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The Effect of Temperature and Storage Time on the Concentration of Vitamin D in Human Serum

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ABSTRACT
Vitamin D is one of the most important types of vitamins that are soluble in fats. The concentration of this vitamin must be maintained in order to work for the immune system, metabolism and skeletal health. The increase or decrease in the concentration of this vitamin in the blood directly affects the health system of men and women at all ages. In this research, 22 blood samples were examined at different times and temperatures in four cases. The first two cases, vitamin D was stored at 40 ˚C and 25 ˚C and tested every day within one week. The second two cases, when vitamin D was stored at 0 ˚C and -8 ˚C for 12 weeks and tested every two weeks and it was noted that the average vitamin D concentration at 40 ˚C and 25 ˚C for 7 days decreased to ~ 14% and ~ 2 % Respectively, while the mean concentration at 0 ˚C and -8 ˚C for 12 weeks decreased to ~ 22% and ~ 28%, respectively. These results revealed that 25 ˚C is the best temperature for storing vitamin D in liquid form for one week, and that the concentration of vitamin D is directly affected when it is stored in high temperatures (40 ˚C) or when it is frozen for long periods.
Keywords: vitamin D, temperature, serum; storage, stability.
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INTRODUCTION
Vitamin D is one of the most important vitamins for human health due to its ability to prevent bone fractures, reduce the risk of osteomalacia, and it is important for immune function.(1-3) Vitamin D is a fat soluble vitamin and there are many types of this vitamin in human body.(4) The most important kinds of vitamin D are D2 (ergocalciferol) and D3 (cholecalciferol). Both of them have the same chemical structure, but differ greatly in the chemical side chain. In general, vitamin D can be found in a few types of foods, such as, oily fish, red meat and egg yolks, also can be generated from human skin after sun exposure at wavelength between 290-315 nm.(5) Vitamin D2 and D3 are bound to the vitamin D binding protein and moved to the liver where both vitamins are hydroxylated to form 25-hydroxyvitamin D (25(OH)D, calcidiol). It is one of the most important types which is measured in the blood to determine the level of the vitamin D in the body.(6,7) The change in vitamin D concentration in blood can be directly effect on human health,(5,8) for instance the deficiency of vitamin D causes rickets in children, osteomalacia in adults, risk of cancer (colon, breast and prostate cancer), heart disease and depression.(1,9-11) Recently, it is estimate that one billion people in the world have vitamin D deficient.(5) However, the high concentration of vitamin D in human body is toxic and can cause hypercalcemia and sometime leads to organ damage due to the high level of calcium in soft tissues such as liver, heart and kidney.(2,12) Vitamin D is stable to oxidation, acid and unstable to heat and moisture. It decomposes by over treatment with UV light.(13) The stability of vitamin D is affected by chemical and physical factors, including the light, PH, storage time, temperature and humidity.(13,14) There are few studied available about the stability of vitamin D in different storage time and temperature. Previous studies have reported that the storage time affects to 25(OH)D concentration. For instance, when the serum is stored at -20 ˚C for 3 months, the concentration of 25(OH)D was decreased about 10%.(15) While another study has found that the concentration of 25(OH)D is not affected by temperature decrease within the time at room temperature for 4 hours, at 2 ˚C to -8 ˚C for 24 hours, seven days at -20 ˚C, also have been tested again for another 3 months at -80 ˚C, in all these storage conditions vitamin 25(OH)D was stable.(16) All these studies have some limitations such as storage conditions investigated and samples limitation.
The purpose of this study was to determine the effect of high and relatively low temperatures (40 ˚C, 25 ˚C, 0 ˚C, -8 ˚C) with the time on the concentration of vitamin 25(OH)D.

MATERIALS AND METHODS
Subjects
This study was carried out in the department of microbiology, college of science, Al-Karkh University of science, Baghdad, Iraq. Twenty two apparently healthy subjects (twelve men and ten women) have participated in this project. All volunteers are aged between 18 and 45 years, with no known disease such as osteomalacia, cancer, hypercalcemia and heart disease. Also all participants were not receiving any medication and food supplements such as vitamins or support supplements. The study protocol was approved by the committee ethics at the college of the science/ Al-Nahrain university, Baghdad, Iraq.

Method
All samples (5mL of blood) were collected within two days from 22 apparently healthy Iraqi people, 12 male and 10 female. The participants' age range was between 18 to 45 years old. The blood samples were collected in a gel tubes. Then, all samples were centrifuged at 3000 R.P.M. for 5 minutes to get the serum. After that, each serum sample was divided into four parts in Eppendorf tube. The first and

second parts were stored at 40 °C and 25 °C respectively and the samples were tested every day for one week to measure the reduction in vitamin D level with time at these two temperatures. The third and fourth parts of each sample were stored at 0 °C and -8 °C respectively and the samples were tested once a week for three months to measure the effect of freezing on vitamin D level. A schematic diagram of method is shown in Figure (1).

The measurement of vitamin 25(OH)D concentration was performed according to the Cobas (vitamin D total II, Roche Diagnostics, Germany) instructions, using Cobas E 411 analyser. The test is able to measure a very low concentrations of vitamin D (up to 3 *ng/dl*).

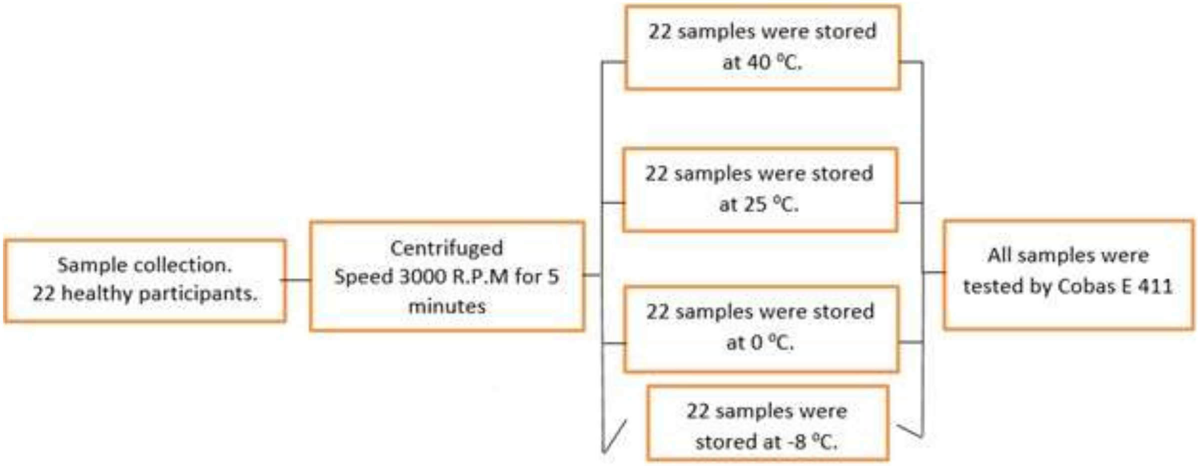


Figure 1: Flowchart of the method

Statistical analysis

Data were expressed as mean ± SE. Student's t-test and ANOVA test were used to compare mean values and calculate significance, all statistical analyses were performed using SPSS version 23.0 software and Microsoft Office Excel 2010. Data were considered statistically significant if p-values were < 0.05.

RESULTS

The investigation of Vitamin D levels in the tested serum samples revealed that vitamin D levels were reduced within time under all temperatures used (40 °C, 25 °C, 0 °C and -8 °C). Table 1 and 3 shows the concentration and the average lost concentration of vitamin D in serum during the times.

Vitamin D stability at 40 °C and 25 °C

The average of daily reduction of vitamin D levels in serum samples at 25 °C for 7 days is low (2.11%) while it is much higher at 40 °C (13.8%) (Table, 1).

The reduction of vitamin D levels at 25 °C during 7 days was not significant at p = 0.02, while at 40 ºC, the reduction of vitamin D levels was significant at p = 0.037 on day 7 (Table, 2). The difference between daily vitamin D levels reduction at 40 °C and 25 °C was not significant on the first day at p = 0.861, despite that the reduction was higher at 40 °C as shown in table 2. Vitamin D level in sample number 1 was reduced by 0.29 ng/mL at 40 °C while 0.06 ng/mL was lost at 25 °C (Table, 1). However, the difference increased gradually and became significant at p = 0.029 on day 7 (Table, 2). In general, the range of vitamin D levels reduction at 40 °C in all serum samples is between 14% to 16% during 7 days. While it is between 2% to 3% at 25°C during 7 days (Figures 2a, and 2b). The average percentage of vitamin D decomposition at 40 °C is 14%. In contrast, it was much lower 2% at 25 °C with 12% difference in comparison with 40 °C.

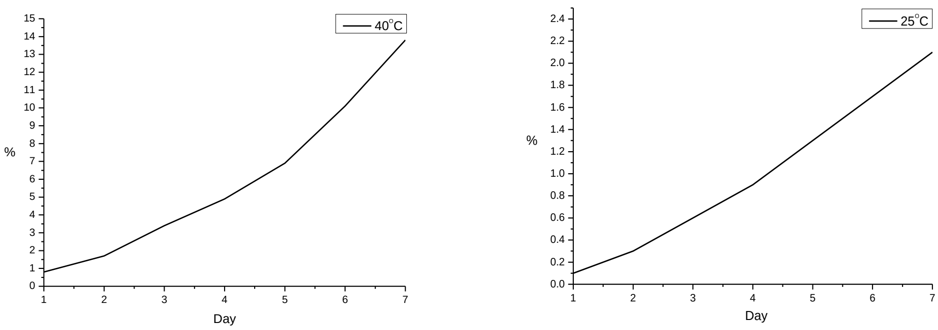
Table 1: The concentrations (ng/dl) and the average lost concentrations (%) of vitamin D in blood samples during one week at 40 °C and 25 °C.

Sample no.	Hour (0)	Day 1		Day 2		Day 3		Day 4		Day 5		Day 6		Day 7	
		40 °C	25 °C	40 °C	25 °C	40 °C	25 °C	40 °C	25 °C	40 °C	25 °C	40 °C	25 °C	40 °C	25 °C
1	38.51	38.22	38.45	38.18	38.40	37.51	38.22	36.92	38.01	36.12	37.90	34.90	37.71	33.19	37.52
2	32.11	31.88	32.00	31.00	31.90	30.21	31.75	29.72	31.61	28.90	31.49	27.25	31.30	25.98	31.15
3	42.30	42.10	42.25	41.82	42.11	41.05	41.91	40.30	41.75	39.54	41.60	37.99	41.44	36.50	41.31
4	52.82	52.32	52.80	51.99	52.71	51.05	52.61	50.71	52.49	49.88	52.40	48.69	52.22	47.02	52.05
5	35.42	35.00	35.33	34.76	35.30	33.92	35.23	33.01	35.12	32.18	35.00	31.00	34.88	29.92	34.71

6	37.91	37.88	37.80	37.12	37.72	36.54	37.61	35.89	37.47	34.98	37.38	33.82	37.22	32.33	37.02
7	32.74	32.25	32.73	31.98	32.65	31.14	32.50	30.25	32.33	29.66	32.21	28.51	32.00	27.69	31.81
8	37.22	37.01	37.20	36.79	37.11	36.02	37.00	35.18	36.80	33.81	36.69	32.72	36.50	31.52	36.39
9	51.02	50.82	50.94	50.06	50.88	49.39	50.72	48.88	50.63	47.75	50.51	46.01	50.35	44.72	50.21
10	37.62	37.33	37.59	37.01	37.50	36.09	37.39	35.33	37.39	34.21	37.25	34.11	37.11	32.40	37.00
11	30.95	30.52	30.89	30.21	30.82	29.88	30.69	29.21	30.60	28.52	30.44	27.22	30.32	25.92	30.19
12	71.01	69.82	70.99	69.51	70.91	68.96	70.74	68.09	70.66	67.10	70.50	65.91	70.41	64.01	70.29
13	36.59	36.22	36.56	35.89	36.50	35.40	36.34	34.79	36.24	34.00	36.13	33.89	36.00	32.22	35.88
14	48.09	47.75	48.00	47.43	47.90	46.91	47.79	46.24	47.61	45.44	47.41	43.98	47.29	42.44	47.01
15	30.59	30.22	30.55	29.89	30.50	29.42	30.41	28.74	30.33	28.09	30.12	26.75	29.99	25.24	29.71
16	34.92	34.74	34.88	34.41	34.83	33.89	34.69	33.21	34.50	32.65	34.35	31.01	34.22	29.51	34.02
17	32.11	31.90	32.10	31.72	32.00	31.22	31.88	31.05	31.71	30.43	31.59	28.22	31.40	26.98	31.29
18	37.89	37.52	37.80	37.49	37.73	37.01	37.69	36.74	37.55	35.98	37.40	34.88	37.22	33.23	37.00
19	41.32	41.00	41.30	40.57	41.25	39.92	41.11	39.44	41.00	38.59	40.88	37.00	40.70	35.51	40.59
20	48.92	48.71	48.88	48.39	48.80	47.80	48.69	47.10	48.51	46.55	48.39	45.22	48.20	43.00	48.02
21	36.90	36.62	36.80	36.00	36.72	35.41	36.55	34.89	36.42	34.01	36.22	32.75	36.09	31.00	35.81
22	42.28	41.97	42.25	41.46	42.19	39.89	42.03	39.29	41.89	38.71	41.70	37.11	41.59	35.90	41.44
%		0.83%	0.12%	1.74%	0.31%	3.44%	0.63%	4.97%	0.96%	6.98%	1.31%	10.1%	1.96%	13.8%	2.11%

Table 2: The significance of Vitamin D levels reduction in blood samples at different times and temperatures (day 1-7, 40 °C, 25 °C).

Time	Mean ± SE		P-value
	40°C	25°C	
0	40.42 ± 1.99	40.42 ± 1.99	---
Day 1	40.08 ± 1.96	40.36 ± 1.99	0.861 NS
Day 2	39.71 ± 1.96	40.29 ± 1.99	0.625 NS
Day 3	39.02 ± 1.99	40.16 ± 1.99	0.694 NS
Day 4	38.40 ± 1.95	40.02 ± 1.94	0.438 NS
Day 5	37.59 ± 1.94	39.88 ± 1.99	0.592 NS
Day 6	36.31 ± 1.94	39.73 ± 1.99	0.218 NS
Day 7	34.83 ± 1.91	39.56 ± 2.00	0.0287 *
P-value	0.0372 *	0.2528 NS	----
* ($P \leq 0.05$), NS: Non-Significant.			



(a) (b)

Figure 2: The average percentage of vitamin D lost in blood samples at different times (a) 40 °C. (b) 25 °C.

Vitamin D stability at 0°C and -8°C.

Vitamin D is not stable in both 0 °C and -8 °C. The average of daily reduction of vitamin D levels in serum samples at 0 °C for 12 weeks is 23.6% while it is 28.7% at -8 °C (Table, 3). These reductions are statistically highly significant at p = 0.0044 and p = 0.0052 respectively. The reduction of vitamin D levels at 0 °C and -8 °C after one cycle of freezing-thawing within 2 weeks was higher than that of samples stored at 25 °C for one week.

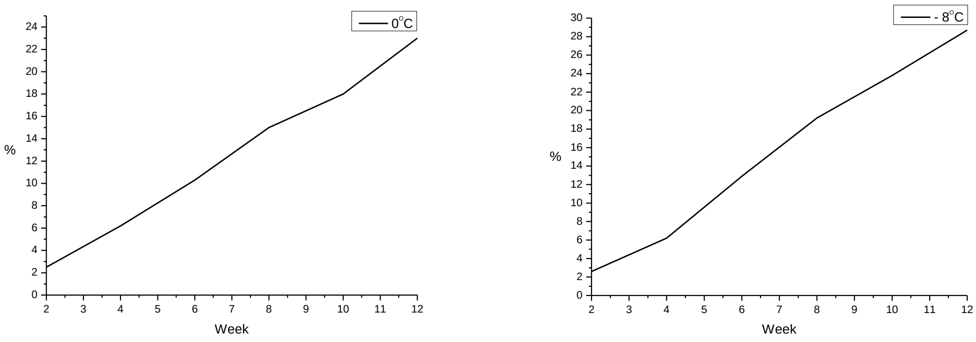
Mostly concentration of vitamin D in serum at low temperatures (-8 °C and 0 °C) showed highly significant increase through 12 weeks (p = 0.0044), (p = 0.0052) respectively, as shown in (Table 4). Figure 3a and 3b, showed that average lost concentration percentage of vitamin D at 0 ˚C and -8 ˚C for all samples were increased gradually to ~22% and ~28% respectively. The concentration of vitamin D in sample number 1 and 2 at 0 ˚C in week 12 were decreased about 26% and 25% respectively, as well as the concentration of vitamin D for the same samples at -8 ˚C were decreased about 20% and 26% respectively (Table, 3). In general, the average lost concentration at 0 ˚C for week 12 was increased from 25% to 31% for all samples, while the average lost concentration of vitamin at -8 ˚C was increased from 22% to 34%.

Table 3: The concentrations (ng/dl) and average lost concentrations (%) of vitamin D in blood samples during 12 weeks at 0 ˚C and -8 ˚C.

Sample no.	Hour (0)	Week 2		Week 4		Week 6		Week 8		Week 10		Week 12	
Temp.		**0 ˚C**	**-8 ˚C**	**0 ˚C**	**-8 ˚C**	**0 ˚C**	**-8 ˚C**	**0 ˚C**	**-8 ˚C**	**0 ˚C**	**-8 ˚C**	**0 ˚C**	**-8 ˚C**
1	38.51	37.22	37.50	35.80	36.96	33.91	35.04	32.00	33.98	30.72	32.19	28.75	30.98
2	32.11	30.98	30.86	29.16	28.00	27.18	26.23	25.30	22.19	32.71	20.90	30.80	18.91
3	42.30	41.22	41.45	40.00	39.76	38.06	37.44	36.22	33.31	34.89	30.32	31.79	29.21
4	52.82	51.43	51.56	50.14	49.32	47.93	47.78	45.09	42.78	43.87	40.65	40.67	37.43
5	35.42	34.21	34.32	32.98	33.56	31.32	31.90	29.54	27.91	27.65	25.23	25.01	23.33
6	37.91	36.77	36.89	35.12	35.32	33.86	33.32	31.90	29.54	30.04	27.12	27.88	25.19
7	32.74	31.42	31.21	30.01	30.23	28.75	29.55	26.12	27.61	24.88	25.67	23.09	23.06
8	37.22	36.03	36.72	34.89	35.89	32.98	33.64	30.33	30.92	29.11	28.33	26.98	26.27
9	51.02	49.99	49.90	48.23	48.55	46.62	46.76	44.43	43.78	43.00	42.28	41.90	40.89
10	37.62	36.60	36.88	35.03	35.12	33.83	33.21	31.91	30.13	30.18	29.77	27.73	27.91
11	30.95	30.04	29.12	28.81	28.89	27.02	26.88	25.39	25.33	24.12	22.52	21.64	20.38
12	71.01	70.19	70.46	68.45	69.18	66.93	64.76	65.04	61.00	63.54	58.99	60.91	56.97
13	36.59	35.89	35.53	34.13	34.43	32.90	30.01	30.73	28.92	29.02	27.21	27.30	25.29
14	48.09	47.18	47.12	45.54	44.21	43.50	41.00	41.95	39.65	40.13	37.17	37.71	35.43
15	30.59	29.77	29.98	28.07	27.78	26.19	24.21	25.09	23.21	23.18	21.32	21.14	19.54
16	34.92	33.89	33.31	32.12	32.95	30.75	28.38	29.00	27.49	27.73	25.19	25.99	23.07
17	32.11	31.17	31.53	29.89	30.31	28.05	26.90	26.43	24.97	25.03	22.44	23.21	20.80
18	37.89	36.83	36.41	35.21	35.17	33.64	32.41	31.60	30.12	29.88	27.30	26.90	26.32
19	41.32	40.56	40.22	39.35	39.21	37.87	35.88	35.74	32.87	34.02	32.11	32.09	30.96
20	48.92	47.88	47.90	46.02	45.90	44.32	41.42	42.98	39.44	41.12	39.73	38.82	36.88
21	36.90	36.00	35.11	34.78	33.81	33.00	30.60	31.70	28.31	28.99	26.86	26.53	24.37
22	42.28	41.58	41.62	40.03	39.33	38.79	36.32	36.92	34.98	34.80	33.03	31.95	30.56
%		2.50%	2.65%	6.23%	6.28%	10.32%	12.99%	15.04%	19.22%	18.06%	23.94%	23.66%	28.73%

Table 4: The significance of vitamin D levels reduction in blood samples at different times and temperatures (week 1-12, 0 ˚C, -8 ˚C).

Time	Mean ± SE		P-value
	0 ˚C	**-8 ˚C**	
Hour 0	40.42 ± 1.99	40.42 ± 1.99	---
Week 2	39.40 ± 2.00	39.34 ± 2.02	0.923 NS
Week 4	37.89 ± 1.98	37.90 ± 1.99	0.933 NS
Week 6	36.24 ± 1.98	35.16 ± 1.94	0.802 NS
Week 8	34.33 ± 1.97	32.65 ± 1.84	0.526 NS
Week 10	33.11 ± 1.92	30.74 ± 1.87	0.0689 NS
Week 12	30.85 ± 1.90	28.80 ± 1.86	0.0893 NS
P-value	0.0052 **	0.0044 **	----
** (P≤0.01), NS: Non-Significant.			



(a) (b)

Figure 3: The average lost concentration percentage of vitamin D in blood samples at different measured times at (a) 0 °C. (b) -8 °C.

DISCUSSION

This study has investigated four storage conditions for vitamin D. seven days at 40 °C, seven days at room temperature, twelve weeks at 0 °C and twelve weeks at -8 °C. There was significant decrease in concentration of vitamin D at 40 °C, while there was no significant difference in concentration at 25 °C during seven days. The concentration was decrease because vitamin D could be decompose during the time when stored at high (40 °C) and was decreased about 1.8% when stored in refrigerator during the same period and that was consistent with this study.(17) The limitation of this study was related with just the healthy participants and there was not ill subjects to evaluate the possible differences of concentration stability of vitamin D between them. Furthermore, the freeze-thawing cycles can largely affect vitamin D concentration when stored at 0 °C and -8 °C.

CONCLUSION

Our results confirmed that the concentration of vitamin D is effected directly by storage time and temperatures. The high common degree of temperature during investigated time. At low temperature (0 °C and -8 °C), the average concentration of vitamin D was decrease as a maximum to 23% and 28% respectively during 12 weeks. The high temperature (40 °C) was chosen in this project because the weather in Middle East and especially in summer is hot and the temperature can exceed 50 °C. These findings are in agreement with previous reports,(Wielders etal.,2009) that the vitamin D level at 25 °C was slightly changed during 7 days and temperatures (40 °C) has significantly effect on the vitamin D level in serum, while there is not big deference in concentration of this vitamin at 25 °C. The concentration level at low temperatures (0 °C and -8 °C) is highly decreased and that about 23%to 28% respectively.

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