ISSN 0975 - 6299 Vol 2 / Issue 3 / Jul - Sept 2011

International Journal of Pharma and Bio Sciences

RESEARCH ARTICLE BIOTECHNOLOGY

PLASMA HOMOCYSTEINE LEVELS AND EFFICACY OF VITAMIN SUPPLEMENTATION AMONG PATIENTS WITH ATHEROSCLEROSIS – A SPECTRAL AND CLINICAL FOLLOW UP

A. BRIGHT[#*], T. S. RENUGA DEVI[*] AND S. GUNASEKARAN [**]

[#]Research Scholar, SCSVMV University, Kanchipuram.
[*]Post Graduate Dept. of Physics, Women's Christian College, Chennai.
[**] Registrar, Periyar University, Salem.

A. BRIGHT
Research Scholar, SCSVMV University, Kanchipuram.
[*]Post Graduate Dept. of Physics, Women's Christian College, Chennai.

ABSTRACT

Homocysteine is an amino acid, which is metabolized either by the remethylation pathway to methionine or the trans-sulfuration pathway to cysteine. The former pathway is dependent on the proper functioning of the enzymes methionine synthetase and methylene tetrahydrofolate reductase as well as adequate blood levels of vitamin B12 and folic acid. The later pathway is dependent on the enzyme cystathionine beta synthetase and adequate blood levels of pyridoxine (vitamin B6). A genetic defect in the enzyme or a dietary deficiency of the vitamins involved in the metabolism of homocysteine can result in hyperhomocysteinemia. It is a strong, graded, independent risk factor for stroke, myocardial infarction and other vascular events.

The present work aims at the application of Fourier-Transform Infrared Spectroscopy for the analysis of blood plasma of patients with atherosclerosis inorder to detect spectral parameters which might serve as biomarker for identifying and detecting homocysteine levels. The analysis led to the identification of specific modes of vibration pertaining to homocysteine in blood plasma. The absorbance values at these specific modes of vibration were significantly increased for the diseased when compared to healthy individuals. For five patients, before the initiation of vitamin supplements along with their regular medication the FTIR spectra of the blood plasma was recorded and their homocysteine levels were clinically tested. They were orally administered a daily dosage of folic acid(5 mg), vitamin B12(250mcg) and vitamin B6(25mg) supplements for a period of two months. Efficacy of these vitamin supplements were analyzed both clinically and spectroscopically at the end of the first and the second month and also the homocysteine levels were clinically tested. The absorption values of the specific modes of vibration pertaining to homocysteine of both pre and post-treatment spectra were noted and the percentage of efficacy of the multivitamins was calculated. The spectral and clinical investigation showed that the addition of these vitamins can markedly reduce the homocysteine levels in blood plasma.

KEYWORDS

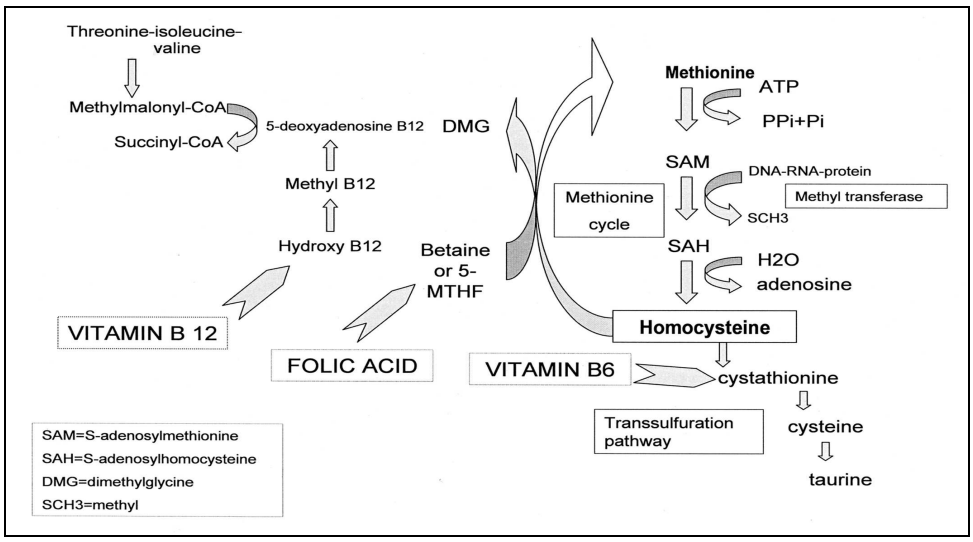
FTIR spectroscopy, plasma homocysteine, vitamin supplementation, atherosclerosis.

1. INTRODUCTION

Hyperhomocysteinemia is now recognized to be an independent risk factor for atherosclerosis [1]. Homocysteine is an unstable amino acid, which undergoes auto oxidation to produce oxygen free radicals [2]. Hyperhomocysteinemia thus causes increased production of free oxygen radicals and an oxidative stress. This contributes to atherosclerosis in two ways. The free oxygen radicals convert the Low Density Lipoprotein (LDLc) deposited in the sub-endothelial tissue to oxidized LDLc (oxLDLc). The oxLDLc then acts as the key mediator of the inflammatory process in atherosclerosis [3]. OxLDLc causes the release of vascular cell adhesion molecule and monocyte chemoattractant protein, which in turn causes monocyte adhesion and penetration respectively. The monocytes then get converted to macrophages, which take up oxLDLc to get converted to foam cells. The foam cells get deposited below the endothelium to form a fatty streak, the first lesion in atherosclerosis. The free oxygen radicals also combine with nitric oxide, inactivating it to peroxynitrite. The resulting endothelial dysfunction, also contributes significantly to atherosclerosis [4].

The internationally accepted treatment for hyperhomocysteinemia involves the use of three homocysteine lowering vitamins viz. folic acid, vitamin B12 and pyridoxine [5]. Folic acid and B12 act predominantly under fasting conditions and pyridoxine acts after meals [6]. In patients with hyperhomocysteinemia, folic acid alone was shown to reduce homocysteine levels by 22% and vitamin B12 by 11%. However when both were administered together, they acted synergistically to cause a reduction in the homocysteine levels by 38.5% [7]. The schematic of homocysteine metabolism is shown in Fig 1. There have been a number of clinical reports about the role of vitamin supplementation in normalizing homocysteine levels among patients with cardiovascular disease [8]. Only a very few researchers have analyzed the efficacy of drugs spectroscopically [9, 10]. The aim of this study is to determine the percentage of efficacy spectroscopically and substantiate it with the clinically obtained results. The former has lot of advantages and hence can be implemented as a prospective tool for the diagnosis and monitoring of plasma homocysteine levels.

Fig 1
Homocysteine Metabolism



2. Subjects and Methods

A group of ten female patients of the same age and blood group with atherosclerosis were enrolled for the study. They were undergoing treatment in the Cardiology Department of the Government General Hospital, Chennai. A group of five female patients were

enrolled for a period of two months for study of the efficacy of multivitamins on plasma homocysteine levels. They were undergoing treatment in the Cardiology Department of the Perambur Railway Hospital, Chennai. Also ten healthy individuals of the same age and sex group were chosen. The FTIR spectra of the blood plasma were recorded and their homocysteine levels were clinically tested.

2.1 Clinical Analysis

2 ml of blood of each individual were collected in EDTA vacutainers. The blood was centrifuged immediately and the plasma was separated. It was subjected to a clinical test (Immunoassay-chemiluminescence) and the homocysteine levels were measured clinically in the reference range of 10µmol/l to 12µmol/l [11]. Only two out of the ten patients enrolled for the study had homocysteine levels much greater than 30µmol/l.The initial homocysteine levels were greater than 20µmol/l for the five patients enrolled for the study of efficacy of multivitamins on plasma homocysteine level. There was a marked reduction in the plasma homocysteine levels at the end of the first month (Day 30) and second month (Day 60) after administering vitamin supplements. The clinical values of the measured homocysteine levels are shown in Table 3.

2.2 FT-IR spectra acquisition

The capillary blood samples (approximately 2ml) of the patients were collected. The blood was immediately centrifuged to separate plasma from erythrocytes. The samples were then stored at -20ºC before analyses. After the samples returned to room temperature (around 25 ºC - 30 ºC) a volume of 1ml of serum was spread evenly over the surface of a thallium chromide pellet. The specimen was air dried for thirty minutes prior to measuring the spectra [12]. The strong absorption band of water in the

mid IR – region poses hindrance and hence to eliminate this, the serum samples were air dried. The dried serum forms a thin uniform film on the pellet [13]. Infrared transparent thallium chromide without the sample was scanned as background for each spectrum and 16 scans were co-added at a spectra resolution of ± 1 cm^{-1}.

The spectra were baseline corrected and they were normalized to acquire identical area under the curves. The spectra were recorded in the wave number range of 400cm^{-1} – 4000cm^{-1} on a Perkin-Elmer FTIR spectrometer at Sophisticated Analytical Instrumentation Facility, Indian Institute of Technology, Chennai, India. The spectra of the five patients chosen for efficacy studies of multivitamins were recorded again at the end of the first month (Day 30) and second month (Day 60) after administrating the vitamin supplements.

3. RESULTS AND DISCUSSION

3.1 Assignment of absorption bands of plasma homocysteine

By careful inspection of the obtained spectra, several spectral parameters can be identified as possible biomarkers for the detection of elevated levels of plasma homocysteine. The wide multiple bands between 3300 and 2300 cm^{-1} corresponds to the anti-symmetric and symmetric stretching frequencies of N-H [14]. An absorbance peak was noticed at 3295 cm^{-1} due to N-H stretching vibrations. The spectra were dominated by absorbance bands at 1542 and 1656 cm^{-1}i.e the amino acid and amide I bands, respectively [15]. The peak at 1542 cm^{-1} was due the bending vibration of NH_2. The amide I band showing a peak at 1656 cm^{-1} was due to stretching vibrations of C=O. The absorbance at 2930 and 1456 cm^{-1} were due to the asymmetric bending and asymmetric stretching vibrations of the CH_2 molecule. The bands at 2996 – 2819 cm^{-1} were assigned to symmetric and asymmetric stretching vibrations of CH_2. The absorbance peak at 1480 –1360 cm^{-1} was attributed to stretching vibrations characteristic of amino acids (COO^-) [16]. The C-S vibrations resulted in a band at 570 – 710 cm^{-1} with a maximum absorption at 698 cm^{-1}. No significant peaks could be detected for the weak vibrations corresponding to the disulphides (S-S) at 500 – 540 cm^{-1} [16-17]. The absorption bands corresponding to the weak stretching vibrations of thiols (S-H) were also insignificant due to its dimeric nature [14].

3.2 *Calculation of Internal Ratio parameter*

Among the various mathematical methods applied for classification in biology and medicine internal standard parameter calculation is one of the simplest procedures. In our study this technique was used to classify the atherosclerotic patients with elevated homocysteine level from that of healthy individuals with the help of the FTIR spectra of corresponding groups. The internal standards for the specific modes of vibration of a healthy volunteer and those atherosclerotic patients with elevated plasma homocysteine levels (>30µmol/l) are shown in Table 1. Also the internal standards for the specific modes of vibration of atherosclerotic patients and healthy volunteers were calculated and the results obtained are shown in Table 2 and Table 3 respectively.

Fig.1

An overlaid spectrum to show the efficacy of vitamin supplements on homocysteine in a atherosclerotic patient.

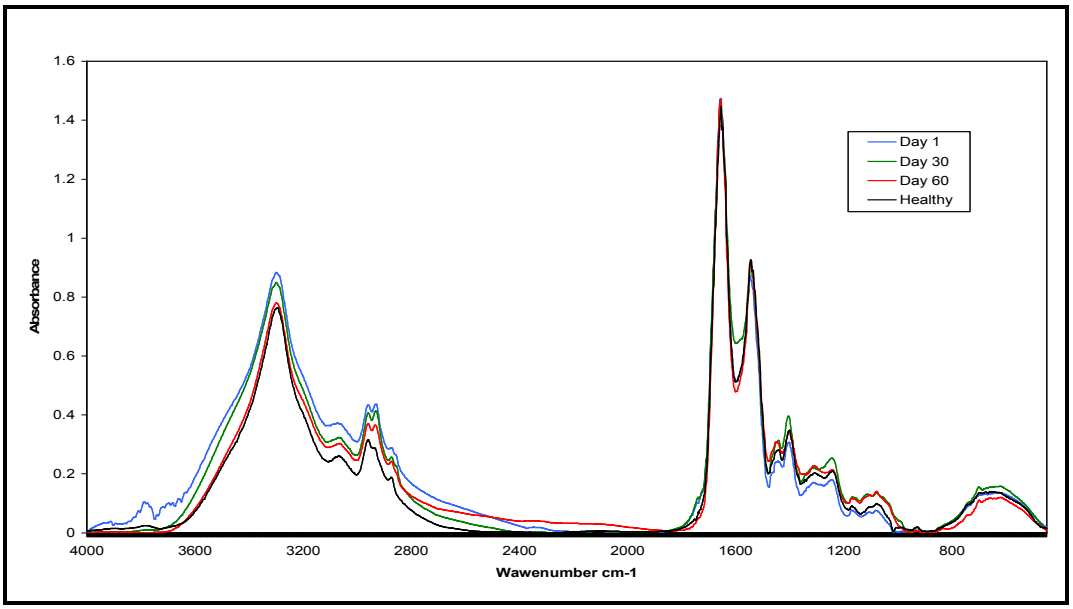


Table 1

Internal Standard evaluation of plasma homocysteine for a healthy volunteer and atherosclerotic patients with elevated homocysteine levels (> 30µmol/l)

Sample	Internal Standard of specific modes of vibration at 3295 cm^{-1}							
	$A_{3295/3295}$	$A_{2930/3295}$	$A_{2848/3295}$	$A_{1656/3295}$	$A_{1542/3295}$	$A_{1456/3295}$	$A_{1402/3295}$	$A_{698/3295}$
Healthy	1.0000	0.5479	0.3851	1.7949	0.0567	0.7350	0.8929	0.3249
Patient 1	1.0000	0.3355	0.1472	1.5165	0.9377	0.3238	0.3973	0.2078
Patient 2	1.0000	0.3928	0.1488	1.5165	1.0063	0.4431	0.5056	0.2658
	Internal Standard of specific modes of vibration at 2930 cm^{-1}							
	$A_{3295/2930}$	$A_{2930/2930}$	$A_{2848/2930}$	$A_{1656/2930}$	$A_{1542/2930}$	$A_{1456/2930}$	$A_{1402/2930}$	$A_{698/2930}$
Healthy	1.8245	1.0000	0.6969	3.2757	2.3239	1.3414	1.6296	0.5929
Patient 1	2.9805	1.0000	0.4386	4.5198	2.7949	0.9652	1.1840	0.6193
Patient 2	2.5458	1.0000	0.5316	4.4608	2.5618	1.1281	1.2870	0.6767
	Internal Standard of specific modes of vibration at 2848 cm^{-1}							
	$A_{3295/2848}$	$A_{2930/2848}$	$A_{2848/2848}$	$A_{1656/2848}$	$A_{1542/2848}$	$A_{1456/2848}$	$A_{1402/2848}$	$A_{698/2848}$
Healthy	2.6188	1.4358	1.0000	4.7305	3.3551	1.9249	2.3750	0.8511
Patient 1	5.7950	2.2798	1.0000	7.3044	6.3719	2.2005	2.6994	1.4119
Patient	4.7891	2.0811	1.0000	7.2627	4.8190	2.1222	2.4211	1.2730

2

	Internal Standard of specific modes of vibration at 1656cm^{-1}							
	$A_{3295/1656}$	$A_{2930/1656}$	$A_{2848/1656}$	$A_{1656/1656}$	$A_{1542/1656}$	$A_{1456/1656}$	$A_{1402/1656}$	$A_{698/1656}$
Healthy	0.5536	0.3035	0.21139	1.0000	0.7093	0.4069	0.5021	0.1799
Patient 1	0.6594	0.2212	0.0970	1.0000	0.6184	0.2735	0.2620	0.1370
Patient 2	0.6694	0.2590	0.1377	1.0000	0.6635	0.2922	0.3333	0.1352
	Internal Standard of specific modes of vibration at 1542 cm^{-1}							
	$A_{3295/1542}$	$A_{2930/1542}$	$A_{2848/1542}$	$A_{1656/1542}$	$A_{1542/1542}$	$A_{1456/1542}$	$A_{1402/1542}$	$A_{698/1542}$
Healthy	0.7805	0.4279	0.2981	1.4099	1.0000	0.5737	0.7079	0.2520
Patient 1	1.0615	0.3578	0.1569	1.6172	1.0000	0.3453	0.4236	0.2216
Patient 2	0.9938	0.3904	0.2075	1.5071	1.0000	0.4404	0.5024	0.2142
	Internal Standard of specific modes of vibration at 1456 cm^{-1}							
	$A_{3295/1456}$	$A_{2930/1456}$	$A_{2848/1456}$	$A_{1656/1456}$	$A_{1542/1456}$	$A_{1456/1456}$	$A_{1402/1456}$	$A_{698/1456}$
Healthy	1.3605	0.7459	0.5195	2.4576	1.7430	1.0000	1.2338	0.4421
Patient 1	3.0880	1.0361	0.4544	4.6828	2.8957	1.0000	1.2267	0.6416
Patient 2	2.5566	0.8864	0.4712	3.4222	2.2708	1.0000	1.1408	0.5998
	Internal Standard of specific modes of vibration at 1402 cm^{-1}							
	$A_{3295/1402}$	$A_{2930/1402}$	$A_{2848/1402}$	$A_{1656/1402}$	$A_{1542/1402}$	$A_{1456/1402}$	$A_{1402/1402}$	$A_{698/1402}$
Healthy	1.1027	0.6045	0.4211	1.9918	1.4127	0.8105	1.0000	0.3584
Patient 1	2.0172	0.8446	0.3705	3.8173	2.3605	0.8152	1.0000	0.5230
Patient 2	1.9780	0.7770	0.3530	3.7007	1.9904	0.8765	1.0000	0.5257
	Internal Standard of specific modes of vibration at 698 cm^{-1}							
	$A_{3295/698}$	$A_{2930/698}$	$A_{2848/698}$	$A_{1656/698}$	$A_{1542/698}$	$A_{1456/698}$	$A_{1402/698}$	$A_{698/698}$
Healthy	3.0770	1.6870	1.1750	5.5583	3.9422	2.2617	2.7906	1.0000
Patient 1	4.8127	1.4147	0.7083	7.2984	2.8957	1.5585	1.9119	1.0000
Patient 2	4.7620	1.4777	0.7855	6.7051	2.7855	1.6671	1.9019	1.0000

Table 2
Internal Ratio Parameter of the specific modes of vibration of plasma homocysteine among patients with atherosclerosis

Samples	A_{3295}/A_{2930}	A_{2930}/A_{2848}	A_{1656}/A_{1542}	A_{1542}/A_{1456}	A_{1456}/A_{1402}
1	2.2594	2.0546	1.61224	3.1719	0.8047
2	1.9892	1.9214	1.5962	2.9715	0.9146
3	2.6312	2.0774	1.6358	2.9992	0.8751
4	2.4302	2.0392	1.5805	2.9409	0.8226
5	2.4587	2.0654	1.5856	3.0667	0.8123
6	2.3921	2.0177	1.5418	2.7060	0.8415
7	2.2629	1.8695	1.5441	2.8926	0.8096
8	2.3932	2.1601	1.5902	2.7971	0.8419

Table 3
Internal Ratio Parameter of the specific modes of vibration of plasma homocysteine among Healthy volunteers

Samples	A_{3295}/A_{2930}	A_{2930}/A_{2848}	A_{1656}/A_{1542}	A_{1542}/A_{1456}	A_{1456}/A_{1402}
1	1.8239	1.4357	1.4021	1.7430	0.8094
2	1.8323	1.4103	1.4001	1.7229	0.7947
3	1.8976	1.4669	1.4169	1.6464	0.8043
4	1.9536	1.4951	1.4785	1.8129	0.7849
5	1.8552	1.4406	1.4545	1.8688	0.8061
6	1.9831	1.4505	1.4229	2.1368	0.8033
7	1.9374	1.5931	1.5189	2.1900	0.7435
8	2.0820	1.4452	1.5214	2.1536	0.7025

3.3 *Calculation of percentage of efficacy*

In order to find the efficacy of folic acid, vitamin B6 and vitamin B12 in bringing down the homocysteine levels, the absorption values of the vibrational bands at 3295cm^{-1}, 2930 cm^{-1}, 2848 cm^{-1}, 1656 cm^{-1}, 1542 cm^{-1}, 1456 cm^{-1}, 1402 cm^{-1} and 698 cm^{-1} corresponding to plasma homocysteine of both pre- and post treatment spectra were noted. The percentage of efficacy was calculated using the formula,

% of efficacy of vitamin supplements = ((Pre – Post)/Pre) x100

The results are shown in Table 4.

Table 4

Sample	Status	Clinical values µmol/l	Absorbance of specific modes of vibration (cm^{-1})							
			3295	2930	2848	1656	1542	1456	1402	698
1	Day 1	24.40	0.8134	0.3376	0.1476	1.4733	0.8856	0.2506	0.3445	0.1539
	Day 30	19.00	0.7453	0.2991	0.1359	1.4551	0.8750	0.2384	0.3158	0.1345
	% of efficacy	**-22.13**	**-8.37**	**-11.40**	**-7.93**	**-1.24**	**-1.20**	**-4.90**	**-8.33**	**-12.61**
	Day 60	15.52	0.7137	0.2728	0.1098	1.4123	0.8208	0.2029	0.2809	0.1272
	% of efficacy	**-36.39**	**-12.26**	**-19.19**	**-25.61**	**-4.14**	**-7.32**	**-19.03**	**-18.46**	**-17.35**
2	Day 1	24.14	0.9799	0.3700	0.1830	1.4619	0.9309	0.3368	0.4095	0.1427
	Day 30	20.32	0.9252	0.2986	0.1453	1.4221	0.9045	0.2700	0.3467	0.1411
	% of efficacy	**-15.82**	**-5.58**	**-19.30**	**-20.60**	**-2.72**	**-2.92**	**-19.86**	**-15.33**	**-1.12**
	Day 60	16.53	0.9042	0.2755	0.1198	1.4001	0.8924	0.2444	0.3059	0.1012
	% of efficacy	**-32.35**	**-7.73**	**-25.54**	**-34.53**	**-4.22**	**-4.14**	**-27.43**	**-25.30**	**-29.08**
3	Day 1	22.52	0.8216	0.3663	0.1642	1.4714	0.9413	0.2959	0.3669	0.1500
	Day 30	18.32	0.7839	0.3450	0.1563	1.4317	0.9243	0.2733	0.3450	0.1390
	% of efficacy	**-18.65**	**-4.59**	**-5.84**	**-4.81**	**-2.70**	**-1.80**	**-7.64**	**-5.97**	**-7.33**
	Day 60	14.40	0.7643	0.3300	0.1404	1.4012	0.8973	0.2525	0.3013	0.1142
	% of efficacy	**-36.06**	**-6.97**	**-18.04**	**-14.49**	**-4.77**	**-4.67**	**-14.67**	**-17.88**	**-23.87**
4	Day 1	22.13	0.8038	0.4144	0.2023	1.4770	0.9549	0.3319	0.4143	0.1400
	Day 30	17.52	0.7789	0.3252	0.1255	1.4385	0.8770	0.2723	0.3255	0.1190
	% of efficacy	**-20.83**	**-3.10**	**-21.50**	**-37.96**	**-2.61**	**-8.16**	**-17.96**	**-21.43**	**-15.00**
	Day 60	14.11	0.7370	0.2745	0.1015	1.4068	0.8277	0.2478	0.3044	0.1090
	% of efficacy	**-36.24**	**-8.31**	**-33.76**	**-49.83**	**-4.75**	**-13.32**	**-25.34**	**-26.53**	**-22.14**
5	Day 1	21.4	0.8834	0.4367	0.2414	1.4843	0.9260	0.3097	0.3961	0.1555
	Day 30	17.80	0.7633	0.4140	0.2157	1.4629	0.9015	0.2747	0.3456	0.1362
	% of efficacy	**-16.8**	**-13.60**	**-5.20**	**-10.65**	**-1.44**	**-2.65**	**-11.30**	**-12.77**	**-12.41**
	Day 60	15.15	0.7500	0.3879	0.1850	1.4453	0.8561	0.2397	0.3067	0.1278
	% of efficacy	**-29.21**	**-15.11**	**-11.17**	**-23.36**	**-3.90**	**-7.55**	**-22.60**	**-22.57**	**-17.81**

Efficacy of vitamin supplements on homocysteine among CVD patients

4. CONCLUSION

The present study was undertaken to utilizing the potential of FTIR spectroscopy in analyzing the efficacy of vitamin supplementation on plasma homocysteine levels among atherosclerotic patients. The specific modes of vibrations pertaining to plasma homocysteine were identified. The percentage of efficacy after 30 days and 60 days of initialization of vitamin supplementation were calculated using the absorption values at the specific modes of vibration. The plasma homocysteine levels had decreased with the progress of the treatment. The spectroscopical outcome was substantiated with the clinical results. This study forms a promising basis for employing spectroscopy in the follow-up of patients undergoing treatment for various ailments. This technique requires a small amount of plasma and the results can be obtained in a short duration. It is much cost effective when compared to clinical tests. It is therefore worthwhile to continue developing spectroscopy as an effective and reliable tool for the diagnosis and follow-up of disease.

REFERENCES

[1] Welch G., Loscalzo J. Homocysteine and Atherothrombosis, NEJM 1998;338: 1042-50.
[2] McCully K. Vascular pathology of homocysteinemia: implications for the pathogenesis of arteriosclerosis.
[3] Kopprasch S, etal. In vivo evidence for increased oxidation of circulating LDL in impaired glucose tolerance, Diabetes 2002; 51: 3120-6.
[4] Kobori Y, etal. Influence of Serum Homocysteine Level on Coronary Atherosclerosis, J Cardiology 2004; 43:223-9.
[5] Misra A., etal. Hyperhomocysteinemia and low intakes of folic acid and vitamin B12 in urban North India, Eur J Nutr 2002; 41: 68 - 77.
[6] Lakshmi A, etal. Plasma Homocysteine level in relation to folate and vitamin B6 status in apparently normal men. Asia Pacific J Clin Nutr 2001; 10:194-5.
[7] Clarke R, Armitage J. Vitamin supplements and cardiovascular risk; review of the randomized trials of homocysteine-lowering vitamin supplements. Semin Thromb Hemost 2000; 26:341-8
[8] Siri PW, Verhoef P, Kok FJ, Vitamins B6, B12 and folate; association with plasma total homocysteine and risk of coronary atherosclrosis. J Am Coll Nutr 1998; 17; 435-41.
[9] Gunasekaran S., Renuga Devi T.S, Sakthivel P.S., Asian J Clinical Cardiology vol 10, 5 2007; 19-29.
[10] Gunasekaran S., Renuga Devi T.S, Sakthivel P.S., Asia Journal of Chem Col 20, 1, 2008, 167-176.
[11] Frantzen F, Faaren A. L, Alfheim I, Nordhei A. K, Clin Chem,1998; 44:311-6.
[12] Heise H. M, Bilter A. J; Molecular structure 1995; 348:21.
[13] Budinova G., Salva J., Volka K., Applied Spectroscopy.
[14] B. B. Ivanova, M. G. Arnaudov, P. R. Bontchev; Spectrochimica Acta Part A 60(2004) 855-862.
[15] Gerard Deleris, Cyril Petribois; Vibrational Spectroscopy, 32 (2003) 129-136.
[16] Gunasekaran S; T. S. Renuga Devi; P. S. Sakthivel, Asian J. Chem. 2008; 20; 167-176
[17] Shaw R. A, Mantseh H. H; Applied Spectroscopy 2000; 54:885.