td>9.5</td>
<td>9.0</td>
<td>8.5</td>
<td>8.0</td>
</tr>
<tr>
<td>HNO₃, mL</td>
<td>0.050</td>
<td>0.050</td>
<td>0.050</td>
<td>0.050</td>
<td>0.050</td>
</tr>
<tr>
<td>Zn Std, mL</td>
<td>0.000</td>
<td>0.5</td>
<td>1.0</td>
<td>1.5</td>
<td>2.0</td>
</tr>
<tr>
<td>Concentration, ppm</td>
<td>50</td>
<td>100</td>
<td>150</td>
<td>200</td>
<td></td>
</tr>
</tbody>
</table>

**Hint:** Pipette 10 mL of water into the tubes first and then pipette out 0.5, 1.0, 1.5, 2.0 mL of water and add the amount of Zn Std. solution prepared in step 8.

14. Press the “leak test” button to initiate the leak test.
15. Observe any warning signal by the instrument indicating a problem. If this occurs check the manual for correction procedures. Error message must be cleaned before proceeding.
16. Ignite the flame by pushing the “flame on/off” button. Flame should light.
17. Repeat 15.
18. Set signal monitor by depressing the “signal monitor” key. The signal monitor screen will appear. Set the absorbance / integrate scale to 0.10 abs units, the reference scale to 0.2 abs units, the reference position to 2, the time-scale to 1000 sec and the time constant to 1 second.
19. Zero the instrument by pushing “auto zero” button. When the instrument has finished establishing its zero, the baseline should be visible on the screen.
20. Aspirate deionized water first to make sure the capillary is free of Zn contamination and baseline remains zero on signal monitor screen.
21. Start the aspiration of the most dilute standard solution to the most concentrated following the instrument prompts on the lower right hand corner of the screen.
22. Observe signal on the monitor screen and check to see if the standard are reproducible and have appropriate absorbance.

After standard curve has been determined, check the correct correlation coefficient. If the correlation coefficient is > 0.995 as shown below and proceed to assay blank, controls and samples.

If controls are out of range, re-prepare and assay them one more time. If this still result in a out of range values, check for other factors (eg. expiration date, contamination).

**Calculations**

\[
\text{Plasma } Zn = \text{ Plasma } Zn \text{ conc. St. curve (mg/L) } \times \text{ dilution factor } \times 0.10 \text{ L/dL} \\
= mg Zn / dL
\]

\[
\text{Erythrocyte } Zn, \mu g/g Hb = \frac{Zn \text{ conc. St. curve (mg/L )} \times 25}{\text{Hemolyzate hemoglobin level (g/L)}}
\]

**Comments**

It is important for the technician to insure a consistent aspiration of the sample and maintain an “fuel, rich, luminescent flame” to maintain a optimal temperature for atomization. Burners must be inspected for aggregated solids and nebulizer system must be check for any deposits that can interfere with the sample flow /1/. Hemogoblin levels may be measured using cyanmethemoglobin assay /2/.

**Reference Interval /1,3/**

Adults and children > 3 month

- Zinc in plasma is 65 to 140 mg/dL (10.2 - 21.9 mmol/dL)
- Zinc in serum is 70 to 150 mg/dL (10.7-22.9 mmol/dL)
- Zinc in erythrocytes 26-49 mg/gHb (0.47 - 0.77 mmol/gHb)

Concentrations in premature infants and neonates have not been determined.
References


APPENDIX B

Selenium in Serum / Plasma and Erythrocytes by Flame Atomic Absorption Spectroscopy

Principle

Serum/plasma or erythrocyte selenium concentration is determined directly in the diluted specimen using a palladium matrix modifier. The metal is assayed using polarized Zeeman-effect flameless atomic absorption spectrophotometry.

Equipment

- Atomic absorption spectrophotometer with Zeeman correction effect (Hitachi Model Z8100) equipped with graphite furnace and an optical pyrometer to control furnace temperature, and autosampling system (Model SS200) all from Hitachi, Ltd.
- Pyrolytic graphite cuvets (180-7444) (Hitachi, Ltd.).
- Electrodeless Discharge Lamp and power supply (Hitachi, Ltd.)
- Autosampler cups, 2.00 and 0.500 ml volume (Fisher Scientific Co. Inc).
- Vortex mixer (Baxter Scientific Co. Inc).
- Variable automatic pippettors (100-1000, 10-100 μL) (Eppendorf, Brinkman Ind., Inc.)
- Fixed volume pippettors (50, 100 μL) (Eppendorf, Brinkman Ind., Inc.).
- 100 mL Nalge volumetric flask. (Nalge Corp. Inc.).

Reagents and Controls

- Selenium Spectrometric Standard ( 1.000 g/L or 1,000 ppm ) (Fisher Scientific Co.)
- Concentrated HCl (Analytical Reagent Ggrade, Fisher Scientific Co.).
- Triton X and Antifoam B (Sigma Chemical, Co.)
- Type I deionized water.
- Palladium (II) Chloride (Analytical Reagent Grade, Aldrich Chemical Co.)
Prepare 9.4 mM solution by dissolving 170 mg of the palladium chloride in about 75 mL of Type I deionized water and 540 μL of concentrated HCl in a 100 mL volumetric, class A flask. Final concentration 9.4 mM solution PdCl₂ in 65 mol/L HCL. Stir overnight, and when dissolved dilute to 100 mL mark and add 100 μL Triton X and Antifoam B.

- Serum Trace Elements Toxicology normal and high controls (UTAK, Lab., Inc.).
- Hemolysate control (erythrocyte assay only). Prepared from pooled human red blood cell specimens and dilute four fold with type I deionized water.

**Specimen Collection and Handling**

Collect serum specimens in a 5 mL Sarstedt monovette syringe. Collect plasma/erythrocyte specimens in a Trace Element microtainer containing 30 μL of 30 mmol/L EDTA (purified). Specimens may also be collected in Becton-Dickinson Trace Elements Vacutainers containing EDTA, heparin or with no additive. Severly lipemic specimens must have chylomicrons removed by centrifugation at 13,000 g for 30 min. Inability to clear the specimen in this manner makes it unacceptable for analysis. Optimal specimen volume: 0.500 mL serum/plasma of 0.5 mL packed erythrocytes. Minimum specimen volume: 25 μL for single analysis.

Separate serum/plasma from erythrocyte within 4 hours of collection by centrifugation at 1000 g for 15 min. Transfer serum/plasma to a microcentrifuge tube and label with patient name, id, collection date. If erythrocyte selenium is to be assayed, add 750 μL of Type I deionized water to packed erythrocytes remaining in microtainer and mix well(approximately fourfold dilution). For vacutainer collections, dilute 250 μL of packet cells with 750 μL of Type I deionized water. Label appropriately. If specimens are to be stored longer than 24 hours before assay, freeze at -20 °C until assay.

**Preparation Prior to Analysis**

1. Insure that all reagents and controls are prepared and that the expiration dates have not been exceeded.
2. Turn on power switch of atomic absorption spectrometer, and allow to initialize.
From memory recall, enter program entitled “serum selenium” into memory. Call up Analytical conditions” screen and verify that correct program is in the memory.

3. Turn on EDL power supply. Switch to HCA mode and adjust wattage dial to read zero.

4. Press “condition set” button, adjust the wattage dial to 5 watt and allow the Se EDL to warm up for at least 45 min. Then follow wavelength calibration, proceed with autosampler and nozzle position check on “test modes” screen. Turn on cooling water, and argon gas. Follow optical pyrometer calibration procedure on “test modes” screen.

Assay Procedure

1. Prepare an intermediate working standard solution by first diluting 100 μL of stock standard (1,000 ppm) with 900 μL of Type I deionized water to give 1.000 mL of solution. Vortex to mix. Transfer 100 μL of this solution to a 100 mL Nalge volumetric flask containing 0.5 mL of 70 % nitric acid and half-filled with Type I deionized water. Mix contents and fill to mark with deionized water. Invert at least 13 times to mix. Label Selenium Standard, 1 ppm.

2. Prepare an aqueous standard curve by adding the following reagents to five labeled, acid-washed autosampler cups:

<table>
<thead>
<tr>
<th>Cup Number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I H₂O, mL</td>
<td>0.600</td>
<td>0.590</td>
<td>0.575</td>
<td>0.550</td>
<td>0.525</td>
</tr>
<tr>
<td>Se Std, 1 ppm, mL</td>
<td>0.000</td>
<td>0.010</td>
<td>0.025</td>
<td>0.050</td>
<td>0.075</td>
</tr>
<tr>
<td>Pd matrix Modifier</td>
<td>0.400</td>
<td>0.400</td>
<td>0.400</td>
<td>0.400</td>
<td>0.400</td>
</tr>
<tr>
<td>Concentration, μL</td>
<td>0.0</td>
<td>10.0</td>
<td>25.0</td>
<td>50.0</td>
<td>75.0</td>
</tr>
</tbody>
</table>
3. Label an appropriate number of cups for specimens, blanks and controls. Fill out specimens manifest listing specimens, id number. Dilute the specimens fourfold and tenfold. Dilute the normal control fourfold and tenfold the high control as follow:

<table>
<thead>
<tr>
<th>Sample, µL</th>
<th>25</th>
<th>50</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I, H₂O, µL</td>
<td>35</td>
<td>70</td>
<td>140</td>
</tr>
<tr>
<td>Pd Matrix Modifier, µL</td>
<td>40</td>
<td>80</td>
<td>160</td>
</tr>
<tr>
<td>Final volume, µL</td>
<td>100</td>
<td>200</td>
<td>400</td>
</tr>
</tbody>
</table>

Place in autosampler wheel position indicated on manifest list. Place standard curve cups followed by one blank and seronorm control in first seven positions and then specimens. Additional controls may be used (i.e. EPA 284; CDC sera, Levels I & II).

4. Depress “Autosampler” button twice and insure that the program includes 10 µL sampling volume for both specimens and standards and default conditions for other parameters.

5. Depress “Analytical Conditions” key and insure that screen parameters are set as shown below:

**Instrumental Analytical Conditions**

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lamp current</td>
<td>14 mA</td>
</tr>
<tr>
<td>Wavelength</td>
<td>196.0 nm</td>
</tr>
<tr>
<td>Slit width</td>
<td>1.3 nm</td>
</tr>
<tr>
<td>Sample volume</td>
<td>10.0 µL</td>
</tr>
<tr>
<td>Cuvette</td>
<td>pyrolitic tube</td>
</tr>
<tr>
<td>Measurement</td>
<td>peak area</td>
</tr>
</tbody>
</table>

6. Depress “Graphite furnace” key and insure that screen parameters are set as shown below:
<table>
<thead>
<tr>
<th>Number</th>
<th>Stage</th>
<th>Temperature Start</th>
<th>Temperature End</th>
<th>Time (Sec)</th>
<th>Carrier gas (mL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>dry</td>
<td>60</td>
<td>90</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>dry</td>
<td>90</td>
<td>120</td>
<td>40</td>
<td>30</td>
</tr>
<tr>
<td>3</td>
<td>dry</td>
<td>120</td>
<td>250</td>
<td>15</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td>dry</td>
<td>50</td>
<td>50</td>
<td>4</td>
<td>30</td>
</tr>
<tr>
<td>5</td>
<td>ash</td>
<td>1200</td>
<td>1200</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>6</td>
<td>atom</td>
<td>2700</td>
<td>2700</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>clean</td>
<td>3000</td>
<td>3000</td>
<td>4</td>
<td>200</td>
</tr>
</tbody>
</table>

RBC specimen were ashed at 1200 °C for 40 sec and the pyrolitic tube cleaned 7 sec.

7. Depress the autozero, and then start button. If the cycle proceeds correctly, allow the sampler to continue beginning with standard curve.

At the completion of the standard curve analyses, check the correlation coefficient of the curve as printed out by the computer. The correlation coefficient must be greater than 0.995, before proceeding with the analysis. Remake the standards and repeat the analysis of the standard curve, if the correlation coefficient is less than 0.995.

8. If the standard curve is satisfactory, continue with the analyses, insuring that blanks and controls are within +/- 2 standard deviation of their respective means or control limits indicated on package instruction of commercial controls. Repeat the analyses of controls if satisfactory values are not obtained before proceeding to assay specimens.

Calculations

Plasma Se, μg/L (ppb) = conc. diluted plasma x dilution factor

Se conc. St. curve (μg/L) x 25

Erythrocyte Se, μg/g Hb =

Hemolyzate Hemoglobin level (g/L)
9. Repeat analysis of specimens whose duplicate pipettings disagree by more than 15%.

Reference Interval

Range of selenium in erythrocytes

<table>
<thead>
<tr>
<th></th>
<th>Range (μg/g Hb)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.4 - 1.2 μg/g Hb</td>
</tr>
</tbody>
</table>

Range of selenium in plasma or serum by age

<table>
<thead>
<tr>
<th>Age Group</th>
<th>Range (μM, μg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preterm infant</td>
<td>0.57 - 1.32 mM (45 - 104 μg/L)</td>
</tr>
<tr>
<td>Term infant</td>
<td>0.77 - 1.19 mM (61 - 94 μg/L)</td>
</tr>
<tr>
<td>1 to 5 years old</td>
<td>1.25 - 1.81 mM (99 - 143 μg/L)</td>
</tr>
<tr>
<td>6 to 9 years old</td>
<td>1.41 - 2.08 mM (111 - 164 μg/L)</td>
</tr>
<tr>
<td>10 years to adult</td>
<td>1.29 - 2.60 mM (101 - 205 μg/L)</td>
</tr>
</tbody>
</table>

References

VITA

Maria Estela Legal de Reid

EDUCATION

   - Teaching assistantship in Pharmacology 1989 ( inhibitors of monoamines oxidase chapter).
   - University team for project in parasitology assaying tests in which children from public school were tested.


PROFESSIONAL EXPERIENCE

1. Laboratory technician

2. Laboratory technician
   Tropical Disease Institute, Asuncion-Paraguay, 1989 (Practicum)

3. Laboratory technician
   Hospital de Clinicas, Asuncion-Paraguay, 1989 (Practicum)

PRESENTATIONS
