STATUS OF VITAMIN D IN PATIENTS WITH TYPE II DIABETES MELLITUS

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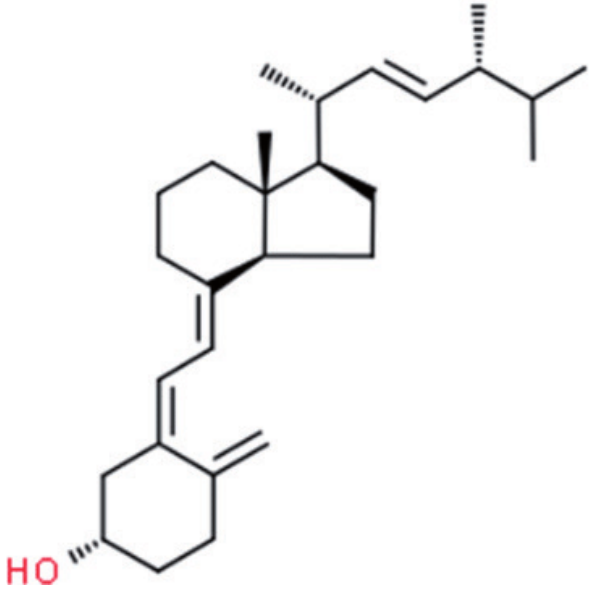
ABSTRACT

Diabetes is a metabolic disorder caused by defective insulin secretion, insulin action or both. The two major actors for the development of diabetes are pancreatic beta cell dysfunction and insulin resistance. In type 2 diabetes mellitus, production and release of insulin is unobstructed but due to factors like ageing or obesity, the produced insulin cannot compensate for the increased demand. Measurement of the serum level of 25-hydroxyvitamin D3 is the most useful parameter in evaluating vitamin D status. The serum level of vitamin D is an essential factor for studying short-term effects upon exposure to ultraviolet light and absorption of the vitamin after oral administration. Method for determination of vitamin D3 is described in this work. The objective of the current study is to analyze the concentration of vitamin D3 in patients with type 2 diabetes mellitus.

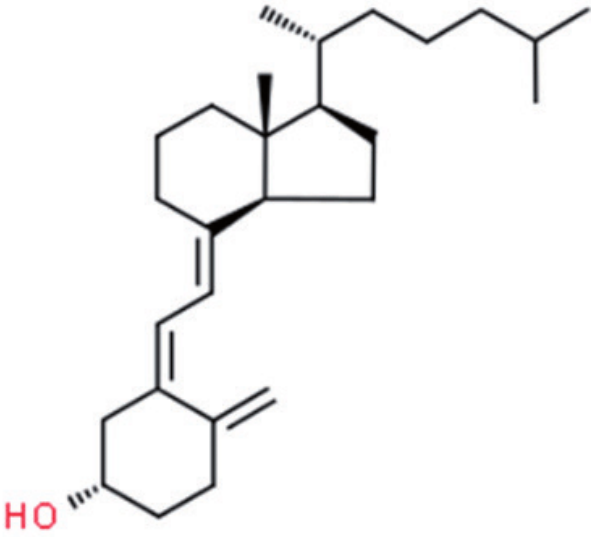
INTRODUCTION

Vitamin D refers to a group of fat-soluble secosteroids responsible for enhancing intestinal absorption of calcium, iron, magnesium, phosphate and zinc. In humans, the most important compounds in this group are vitamin D3 (also known as cholecalciferol) and vitamin D_2 (ergocalciferol).) Cholecalciferol and ergocalciferol can be ingested from the diet and from supplements. The body can also synthesize vitamin D (specifically cholecalciferol) in the skin, from cholesterol, when sun exposure is adequate (hence its nickname, the "sunshine vitamin").

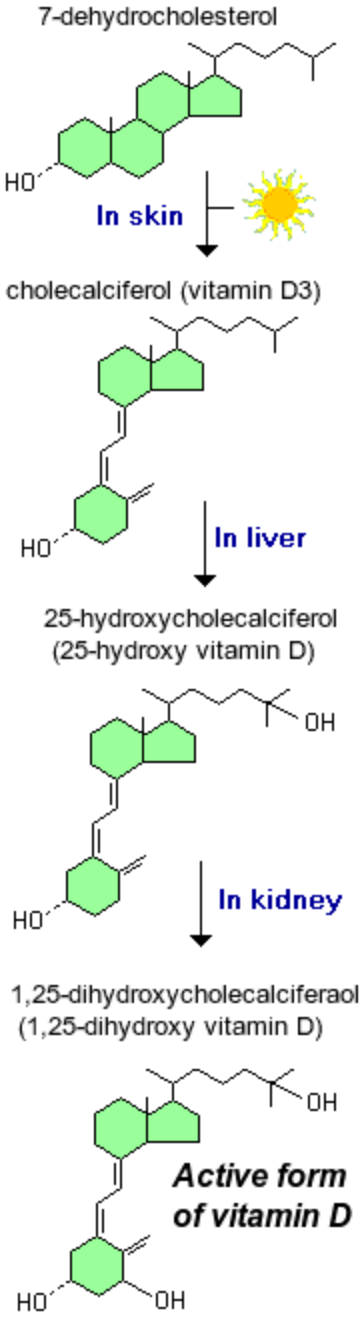
Ergocalciferol (D2)



Cholecalciferol (D3)



In the liver, cholecalciferol is converted to calcidiol which is also known as calcifediol, 25-hydroxycholecalciferol or 25-hydroxyvitaminD3 abbreviated as 25(OH) D3.Ergocalciferol is converted in the liver to 25-hydroxyergocalciferol, also known as 25-hydroxyvitamin D2 abbreviated as 25(OH) D2.These are the two specific vitamin D metabolites that are measured in serum to determine a person's vitamin D status. Part of the calcidiol is converted by the kidneys to calcitriol, the biologically active form of vitamin D. Calcitriol circulates as a hormone in the blood, regulating the concentration of calcium and phosphate in the bloodstream and promoting the healthy growth and remodeling of bone. Calcitriol also affects 1neuromuscular and immune function.



The chemical structure of vitamin D3 was established and proven to result from the ultraviolet irradiation of 7-dehydrocholesterol .When 7-dehydrocholesterol or ergocalciferol in the skin are exposed to solar ultraviolet B [UV B, 290 to 320nm], chole calciferol [Vitamin D3] is formed. Concentration of vitamin D varies according to the geographic region, clothing, climate, dietary deficiency, age,

gender etc. People with dark skin do not absorb sunlight as easily as those with light skin, so their risk of low vitamin D is even higher. The status of vitamin D is known to be lower in the elderly compared with the young because of the reduced subcutaneous concentration of 7-dehydrocholesterol and reduced absorption of oral vitamin D. People living in high latitude will have very limited vitamin D and this is very true in winter months. It has been reported that vitamin D deficiency may predispose to glucose intolerance, altered insulin secretion and type 2 diabetes mellitus. Vitamin D replenishment improves glycaemia and insulin secretion in patients with type 2 diabetes with established hypovitaminosis D, thereby suggesting a role for vitamin D in the pathogenesis of type 2 diabetes mellitus 4,5). The mechanism of action of vitamin D in type 2 diabetes is thought to be mediated not only through regulation of plasma calcium levels, which regulate insulin synthesis and secretion ,but also through a direct action on pancreatic beta cell function.Therefore owind to its increasing relevance,one can very well understand the important role of vitamin D in the pathogenesis of type 2 DM.

Type 2 DM results from insulin resistance, a condition in which cells fail to use insulin properly,sometimes also with an absolute insulin deficiency.In type 2 DM, though the production and release of insulin is unobstructed; the produced insulin cannot compensate for the increased demand due to factors like ageing or obesity. Type 2 DM is a polygenic disease resulting from the combined effect of peripheral insulin resistance and a genetically determined susceptibility to B cell dysfunction caused also by the defective ion channel function.

High-performance liquid chromatography (HPLC; formerly referred to as high-pressure liquid chromatography), is a technique in analytic chemistry used to separate the components in a mixture, to identify each component, and to quantify each component. It relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material.Each component in the sample interacts slightly differently with the adsorbent material,causing different flow rates for the different components and leading to the separation of the components as they flow out the column.HPLC has been used for medical (eg:detecting vitamin D levels in blood serum),legal(eg:detecting performance enhancement drugs in urine),research (eg:separating the components of a complex biological sample or of similar synthetic chemicals from each other)

and manufacturing (eg:during the production process of pharmaceutical and biological products) purposes.

Retention time :

The time taken for a particular compound to travel through the column to the detector is known as its retention time. This time is measured from the time at which the sample is injected to the point at which the display shows a maximum peak height for that compound.

Interpreting the output from the detector:

The output will be recorded as a series of peaks – each one representing a compound in the mixture passing through the detector and absorbing UV light. Retention times are used to identify the compounds present. The area under the peak is proportional to the amount of the compound(X) and this area can be calculated automatically by the computer linked to the display.

MATERIALS AND METHOD

The analysis of vitamin D metabolites a unique challenge as the lipophilic compounds strongly associate with vitamin D-binding protein. There are different methods for the determination of 25-hydroxy vitamin D. The Competitive Protein Binding Assay (CPBA), a fully automated chemi luminescence method, the HPLC method are some of them. HPLC estimation has an advantage that 25(OH)D2,25(OH)D3 and some other vitamin metabolites can be estimated separately.

- *COLLECTION OF SERUM SAMPLES FOR ANALYSIS*

5 ml of serum samples irrespective of the gender was collected from the patients of a clinic lab who were identified having Type II D.M during July-August 2013. 250µL of serum was extracted by 250µL methanol isopropanol (90:10V/V) solution. The amount of vitamin D was estimated at the Biochemistry laboratory of the college.

- *CHEMICALS*

All solvents were of analytical grade. n-Hexane, methanol and isopropyl alcohol were of HPLC grade (Merck). Rectified spirit (Travancore sugars) was purchased from Sigma-Aldrich (99%, HPLC). Hydrochloric acid was of analytical grade.Glycerol was of analytical grade (Merck). Water quality was secure by treatment on millipore synergy U.V water treatment system.

Calibrator stock (1mg/100ml) of 25 (OH)D3 was prepared by dissolving the compound in rectified spirit and the concentration was verified on a Perkin Elmer spectrophotometer using molar absorptivities at 265nm(1cm path length) of 18000 and the concentration of the solution corresponds to 1000ng/ml.The multiple working standards containing 100ng,50ng,25ng,15ng,10ng and 5 ng per 20ml were prepared by appropriate dilution using rectified spirit.Stocks solution and working standards were always protected from light during preparation.

- *CALIBRATION OF THE INSTRUMENT*

Inorder to analyse the level of vitamin D in serum sample in patients with diabetes mellitus, it should be noted that the instrument is calibrated using the standard solutions of Vitamin D.

- *SERUM SAMPLE PREPARATION FOR HPLC*

500µL of serum brought to the room temperature was taken in a 2ml microvial and precipitated by mixing with 500µl of methanol-isopropanol (90:10 v/v). It was kept as such for one minutes and vortex mixed for 30s. 500µl n-hexane was added to it and vortex mixed for one minute and centrifuged at 450 RPM for 3 minutes. The hexane layer was carefully separated using a micropipette[17]. The extract was evaporated to dryness under vacuum in a rotary evaporator. Residue was reconstituted in 50ml of methanol and 20ml was injected.The solvent system used for elution consisted of a mixture of methanol and water (87.5:12.5v/v)and detected at 265nm at 11.9min.Very slight variation in the retention time was observed.Peak heights and area were recorded.The concentration in the sample was calculated using the regression equation obtained with known concentrations and peak heights of authentic sample.

- *REGRESSION FORMULA*

A regression is a statistical analysis assessing that the association between two variables. It is used to find the relationship between two variables.

Linear Regression: $Y = a + bX$

Where:

X and Y are the variables.

Y= the variable that we are trying to predict

X= the variable that we are using to predict Y.

a= the intercept.

b = the slope

RESULT AND DISCUSSION

The aim of the project was to study the status of vitamin D of T2DM patients of the age-group from the South Indian population and to check whether any significant correlation exist among the parameters.T2DM patients were defined on the basis of serum HbAlc percentage (greater than or equal to 6.5). Out of 80 cases participated we could get completed data from only for 73 cases. Insufficient serum for determination of vitamin D status was a limiting factor for deriving data from the rest of the patients.For the calculation of vitamin D, 20 µl of solutions containing 100, 125, 250, 500, and 1000 ng of 25(OH)D3 monohydrate per ml were separately evaporated in microvials. The dried samples were then reconstituted in 20 µl n-hexane and separately injected and eluted under the same experimental conditions as in the estimation procedure.The peak heights were recorded and the regression equation was obtained.The data for the estimation is given in the given table:

Standardisation peaks and concentrations

Peak height (X)	*Concentration (ngml) (Y)*
509	*100*
751	*125*
912	*250*
1372	*500*
3076	*1000*

The regression equation produced from the data Y= -80.263 +0.359X was used to find the unknown concentrations of the serum samples. The actual concentration of 25(OH)D3 of serum would then be 1/5 the value so obtained.The data is given in table below.

Data produced

Sl.No.	*25(OH)D [8-60ng/ml]*
1	*0*
2	*3.83*
3	*7.35*
4	*9.8*
5	*23.86*
6	*20.56*
7	*22*
8	*3.6*
9	*30*
10	*5.4*
11	*14.1*
12	*15*
13	*12.45*
14	*27.1*
15	*25.4*
16	*0*
17	*14.5*
18	*16.96*
19	*0*
20	*0*
21	*16.68*

22	*1.9*
23	*12.2*
24	*51.3*
25	*74*
26	*116.7*
27	*37.6*
28	*17.9*
29	*3.4*
30	*34.6*
31	*34.2*
32	*162.7*
33	*33.3*
34	*8.7*
35	*58.6*
36	*3*
37	*0*
38	*0*
39	*0*
40	*0*
41	*3.7*
42	*61.6*
43	*72.8*
44	*62.4*
45	*35.4*
46	*78.4*
47	*40*
48	*33.8*
49	*13.5*
50	*12.88*
51	*16.4*
52	*0*
53	*0*
54	*8.2*
55	*17.62*
56	*0*
57	*26.32*
58	*49.71*
59	*0*
60	*64.78*

61	*36.6*
62	*58.4*
63	*33.7*
64	*0.03*
65	*23.51*
66	*5.84*
67	*0*
68	*23.94*
69	*29*
70	*25.52*
71	*3.55*
72	*7.86*
73	*23.14*
74	*17.12*

DISCUSSION

Though, historically a 'normal' nutritional vitamin D status has been defined as just about any circulating level of 25(OH) D in asymptomatic subjects, a range of 8-60 ng/ml is generally accepted for it. It is also reported that 32ng/ml is needed for normal functions .Low vitamin D status is reported associated with markers of impaired glucose metabolism such as glycosylated haemoglobin (HbA1c).In a study on a heterogeneous group of middle aged subjects,HbA1c concentration is reported decreased at higher serum vitamin D concentration independent of covariates. Absence of any association between vitamin D deficiency and other parameters in that study including HbA1c was accounted due to vigorous clinical management of the disease and the longer time interval between the disease development and the assessment of vitamin D status.

Out of the 74 cases studied in the project, we have noticed 48 cases (65%) have lesser than the required minimum. Vitamin D intoxication is referred to as a hypercalcemic state.Vitamin D formation in skin depends on sunlight and the main dietary source of it is fatty ocean fish and other marine foods.It is interesting to note that inspite of both the favourable factors,the relative vitamin D level of local inhabitants is very low. Ie,The concentration of vitanibn D3 in type 2 diabetic patient is very low

AMOUNT OF 25(OH)D3[ng'ml]	*NO.OF SAMPLES ANALYSED*
Deficient (<20)	*42*
20-50	*20*
50-80	*10*
80-100	*0*
>>>100	*2*

CONCLUSION

T2DM is a chronic disease that occurs either when the pancreas does not produce enough insulin or when the body cannot effectively use the insulin it produces. Mechanisms for the onset of this disease remain under extensive investigations. Abnormal homeostasis of vitamin status may contribute to certain diabetic outcomes. Any information regarding the status of the vitamin D would be a contribution to the field of health care. This becomes all the more important as India is expected to have the maximum diabetic patients by 2020.Normal range of Vitamin D level in human serum is found to be 8-60 ng/ml. From the analysis,we can conclude that :-

- *Vitamin D may play a role in type 2 diabetes.*
- *Majority of the diabetic patients were suffered by vitamin deficiency and it constitute about 41% of the total.*
- *A very small percentage of the patients (about 2%) shows abnormal increase in vitamin D level.*
- *The concentration of vitamin D3 in type 2 diabetic patient is very low.*
- *A relationship is found in between level of vitamin D and diabetes mellitus.*

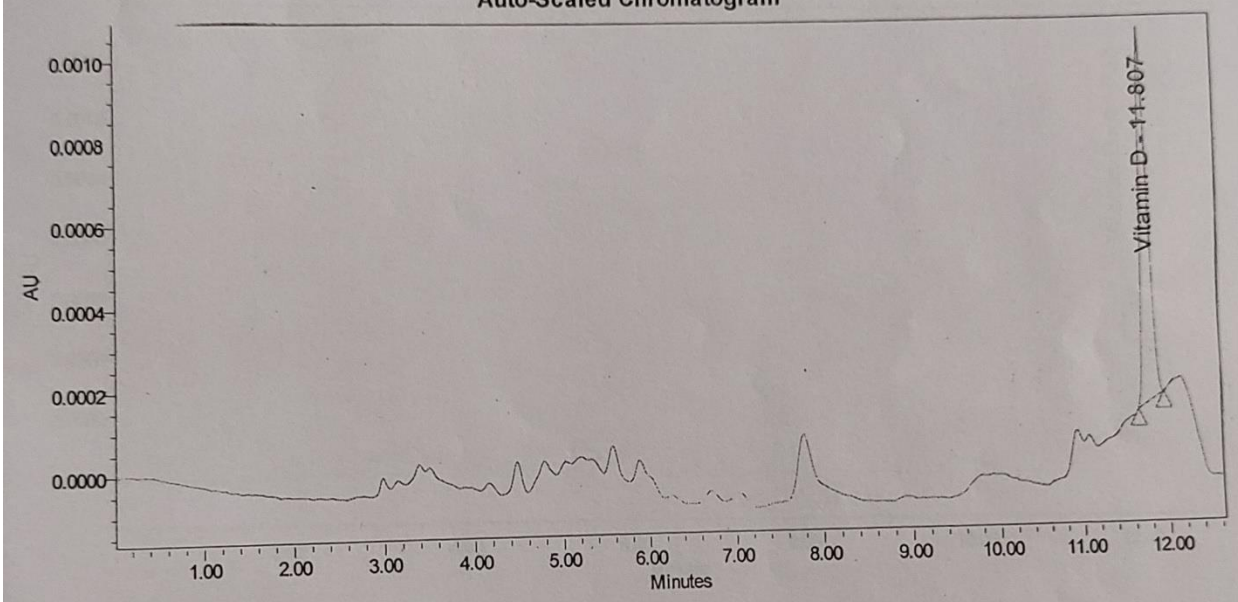
Some sample informtions and corresponding chromatograms are randomly listed below :

Sample 1

SAMPLE INFORMATION

Sample Name:	A/40 new (as such -no dil)	Acquired By:	System
Sample Type:	Standard	Sample Set Name:	
Vial:	1	Acq. Method Set:	25 OHD3
Injection #:	9	Processing Method	Vitamin D
Injection Volume:	20.00 ul	Channel Name:	2487Channel 1
Run Time:	20.0 Minutes	Proc. Chnl. Descr.:	265
Date Acquired:	6/28/2010 12:40:40 AM IST		
Date Processed:	3/25/2014 2:56:56 PM IST		

Auto-Scaled Chromatogram



Peak Results

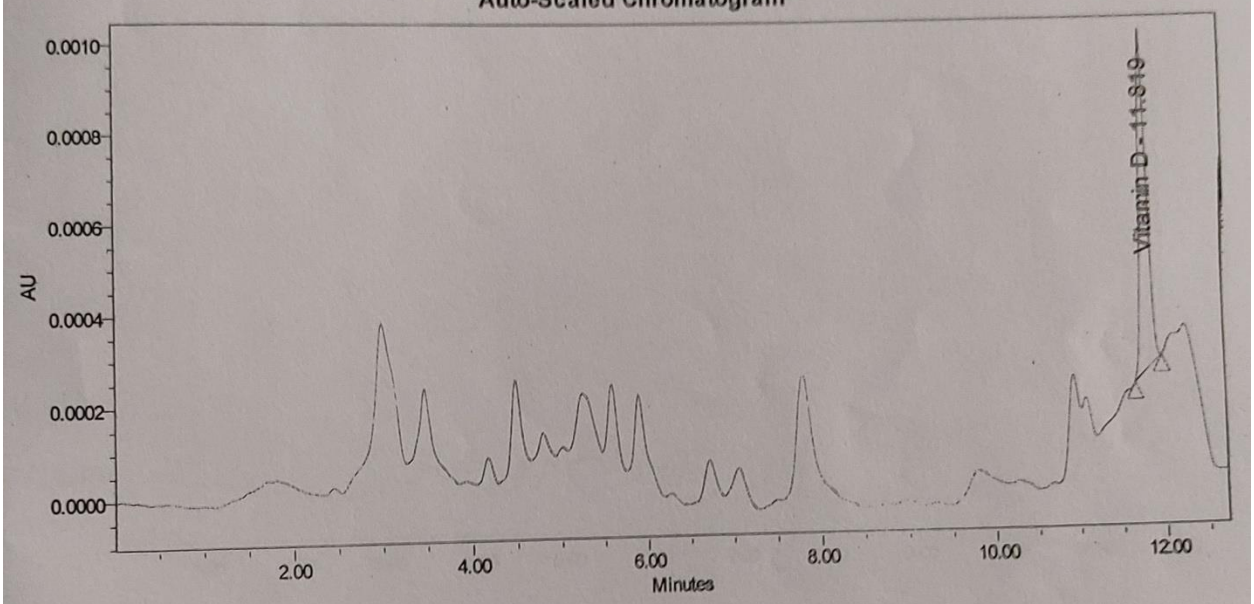
	Name	RT	Area	Height	Amount	Units
1	Vitamin D	11.807	5867	912		

Sample 2

SAMPLE INFORMATION

Sample Name:	A/80 new	Acquired By:	System
Sample Type:	Standard	Sample Set Name:	
Vial:	1	Acq. Method Set:	25 OHD3
Injection #:	8	Processing Method	Vitamin D
Injection Volume:	20.00 ul	Channel Name:	2487Channel 1
Run Time:	20.0 Minutes	Proc. Chnl. Descr.:	265
Date Acquired:	6/28/2010 12:24:27 AM IST		
Date Processed:	3/25/2014 2:58:51 PM IST		

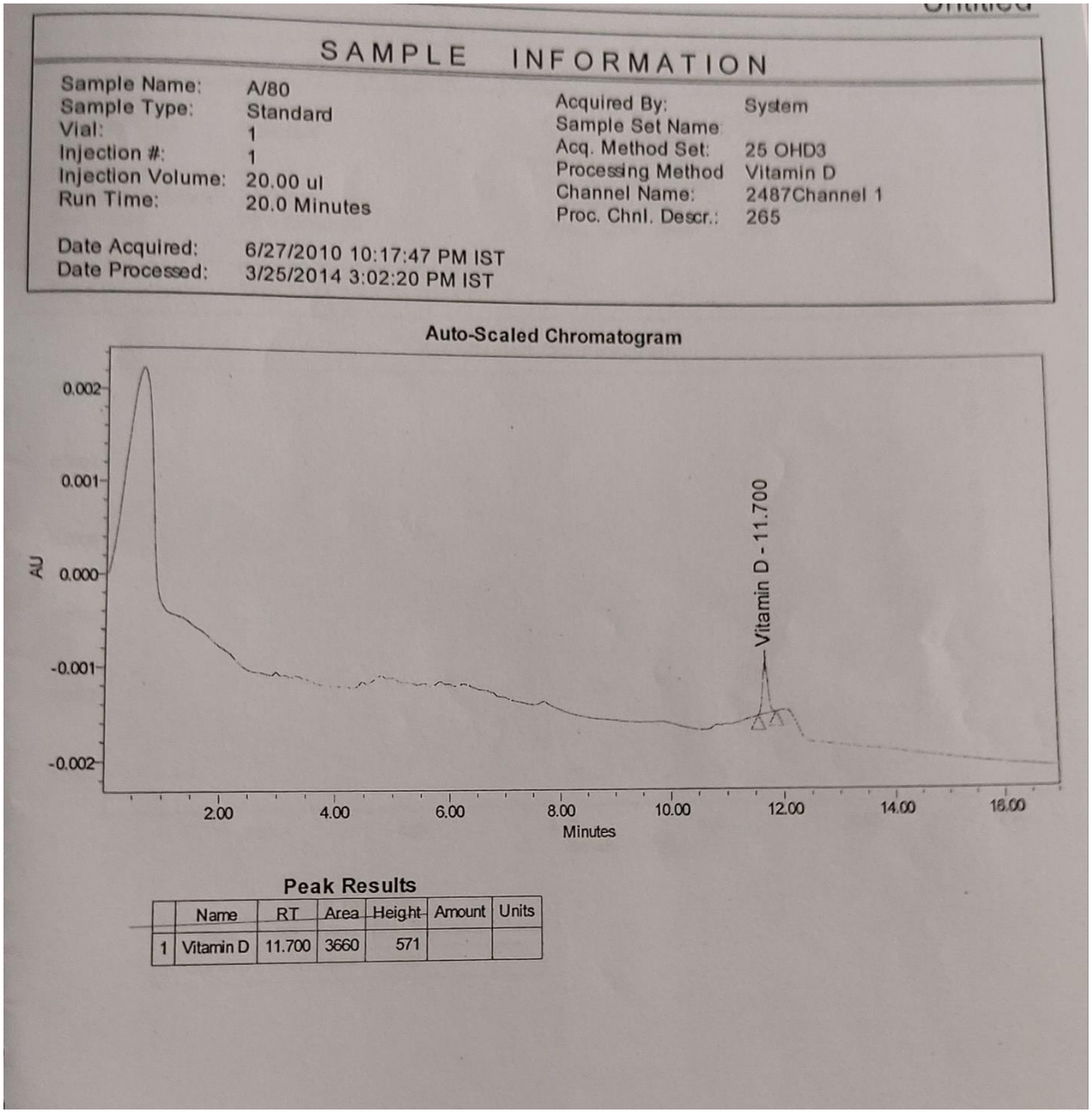
Auto-Scaled Chromatogram



Peak Results

	Name	RT	Area	Height	Amount	Units
1	Vitamin D	11.819	4895	748		

Sample 3



SAMPLE INFORMATION

Sample Name:	A/80	Acquired By:	System
Sample Type:	Standard	Sample Set Name:	
Vial:	1	Acq. Method Set:	25 OHD3
Injection #:	1	Processing Method	Vitamin D
Injection Volume:	20.00 ul	Channel Name:	2487Channel 1
Run Time:	20.0 Minutes	Proc. Chnl. Descr.:	265
Date Acquired:	6/27/2010 10:17:47 PM IST		
Date Processed:	3/25/2014 3:02:20 PM IST		

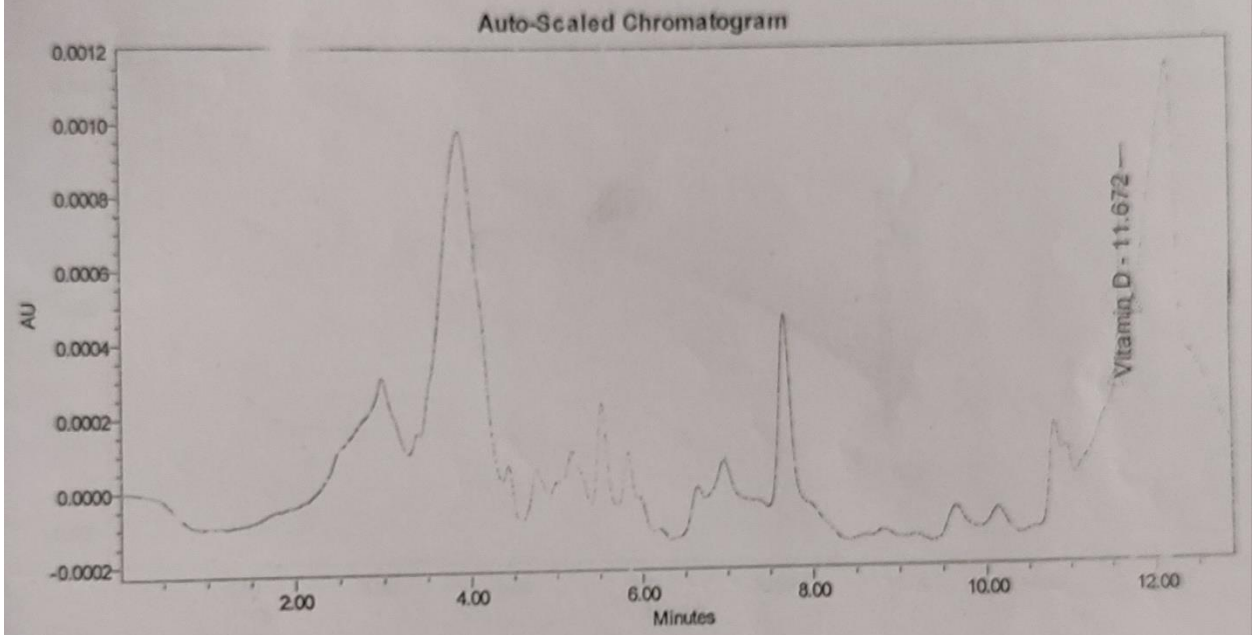
Peak Results

	Name	RT	Area	Height	Amount	Units
1	Vitamin D	11.700	3660	571		

Sample 4

SAMPLE INFORMATION

Sample Name:	A/100	Acquired By:	System
Sample Type:	Standard	Sample Set Name:	
Vial:	1	Acq. Method Set:	25 OHD3
Injection #:	17	Processing Method	Vitamin D
Injection Volume:	20.00 ul	Channel Name:	2487Channel 1
Run Time:	20.0 Minutes	Proc. Chnl. Descr.:	265
Date Acquired:	6/28/2010 2:52:58 AM IST		
Date Processed:	3/25/2014 3:12:15 PM IST		



Peak Results

	Name	RT	Area	Height	Amount	Units
1	Vitamin D	11.672	2967	494		

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