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Comparison Between two Techniques HPLC, ELISA in the Estimation of Levels of Vitamin D in Plasma

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ABSTRACT

Background: Vitamin D is very important in the metabolism of calcium and phosphate in the body. The deficiency of that vitamin may cause different cases like osteoporosis, osteomalacia, cancers and etc. And because of the importance of that vitamin, there are various methods used to determine their levels in the blood. Objectives: to evaluate whether HPLC or ELISA techniques in the estimation of levels of vitamin D in plasma are better by comparison between them in accuracy and clinical implications. Material and Methods: Blood samples were taken in the private clinical laboratory (Al-Jawadeen Laboratory) for people with suspected vitamin D deficiency, their ages ranged from (20-45 years), and the samples were tested in the laboratory in an automated device for vitamin D level then the same remained samples were used to determine levels of the vitamin by ELISA and HPLC. Results: The results reveal that there were no significant differences between Laboratory results and ELISA results for vitamins. There was a significant difference between laboratory and HPLC results and significant differences between ELISA and HPLC results. Conclusions: The results of the HPLC method reveal a lower level of 25-OH-hydroxyvitamin D compared to the other techniques used here. These results need additional studies with high sample sizes to be more accurate in deciding which method is most appropriate in estimating vit D.

Introduction: Vitamin D is a very important vitamin in the homeostasis of calcium and phosphate in the body and it has a critical role in immune response, and insufficiency of vitamin D is recorded as a risk factor for many diseases like rickets, osteoporosis, osteomalacia (WANG, *et al*., 2011), decreases muscle strength (Bischoff-Ferrari, *et al*., 2004), cancers (Holick, 2004), cardiovascular disease (Forman, *et al*., 2005), type I diabetes mellitus (Hypponen, *et al*., 2001) and overall mortality (Melamed, *et al*., 2008).

Because of these diseases caused by vitamin D deficiency, the need for the use of more accurate methods for determining vitamin D status has increased.

The most frequently studied and measured form in the plasma or serum of vitamin D metabolites is 25-hydroxyvitamin D, which includes 25- hydroxyvitamin D2 and 25-hydroxyvitamin D3 which are used as a biomarker for Vitamin D status (STOKES, *et al*., 2018).

Vitamin D and its metabolites are very lipophilic and bind tightly to vitamin D binding protein, also these metabolites are found at very low levels, and because of that the quantification of vitamin D in plasma or serum is a challenging task (WANG, *et al*., 2011).

Vitamin D deficiency is a widely common problem in the world and the determination of circulating level of 25(OH)D become highly requested, and for this reason, developed many analytical performances of the determination by using immunoassays like ELISA, also mass spectrophotometric methods, which considered today the gold standard for the quantitative determination of 25(OH)D (ALTIERI, *et al*., 2020).

Objective: This study aimed to evaluate whether HPLC or ELISA techniques in the estimation of levels of vitamin D in plasma are better by comparison between them in accuracy and clinical implications.

Material and Methods: Blood samples were taken in the private clinical laboratory (Al-Jawadeen Laboratory) for people with suspected vitamin D deficiency, their ages ranged from (20-45 years), and we delivered an application to be filled out by the subjects, containing the subject's information and symptoms, to ensure that the subjects suffer from vitamin D deficiency, the period of collecting samples taken from (10-17) days, and the number of samples taken was 63 samples, the number of males was 24 and the number of females was 39, and they were kept until reaching (Al-Fadhel Foundation for Study, Development and Training Services) to be read using (ELISA) & (HPLC) devices.

Before reading the samples, we prepared them and added some specific solutions and reagents with known concentrations and specific proportions, and they were washed manually. The preparation of samples differs for each device according to the instructions for each device. After reading the results of both the (ELISA) and the (HPLC), we compared them together, as well as the results of the blood test using (the automated) device used in the blood lab (Al-Jawadeen Laboratory), to obtain the most accurate result among all devices.

ELISA Technique:

According to the sequential Competitive Method (Type 6): An immobilized antibody, enzyme-antigen conjugate, and native antigen were used as the essential reagents for a solid-phase sequential enzyme immunoassay. A binding reaction was produced after mixing immobilized antibody and blood sample which contain native antigen between this native antigen for a limited number of insolubilized sites (Lind, *et al.*, 1997).

Then wash the plate by a wash step to remove unreacted native antigen, then the enzyme-conjugated antigen was introduced. The site of antibody that unoccupied by the native antigen then conjugated with the enzyme.

Then the plate incubated for a short second, after that by using decantation of aspiration, the antibody- bound fraction isolated for the antibody unbound to antigen.

The enzyme activity in the antibody-bound fraction is proportional to the native antigen concentration inversely. After that a dose response curve was generated by using calibrators of known antigen concentration (vitamin D), and this curve was used to calculate the concentration of vitamin.

HPLC Technique (Extraction of Serum): Serum samples (0.3 ml) were putted in 5 ml glass test tubes, then 500 µl of methanol-isopropanol (90: IO. v/v) was added and the tubes were mixed by vortex for 15 second. Then aliquot of hexane (1.5 ml) was added and the tubes were mixed by vortex for 60 second, then separate by centrifuge at 1000 g for 3 min. the then-hexane layer was carefully transferred to a new tube and dried under vacuum at 25 ^{o} C. the precipitate was suspended in 0.3 ml of mobile phase B and filtered through 0.2 um filter to be ready for HPLC analysis (Tsugawa, *et al*., 2005).

Statistical Analysis: Statistical analysis were performed by using Microsoft Office Excel 2019. The results are represented as Number, and mean ± S.D. The analyses of variances were made by using One Way ANOVA and LSD (Less Significant Variance).

Results: Table 1 show the information about subjects which includes the gender (male 24 and female 39) and also it reveals the age of subjects as the mean ± SD (31.59 ± 5.29).

Table 1: Information about Subjects

Parameter	Result
Gender (No.)	
Male	24
Female	33
Age (Mean ± SD)	31.59 ± 5.29

The subjects were measured for the concentration of 25-OH hydroxy vit D in two techniques as well as the results taken from the clinical laboratory and the mean ± SD mentioned in Table (2). The results reveal that there were no significant differences between Laboratory results and ELISA results for vitamins. There was a significant difference between laboratory and HPLC results and significant differences between ELISA and HPLC results. The results of the HPLC method reveal a low concentration of 25-OH hydroxy vit D in comparison with the other techniques used here.

Table 2: comparison of three techniques in the 25-OH hydroxy vit D measurement

	Laboratory results Mean ± SD	ELISA results Mean ± SD	HPLC results Mean ± SD
Subjects n= 63	18.67±6.64	18.47±7.64	11.35±5.81[a,b]
The Results are represented as Mean ±S.D. (Standard Deviation) [a] The results show significant differences at $P \leq 0.05$ in comparison with Laboratory results. [b] The results show significant differences at $P \leq 0.05$ in comparison with ELISA results.			

Discussion: the results of this study show no significant differences between the automated method used by the clinical laboratory and the ELISA test but there were significant differences between laboratory results and HPLC where the results of HPLC were lower than the other techniques.

Another study that compares between the methods used for measuring Vitamin D (chemiluminescent immunoassay, HPLC, and ELISH) was show a higher correlation between HPLA and ACLIA and a lower correlation between HPLC and ELISA, and the use of laboratory method CLIA was more suitable because HPLC is too much lengthy and high cost in comparison with CLIA (Pal, *et al*., 2013).

Another study revealed that HPLC is a reliable method for determining the concentration of 25(OH) D levels in human serum and can be used as a reference test (Çiçek, *et al*., 2019).

Our study revealed that the results of HPLC were significantly lower than that of laboratory and ELISA and this may be because of some limitations of the present study like the small sample size relative to some previous studies. Other research for the comparison between HPLC and ELISA technique shows that HPLC was better for the estimation of vitamin D than ELISA (Abdulla, *et al.*, 2015) while other study shows significant variation between the use of HPLC and other immunochemical methods (Klapkova **et al**., 2017).

Conclusion The results of the HPLC method reveal a lower concentration of 25-OH-hydroxyvitamin D compared to the other techniques used here. These results need additional studies with high sample sizes to be more accurate in deciding which method is most appropriate in estimating vit D.

References:

1. Abdulla, M., Hanafi, R. S., Y., H., & Gad, M. Z. (2015). Design-of-Experiment Approach for HPLC Analysis of 25-Hydroxyvitamin D: A Comparative Assay with ELISA. *Journal of Chromatographic Science*, *53*(1), 66-72. https://doi.org/10.1093/chromsci/bmu017
2. ALTIERI, Barbara, et al. (2020) Vitamin D testing: advantages and limits of the current assays. *European Journal of clinical nutrition*, 74.2: 231-247.
3. Bischoff-Ferrari, H.A., Dietrich, T., Orav, E.J., Hu, F.B., Zhang, Y., et al. (2004) Higher 25-hydroxyvitamin D concentrations are associated with better lower-extremity function in both active and inactive persons aged > or = 60 y. *The American Journal of Clinical Nutrition*, 80, 752-758.
4. Çiçek, H., Aksoy, Ü. G. Ç., and Kul, S. (2019) Consistency of High-Performance Liquid Chromatography, Electrochemiluminescence, and Mass Spectrometry Methods for Vitamin D Measurement. *ACTA SCIENTIFIC MEDICAL SCIENCES*, vol 3, 8: 15-22.
5. Forman, J.P., Bischoff-Ferrari, H.A., Willett, W.C., Stampfer, M.J. and Curhan, G.C. (2005) Vitamin D intake and risk of incident hypertension: Results from three large prospective cohort studies. *Hypertension*, 46, 676-682. http://dx.doi.org/10.1161/01.HYP.0000182662.82666.37.
6. Holick, M.F. (2004) Vitamin D: Importance in the prevention of cancers, type 1 diabetes, heart disease, and osteoporosis. *The American Journal of Clinical Nutrition*, 79, 362-371.
7. Hypponen, E., Laara, E., Reunanen, A., Jarvelin, M.R. and Virtanen, S.M. (2001) Intake of vitamin D and risk of type 1 diabetes: A birth-cohort study. *Lancet*, 358, 1500- 1503. http://dx.doi.org/10.1016/S0140-6736(01)06580-1
8. Klapkova E, Cepova J, Pechova M, et al. (2017) A Comparison of Four Methods (Immunochemistry and HPLC) for Determination of 25-(OH)-Vitamin D in Postmenopausal Women. *Clinical Laboratory* Feb;63(2):385-388. DOI: 10.7754/clin.lab.2016.160509. PMID: 28182348.
9. Lind, C., Chen, J. and Byrjalsen, I. (1997) Enzyme immunoassay for measuring 25-hydroxyvitamin D3 in serum. *Clinical Chemistry*, 43, 943-949.
10. Melamed, M.L., Michos, E.D., Post, W. and Astor, B. (2008) 25-hydroxyvitamin D levels and the risk of mortality in the general population. *Archives of Internal Medicine*, 168, 1629-1637. http://dx.doi.org/10.1001/archinte.168.15.1629.
11. Pal, M., Datta, S., Pradhan, A., Biswas, L., Ghosh, J., Mondal, P., Rahut, R., Chaudhuri, A., Sau, S. and Das, S. (2013) Comparison between different methods of estimation of vitamin D. *Advances in Biological Chemistry*, **3**, 501-504. doi 10.4236/abc.2013.35054.
12. STOKES, Caroline S.; LAMMERT, Frank; VOLMER, Dietrich A. (2018) Analytical methods for quantification of vitamin D and implications for research and clinical practice. *Anticancer Research*, 38.2: 1137-1144.
13. Tsugawa, N., Suhara, Y., Kamao, M. and Okano, T. (2005) Determination of 25-hydroxyvitamin D in human plasma using high-performance liquid chromatography—tandem mass spectrometry. *Analytical Chemistry*, 77, 3001-3007. http://dx.doi.org/10.1021/ac048249c.
14. WANG, Zhican, et al. (2011) Simultaneous measurement of plasma vitamin D3 metabolites, including 4β, 25-dihydroxy vitamin D3, using liquid chromatography–tandem mass spectrometry. *Analytical biochemistry*, 418.1: 126-133.