Concern has been raised about the reliability of test results for 25-OH vitamin D from both serum and dried blood spots samples. Recently a plethora of scientific research has increased knowledge regarding key roles of vitamin D in health and disease, and sparked a tremendous increase in demand for laboratory analysis of vitamin D status. In effort to meet the increased demand several automated high throughput vitamin D assays have become available. Unfortunately several commonly used immunoassays, one of which has been utilized extensively in the past to establish reference ranges, have been found to be inaccurate and associated with high inter-laboratory variability.

The immunoassays are unable to distinguish between serum levels of 25-hydroxy (OH) vitamins D$_2$ and D$_3$. Further, having abandoned the traditional solvent extraction of samples, the immunoassays are prone to non-specific interferences.  

**Does Your Vitamin D Test Measure Up?**

*BY DAVID QUIG, PhD, DACBN and JACK MAGGIORE, PhD, MT(ASCP)*

Concern has been raised about the reliability of test results for 25-OH vitamin D from both serum and dried blood spots samples. Recently a plethora of scientific research has increased knowledge regarding key roles of vitamin D in health and disease, and sparked a tremendous increase in demand for laboratory analysis of vitamin D status. In effort to meet the increased demand several automated high throughput vitamin D assays have become available. Unfortunately several commonly used immunoassays, one of which has been utilized extensively in the past to establish reference ranges, have been found to be inaccurate and associated with high inter-laboratory variability.

The immunoassays are unable to distinguish between serum levels of 25-hydroxy (OH) vitamins D$_2$ and D$_3$. Further, having abandoned the traditional solvent extraction of samples, the immunoassays are prone to non-specific interferences.

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### Table: Vitamin D; blood spot

<table>
<thead>
<tr>
<th>Vitamin D$_2$</th>
<th>Result</th>
<th>Reference Interval</th>
<th>Low</th>
<th>Mod</th>
<th>Optimal</th>
<th>Mean</th>
<th>Mod+</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>25-Hydroxyvitamin D Total</td>
<td>11</td>
<td>40 - 80</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25-Hydroxyvitamin D$_2$</td>
<td>&lt; 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25-Hydroxyvitamin D$_3$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

25-Hydroxyvitamin D is the major circulating form of vitamin D, occurs in 2 forms: vitamin D$_2$ (ergocalciferol) and vitamin D$_3$ (cholecalciferol), and is the precursor of the active form (1,25-dihydroxyvitamin D$_3$). Because of its long half-life, measurement of total 25-Hydroxyvitamin D (D$_2$ plus D$_3$) provides the best assessment of patient vitamin D status and includes vitamin D derived from diet, supplements and exposure to UVB light (e.g. sunlight). Vitamin D is best known for its role in calcium and bone metabolism but emerging research indicates that low levels of vitamin D may be associated with increased risk of some cancers, type 2 diabetes mellitus, multiple sclerosis, cardiovascular disease, rheumatoid arthritis, depression, Alzheimer’s disease, infections, psoriasis, osteopenia and osteoporosis and neurocognitive dysfunction. Vitamin D regulates the expression of a vast array of genes in tissues including immune cells, the vasculature, muscle and reproductive organs. Vitamin D insufficiency is common and deficiency can have adverse health effects at any stage of life.

Many testing methods do not differentiate between the 2 forms of vitamin D and only total concentrations are reported. This LC/MS QQQ method is sensitive and specific for both vitamin D$_2$ and D$_3$, and each form is measured and reported independently.

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### Reference Intervals

Due to geographic location, ethnic background, and seasonal variation, population-based reference values for vitamin D do not correlate well with clinically relevant vitamin D effects and are of limited clinical value. The following reference intervals are similar to those of the 2011 Endocrine Society Practice Guidelines and apply to males and females of all ages.

- < 10 ng/mL (< 25 nmol/L) - severe deficiency. May be associated with osteomalacia or rickets (children). Serum calcium and phosphate may be low and, parathyroid hormone and serum alkaline phosphatase may be abnormally high.
- 10 - < 20 ng/mL (< 25 - 50 nmol/L) - deficiency. Increased risk of osteoporosis and secondary hyperparathyroidism.
- 20 - < 40 ng/mL (< 50 - 100 nmol/L) - moderate deficiency to suboptimal. In addition to insufficient intake and exposure to UVB light, consider malabsorption syndromes (e.g. pancreatic insufficiency, Celiac or Crohn’s disease), hepatic or kidney disease, and prolonged use of medications such as antifungals, antiseizure drugs, cholesterol and glucocorticoids.
- 40 - 80 ng/mL (100 - 200 nmol/L) - optimal levels in a healthy population.
- > 100 ng/mL (> 250 nmol/L) - elevated. Toxicity is usually associated with vitamin D levels > 150 ng/mL (> 375 nmol/L) for prolonged periods of time.

### References


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**Boundaries of Testing:**

- Minimum of 10 ng/mL (25 nmol/L) for total 25-Hydroxyvitamin D
- Maximum of 100 ng/mL (250 nmol/L) for total 25-Hydroxyvitamin D

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**Contributions:**

- David Quig, PhD, DACBN
- Jack Maggiore, PhD, MT(ASCP)

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**In This Issue**

5. Meet Jack Maggiore PhD, Assistant Director – Chemistry, R&D

6. The Vitamin D Receptor — the other half of Vitamin D function.

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**Why does DDI measure D2 and D3 instead of just total vitamin D?**

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**KNOWLEDGE SPOT**

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**CONTINUED ON NEXT PAGE**
Doctor’s Data, Inc. (DDI) has developed very precise and accurate liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods for 25-OH vitamin D$_2$ and D$_3$. The isotope dilution LC-MS/MS approach is currently considered to be the “gold standard” for measurement of vitamin D status. In effort to circumvent the significant intra- and inter-laboratory variability in vitamin D results, the U.S. National Institute of Standards and Technology (NIST) developed a candidate reference procedure that incorporates LC-MS/MS analysis. Further to that end NIST has made available a standardized reference material (SRM 968e, Fat Soluble Vitamins in human serum) for use in standardization of LC-MS/MS methods and results within and across laboratories. The latter is important because the results of one recent study (n = 50) suggest that results obtained using routine LC-MS/MS methods may be about 11% higher than those measured using the NIST candidate reference LC-MS/MS procedure. It is noted that currently no certified reference materials (CRM) are available for 25-OH vitamin D, but the NIST SRM 968e is the best alternative available.

DDI utilizes a proprietary method for the extraction of the 25-OH Vitamin Ds from serum and dried blood spots prior to analysis by LC-MS/MS. To correct for sample extraction and analytical variability, aliquots of deuterated 25-OH D$_2$ and D$_3$ internal standards are added to all samples, control specimens and calibration standards for every analytical run. Intra-assay precision and accuracy of the DDI assay for serum 25-OH D$_2$ was determined by repeat analysis of three different NIST SRM samples over four consecutive days. The values for the three NIST SRM standards were established using the NIST candidate reference LC-MS/MS method. The data presented in Table 1 indicate that the DDI method for serum is very precise and accurate maintaining 97-100% concordance, even at a very low level of 25-OH D$_2$. The data also indicate that there does not appear to be any bias inherent in the DDI assay. We were not able to evaluate the performance of our assay for 25-OH D$_3$ in this study because the NIST reference materials do not contain appreciable levels of that form of vitamin D. Participation in external proficiency testing programs is a means by which laboratories can evaluate the accuracy of analytical methods on a given day, and see how they compare with other labs using similar or different methodologies. The College of American Pathologists (CAP) sends serum specimens to laboratories for analysis and the laboratories that use LC-MS/MS methods (peer group, n = 61). No apparent bias was observed for the DDI results, or among the peer group, against the reference values.

It is very important to clinicians that laboratories are able to reproduce precise values from day-to-day and across extended testing. The data also indicate that there does not appear to be any bias inherent in the DDI assay. We were not able to evaluate the performance of our assay for 25-OH D$_3$ in this study because the NIST reference materials do not contain appreciable levels of that form of vitamin D.

### Table 1. Concordance of serum 25-OH D$_2$ levels in NIST Standard Reference Materials with values obtained from the DDI LC-MS/MS method.

<table>
<thead>
<tr>
<th>CRM 978e</th>
<th>Reference Value (nmol/L)</th>
<th>DDI Result Mean (SD)</th>
<th>% Recovery Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level 1</td>
<td>17.8</td>
<td>17.9 (0.13)</td>
<td>100.4 (0.73)</td>
</tr>
<tr>
<td>Level 2</td>
<td>32.2</td>
<td>31.2 (0.64)</td>
<td>97.0 (1.99)</td>
</tr>
<tr>
<td>Level 3</td>
<td>49.7</td>
<td>48.2 (0.75)</td>
<td>97.0 (1.5)</td>
</tr>
</tbody>
</table>

### Table 2. DDI CAP accuracy-based proficiency test results for 2012.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Reference Target (nmol/L)</th>
<th>DDI Result</th>
<th>+/- From Reference Target</th>
<th>+/- From LC/MS/MS Peer Group Mean</th>
<th>Average of all LC/MS/MS Submissions (n = 61)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABDV-6</td>
<td>73.4</td>
<td>69.9</td>
<td>-4.7 %</td>
<td>-4.5 %</td>
<td>73.2</td>
</tr>
<tr>
<td>ABVD-7</td>
<td>55.4</td>
<td>58.8</td>
<td>+6.1 %</td>
<td>-0.2 %</td>
<td>58.9</td>
</tr>
<tr>
<td>ABVD-8</td>
<td>86.6</td>
<td>85.9</td>
<td>-0.8 %</td>
<td>+1.9 %</td>
<td>84.3</td>
</tr>
<tr>
<td>ABVD-9</td>
<td>57.9</td>
<td>59.7</td>
<td>+3.1 %</td>
<td>+7.2 %</td>
<td>55.7</td>
</tr>
<tr>
<td>ABVD-10</td>
<td>37.2</td>
<td>39.0</td>
<td>+4.8 %</td>
<td>+3.2 %</td>
<td>37.8</td>
</tr>
</tbody>
</table>
periods of time. Table 3 confirms that DDI has excellent precision with respect to measurement of 25-OH D$_3$ in serum and dried blood spots; cumulative interassay CVs of under 3% and 4%, respectively. Such low interassay variability provides confidence that clinicians can rely on the DDI vitamin D tests to monitor the efficacy of therapeutic intervention to affect patients’ 25-OH D$_3$ levels.

There is considerable demand for a precise and accurate method for assessing vitamin D status from dried blood samples (finger prick) collected by patients or clinicians. Dried blood spots have been validated to be an appropriate means of sampling for accurate analysis of several (e.g. insulin), but not all circulating metabolites. In a small study blood spot cards were meticulously prepared under highly controlled conditions in a laboratory setting prior to shipment to a second laboratory. The levels of 25-OH-D from the blood spots and serum from each subject (n = 20) were analyzed by an LC-MS/MS method and statistically similar levels of total 25-OH D were reported for the paired matrices.

There are several inherent problems associated with collection of blood spots that can significantly affect test results. Therefore DDI developed a robust blood spot test for vitamin D that provides results deemed equivalent to those from serum.

In the clinical setting blood spots were collected (finger prick) by 31 subjects and serum was obtained after blood was drawn by a phlebotomist. All samples were sent to DDI for analysis of Total 25-OH D. Figure 1 indicates that there was excellent agreement for the results from the blood spots and serum, and there was no apparent systematic bias. The data provide evidence that substantially equivalent results for 25-OH D status can be obtained in the real life clinical setting for patients from self-collected blood spots or serum.

To further evaluate the concordance of DDI results for the two collection methods similar testing was performed for an additional 46 subjects. In the second study 26 subjects self-collected blood spots and 20 subjects had the finger stick and blood spotting performed by a clinician; all serum samples were prepared by a professional phlebotomist. The self-collection arm of the study was performed under observance of a silent investigator.

CONTINUED ON NEXT PAGE

### Total 25-OH Vitamin D

<table>
<thead>
<tr>
<th>Serum 25-OH D3</th>
<th>Blood Spot 25-OH D3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>Interassay CV</td>
</tr>
<tr>
<td>79.87 nmol/L</td>
<td>2.6%</td>
</tr>
<tr>
<td>193.19 nmol/L</td>
<td>2.4%</td>
</tr>
</tbody>
</table>

Table 3. Interassay cumulative CVs for 25-OH D$_3$ in serum and blood spot specimens.

...DDI has excellent precision with respect to measurement of 25-OH D$_3$ in serum and dried blood spots.
in order to note potential common errors associated with self-collection of blood spots. The observations have been utilized by DDI in preparation of detailed collection instructions that facilitate proper sample collection. Figures 2 and 3 depict and confirm the results of the previous DDI study and indicate excellent agreement between a patient’s levels of Total 25-OH D and 25-OH D$_3$, as determined from dried blood spots and serum. We were unable to perform meaningful correlation analysis for 25-OH D$_2$ because, as expected, too few subjects had reportable levels of D$_2$. As a result of explicit collection instructions, to date DDI has had to reject only about 1% of blood spot card samples.

References:
Meet Jack Maggiore PhD, Assistant Director – Chemistry, R&D

Dr. Jack Maggiore joined the team at Doctor’s Data in 2012, and serves as the Assistant Laboratory Director of Chemistry and Research and Development. Dr. Maggiore is certified as a medical technologist by the American Society for Clinical Pathology, MT(ASCP) and the National Certifying Agency for Medical Laboratory Scientists, CLS(NCA), and has received a Master of Science Degree in Clinical Chemistry and Doctorate Degree in Pathology from the University of Illinois. Previously, Dr. Maggiore managed the clinical laboratory operations at the University of Illinois at Chicago Medical Center and was Chief Scientific Officer of BioSafe Laboratories and Healthy Life Laboratories. His expertise includes dried blood spot analysis, method development and validation, clinical trials and regulatory affairs. Jack’s clinical research interests include disease management and also development of novel biomarkers for chronic diseases, for which he holds several patents for medical devices and companion diagnostic products. Dr. Maggiore has consulted for several in vitro diagnostic, pharmaceutical, and nutraceutical companies, as well as for the Centers for Disease Control and Prevention, the National Institutes for Health, and the National Aeronautic Space Agency. He has authored more than forty peer-reviewed and invited publications and textbook chapters, has lectured extensively both locally and nationally, and has presented his research findings at international scientific society meetings. He is an active member of the American Association for Clinical Chemistry (AACC), where he has chaired the Board of Editors for Clinical Laboratory News and has served the Chicago Section Executive Committee as chair. He has also been recognized by the AACC as the Lemuel J. Bowie Young Investigator for outstanding clinical research. Dr. Maggiore’s mission is to work with health care professionals to empower consumers to take a more active role in their health maintenance.

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The Vitamin D Receptor – the other half of Vitamin D function.

Andrea Gruszcz, ND

The recent resurgence of interest in vitamin D has prompted its use as a therapeutic agent in a variety of settings. A recent meta-analysis indicates that that supplemental cholecalciferol (vitamin D₃) significantly reduces all-cause mortality and emphasizes the importance of promptly diagnosing and adequately treating vitamin D deficiency. What does vitamin D do? (The surprising answer is: very little.) Without the vitamin D receptor, 1,25(OH)₂D₃ is unable to perform most of its functions. Most of the functions ascribed to vitamin D are actually the result of the vitamin and its receptor binding together to form a ligand. If a patient fails to improve on vitamin D therapy, a closer look at VDR might be a logical next step to optimize patient health and wellness.

VDR is a nuclear receptor, i.e., VDR and other nuclear receptor proteins act as intracellular transcription factors. They directly regulate gene expression and protein synthesis and affect a wide variety of cell, tissue and organ functions. VDR is ubiquitous in all the nucleated cells of the body, and is currently known to influence the function of at least 900 genes. VDR mediates most of the known functions of D₃ which include immune function, inflammation, insulin-like growth factor signaling, sex hormone signaling, xenobiotic detoxification, mineral and bone metabolism, cardiovascular function, regulation of cell proliferation and differentiation, calcium metabolism and calcium absorption.

The vitamin D/VDR ligand has an important role in the regulation of blood pressure and the regulation of the rennin-angiotensin system. In addition, inflammatory pathways and immune function are mediated by VDR signaling. VDR has a non-classical role in immunoregulation. The vitamin D/VDR ligand has anti-infective and anti-inflammatory effects through the modulation of T-cell antigen receptors; the antigen receptors are required for T-cell activation. The vitamin D/VDR ligand down regulates the expression of pro-inflammatory cytokines. In the GI tract, the vitamin D/VDR ligand performs multiple critical functions that help regulate the intestinal terrain including modulation of tight junctions, colonization of beneficial or commensal microbes, pathogen resistance, anti-microbe peptide secretion, and mucosal defense. There is also evidence that the microbiome and its metabolites may influence the VDR signaling pathways. Studies have indicated that VDR dysfunction or dysregulation may result in exaggerated inflammatory responses. The regulation of T-cells, B-cells, macrophages, dendritic cells and epithelial cells by the vitamin D/VDR ligand may indicate a link between ligand function, Vitamin D status, and autoimmune disorders such as IBD, juvenile diabetes, multiple sclerosis, asthma and rheumatoid arthritis. VDR function and Vitamin D status are further implicated in several types of cancer proliferation. VDR is expressed by many malignant tissues. Mutant p53 tumor suppressor proteins which are common in cancer cells have been shown to affect the function of VDR.

Since its discovery in 1969 the role of VDR in endocrine and other signaling pathways has been illuminated, and there is much yet to learn. The rise of genomics (the study of the genetic ‘map’ of organisms) has resulted in the mapping of VDR. Studies of the genetic sequences of the VDR protein have demonstrated several single nucleotide polymorphisms, or SNPs, that are associated with specific functions of VDR, and that may affect health and wellness. DNA sequence variations are common in any given population; these variations, termed “polymorphisms” may have perceptible biological effects. Single nucleotide polymorphisms (SNPs) in VDR have been associated with specific variations in patterns of health. Several SNPs in VDR have been discovered and evidence shows that certain SNPs have specific associations:

- The wild type (FF) is associated with an increased risk of hypertension in males and females with a family history of hypertension and a personal history of smoking. Occupational health studies have shown that in workers with lead exposures, VDR/Fok1 mutations are associated with increased white matter brain lesions and are further associated with increased lead-induced hypertension.

A polymorphism in both Fok and Taq may be associated with an increased risk of renal stones, as well as a decreased immune response to Mycobacterium tuberculosis infection. Additional VDR polymorphisms, such as apa and bsm, are currently under investigation.

In conclusion, vitamin D status is only half the story of Vitamin D function. The status of the genotype and phenotype of the vitamin D receptor is equally important. If a patient fails to improve on vitamin D therapy, an assessment of VDR SNPs might be a logical next step to optimize patient health and wellness.

References available upon request.
7-Dehydrocholesterol

Previtamin D3

UV B radiation from sunlight

Spontaneous isomerization

Cholecalciferol

Vitamin D3

Vitamin D from Diet

Vitamin D binding protein

Calcidiol

25-OH-Vitamin D3

Liver (Hepatocytes)

CYP2R1

CYP27A1

Gc-globulin

Calcidiol

25-OH-Vitamin D3

Kidney

Calcidiol

25-OH-Vitamin D3

Inactive 24,25-OH-vitamin D

Parathyroid hormone

CYP27B1

1alpha-hydroxylase

mitochondrial CYP24A1 24-hydroxylase

Endocrine modulators

Vitamin D binding protein

Calciol

25-OH-Vitamin D3

Active 1,25-OH-Vitamin D

Nuclear Membrane

Activation

Tissue-specific regulation

VDR

Retinoid X receptor (modulates transcription and Vit. D activity)

Retinoic Acid Receptor

9-cis retinoic acid

Vitamin A

Decreased cell proliferation, increased cell differentiation, decreased oncogene promotion, increased apoptosis

VDR target binding and transcription modulation
Why does DDI measure D2 and D3 instead of just total vitamin D?

Vitamin D₃ is the active form in the body. Vitamin D precursors, obtained from sun exposure or food, are biologically inert and must be activated in the body. Vitamin D₂ undergoes a metabolic activation involving sequential hydroxylations at 25- and 1α-carbons by cytochrome P450-based hydroxylases to give the main circulating form of 25-hydroxyvitamin D₂, and then, a hormonally active form: 1α,25-dihydroxyvitamin D₃. In humans, mitochondrial enzymes hydroxylate D₂ precursors five times more than D₃. Only one liver enzyme hydroxylates D₂ and D₃ equally.

The plant-derived vitamin D₂ undergoes the same activation steps, however, minor differences in the chemistry of side chains between the two forms of vitamin D lead to the production of unique, biologically active metabolites. The D₂ metabolites have superior affinity for vitamin-D binding proteins and the vitamin D receptor (VDR). Patient responses to D₂ appear far more variable and while D₂ given in large amounts will increase D₂ levels short-term, it does not contribute to long-term reversal of D₃ deficiency—high dose D₂ may actually decrease D₃ levels over time. There is also evidence of age-related impairment in D₃ metabolism.

My patient needs Vitamin D supplementation. What do I look for in a Vitamin D supplement?

Based on pharmacokinetic studies, current research and clinical evidence, vitamin D₂ and D₃ are no longer considered bioequivalent and should not be considered interchangeable. The type of vitamin D is listed on supplement bottles or in the ingredients; look for D₃ (cholecalciferol), not D₂ (ergocalciferol). A growing number of experts believe that the current recommended daily intake levels for vitamin D are too low. Companies using third party quality testing should deliver vitamin D doses consistent with labeling. Assess vitamin D levels during therapy to ensure adequacy. Check again at changes of season when sun exposure is likely to change dose requirements.

What is the relationship between vitamins A & D?

Vitamins A and D are fat-soluble nutrients essential for health. There is evidence from both animal and human studies that Vitamin A may have antagonistic effects on bone metabolism and Vitamin D levels. Animal studies indicate the vitamin A antagonizes vitamin D by affecting calciferol-metabolizing enzymes in the liver. Ingestion of 30,000 IU of vitamin A impairs the rapid intestinal vitamin D-induced calcium response in humans. High doses of vitamin A are known to be hepatotoxic. A study of Swedish women demonstrated that the women with the highest total vitamin A intake had an elevated bone fracture risk. Vitamin A (as retinoic acid) has been shown in animals to suppress osteoblasts, stimulate osteoclasts, and prevent vitamin D from maintaining normal serum calcium levels. Lower bone mass has been documented in patients using retinoid treatments. Retinoid-induced bone resorption does not seem dependent on vitamin D₃, dietary calcium or dietary phosphorus levels.

While both vitamins A and D are essential for health, safe upper limits for retinol supplementation should be observed.

Are there clinically significant interactions between vitamin D and commonly used medications?

Vitamin D interacts with many types of medications:

- **Antiacids** – aluminum is found in most antiacids. Vitamin D may increase the amount of aluminum absorbed
- **Steroids**, such as Prednisone, impair vitamin D metabolism; this will decrease calcium absorption and increase bone loss over time
- **Heart medications** (Digoxin, Diltiazem, Cardizem, Dilacor, Tiazac) – vitamin D affects calcium absorption. Calcium levels affect heart function and may alter the effects of cardiac medications
- **Calcium channel blockers** (Verapamil, Calan, Verelan, Verelan PM, Isoptin, Isoptin SR, Covera-HS) – may decrease vitamin D metabolism
- **Calcipotriene** (Dovonex, Daivonex) - a synthetic derivative of calcitriol; additional vitamin D might increase the effects and side effects of medication
- **Fat-blocking medications** (orlistat, Alli, Xenical, or cholestyramine, questran, locholest, prevaleit) may decrease vitamin D absorption
- **Estrogens** – appears to increase vitamin D levels in the blood; benefits to bone metabolism may be lost with the addition of progesterone
- **Isoniazid** (INH) - used to treat tuberculosis, may raise blood levels of vitamin D
- **Thiazide diuretics** increase the actions of vitamin D and may raise blood calcium levels
- **Anticonvulsants** – phenobarbitol or phenytoin – may decrease vitamin D levels by accelerating its use
- **Mineral oil** – may interfere with vitamin D absorption