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DEVELOPMENT OF A NEW HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR VITAMIN A &B-CAROTENE IN HEALTHY BLOOD SERUM

NOOR MUSTAFA ALI

Chemistry and Biochemistry Department, College of Medicine, Al-Nahrain University, Baghdad-Iraq

ABSTRACT

To develop a new rapid and sensitive HPLC method for measurement of vitamin A and β-carotene in sera of healthy individuals. This study was conducted at chemistry and biochemistry department, college of medicine, Al-Nahrain University, Baghdad, Iraq from July2014 to March 2015; a cross-linked polymer was synthesized by condensation reaction between mixture of glycerol and triethanol amine with maleic anhydride. The resulted polymer have high rigidity and easily grinded, with high stability and used as stationary phase for HPLC column. Vitamin A and β-carotene were measured using by improved gradient reversed phase HPLC technique. Reproducible determination with highly sensitive detection was attained by HPLC with UV-Visible detection. The detection limit of vitamin A and β-carotene were 0.05and 0.1µg/ml respectively. Fifty healthy volunteers aged 25.45±6.54 years were included in this study. All participant and exclude taking supplement of multi vitamins for the last two months. The method gives a good linearity ranges between 0.05 to 15µg/ml for Vitamin A and β-Carotene. The CVs% of within-day precision for vitamin A was 1.41-2.42% and β-carotene was 0.81-1.17%. While the analytical recoveries were98.2% for vitamin A and 96.8% for β-carotene.

KEYWORDS: triethanolamine-glycerol-maleate, vitamin A, β-carotene, HPLC.

NOOR MUSTAFA ALI
Chemistry and Biochemistry Department, College of Medicine,
Al-Nahrain University, Baghdad-Iraq

INTRODUCTION

Fat-soluble vitamins such as Tocopherols, β-carotene, and lycopene are very important antioxidants [1,2&3] Reduced levels of these antioxidants in plasma have been reported to be associated with numerous diseases [4, 5]. Association between high plasma levels of fat-soluble antioxidant vitamins and lower risk of atherosclerosis was described in several studies [6]. Vitamin E is constituted by a group of eight isomers that include both Tocopherols and Tocotrienols. Vitamin A deficiency is one of the major public health nutrition problems in many developing countries (including atoll nations in the South Pacific)[7]. Its main manifestation is exophthalmia, which may lead to irreversible blindness[8]. The major source of vitamin A in most developing countries is carotenoids, which occur in very high levels in a number of green leaves [9]. Recent epidemiological evidence and experimental studies indicate that carotenoids may also be associated with reduced risk of certain types of cancer. This is likely to be related to the antioxidant properties of the extended polyenes system in these compounds. There are several carotenes that have pro-vitamin A activity. Their common feature is the β-ionone ring. Beta carotene, having two such rings, has roughly twice the activity as the other carotenoids. These compounds are transformed into retinol (vitamin A) by enzyme action in the liver and intestine[10] In recent years, several HPLC systems have been developed to analyses carotenoids [11] Numerous methods have been described for the analysis of fat-soluble antioxidant vitamins in various biological matrices[12]. Several reversed-phase high-performance liquid chromatographic (RP-HPLC) methods using or stationary phase coupled with spectrophotometric, flurometric, electrochemical, or mass spectrometric detection were developed [13,14,15&16]. In this work triethanolamine-glycerol-maleate co-polymer was prepared and used as a new stationary phase for HPLC column. It is used for analysis of several material A and β-carotene. Isocratic and gradient elution programs were applied for separation as well as the percent composition of the mobile was studied.

METHODS

This study was conducted in case control study was conducted during period from the 2013 to 2014 in the Al-Khadimiya Teaching Hospital. HPLC technique with new modified method was used to estimate serum vitamin A and β-carotene in samples of 50 male healthy volunteers aged 25.45±6.54 years participated in this study exclusion taking supplement of multi vitamins.

Preparation of Triethanolamine-Glycerol-Maleate Copolymer

In a 100ml round-bottom flask placed in a sand-bath and equipped with a thermometer and stirrer, a mixture of 7.45gm (0.05mole) triethanolamine and 4.61gm (0.05mole) glycerol were placed. The mixture was stirred for 15min. and 14.7gm (0.15mole) of maleic anhydride was added to the mixture. The temperature was rise gradually to 160oC. The reaction was performed under vacuum. Continued heating at this temperature, for 3 hours, caused an increase in viscosity of the solution until crystalline polymer was formed. The final product was washed with warm water and methanol for several times, and then dried in vacuum oven at 50^{o}C overnight[17].

Preparation of calibrations

A stock solution of 15ppm of standard *vitamin A and β-carotene* were prepared by dissolving 1.5mg of vitamin A in acetonitrile and diluted to 100ml. The same procedure for β-carotene was followed in the preparation of their stock solutions. Other standard solutions were prepared by subsequent dilution of the stock solutions. The solvent used to prepare these solutions before injection into HPLC was usually used as the mobile phase employed for their separation[18].

Serum sample preparation

In order to analyze *vitamins A and β*-carotene serum, the samples were prepared by adding 50µl of 15% 5-sulphososalsic to 400µl of deprotienzation frozen serum, then mixed and centrifuge at 3000rpm for 10min. The supernated was taken and diluted three folds with ethanol and filtrated using miniporefilter paper[16.]

Chromatographic conditions

Using solvents as mobile phase; ethanol, methanol, acetonitrile and buffer phosphate 60mM pH 4.5 or mixture of some of them. Five sets of experiment were performed; first was carried out with ethanol/ buffer phosphate 60mM pH 4.5, second was carried with methanol/ buffer phosphate 60mM pH 4.5; third was carried with methanol/ acetonitrile, fourth was carried with ethanol/acetonitrile and fifth was carried with acetonitrile/ buffer phosphate 60mM pH 4.5 the elution polarity was changed to obtain appropriate mobile phase for_mixture of *vitamins A and β-carotene* measurement. The polarity or mobile phase strength was expressed as polarity index ($P'_{A,B}$). In mixture with two components A and B, the polarity index was calculated according to the following equation

$$\mathbf{P'_{A,B} = \Phi A\, P'_A + \Phi B P'_B}$$

Where **A=** ethanol solvent, **B=** acetonitrile solvent. **ΦA=** fraction of ethanol/acetonitrile, $\mathbf{P'_A}$**=** polarity index ethanol/acetonitrile, **ΦB=** fraction of ethanol/acetonitrile, $\mathbf{P'_B}$**=** polarity index ethanol/acetonitrile, the same thinks for other sets.

Samples analysis

All the prepared serum samples, standard solutions, and mixtures of then have been chromatographically analyzed with the triethanolamine-glycerol-maleate column, with 250mm length and 4.6mm internal diameter (i.d.) using different sets of mobile phases. The mobile phase used was gradient acetonitrile: buffer phosphate, 60mM pH 4.5 (75: 25, by volume) at 1.0ml/min flow rate and detection at wavelength 230nm for *vitamins A and β-Carotene.* Quantification was done by comparing the areas under peaks (AUP) of samples with those obtain of standards. The measurement of *vitamins A and β-carotene* depended on the standard addition methods. The HPLC-UV-VIS system used in this work was Shimadzu (Kyoto, Japan) which consisted of a system controller model SCL-10 AVP, a degasser model DGU-12A, two liquid delivery pumps model LC-8AVP, UV-Visible detector model SPD-10AVP, and injector model SIL-10A, equipped with 20 µl sample loop. The HPLC system has been interfaced with computer via a Shimadzu class-VP5 chromatography data system program supplied by the manufacturer; Epson LQ-300 printer model P852A (Japan).

Data analysis

Measurements were repeated three times for each sample and the results were averaged. Results were compared by use of student's t-test for independent variables. Significance was set at $p<0.05$.

RESULTS AND DISCUSION

Synthesis of copolymers used as a stationary phase in this work was done via condensation reaction of triethanolamine-glycerol with maleic anhydride in which produces a hard and rigid. The high degree of cross linking of the co-polymer prevented the solvation process and for this reason it was difficult to determine the molecular weight and the degree of polymerization. The copolymer was identified by FTIR in which the appearance of absorption band at 1732 cm^{-1} due to the stretching vibration of the C = O of the formed ester and a band at 1296 cm^{-1} for C-N as shown in figure 1. Also an X-ray diffraction was used to identify the nature of the polymer whether it is a crystalline or not. The results showed that the polymer have different crystalline forms as shown in figure 2. Swelling test for prepared polymeric stationary phase was performed according to the ASTM procedure[8]. The degree of cross-linking has been measured using polar, moderately polar and non-polar that are usually used in HPLC such as (water, acetone, acetonitrile and hexane). The results of the swelling ranged from 1% to 4% which are theoretically expected, except for unexpected value for water 4% which could be attributed to the presence of hydrogen bonding forming moiety on the polymer surface. The solubility has been examined using different solvents such as acetonitrile, benzene, chloroform, dioxin, DMF, DMSO, hexane, methanol and water. It is found that the polymer insoluble and undecompose in all the above solvents and it is very stable. These results were attributed to high cross-linking of the polymer. However, at pH higher than 10 the polymer being decompose due to hydrolysis of the ester bond of the polymer. Column packing was done by using the slurry formed by mixing the triethanolamine-glycerol-maleate powder with 100 ml acetonitrile and homogenized in an ultrasonic bath and placed in the slurry reservoir and the column was packed using down-flow packing system. The study was carried out for the analysis of vitamins by using the co-polymer triethanolamine-glycerol-maleate column (25 x 0.4 cm).

Figure 1
FTIR spectrum of the copolymer triethanolamine-glycerol-maleate

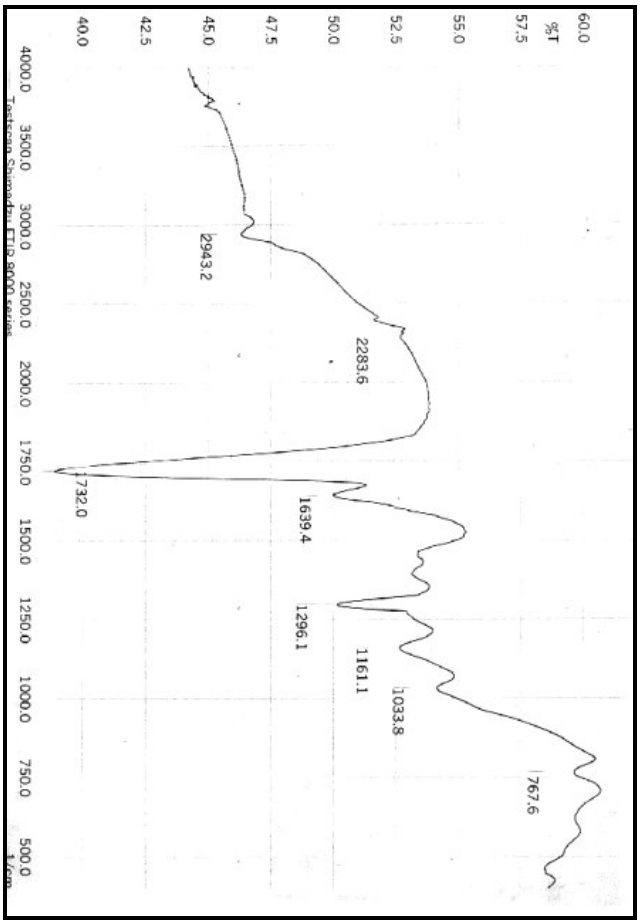
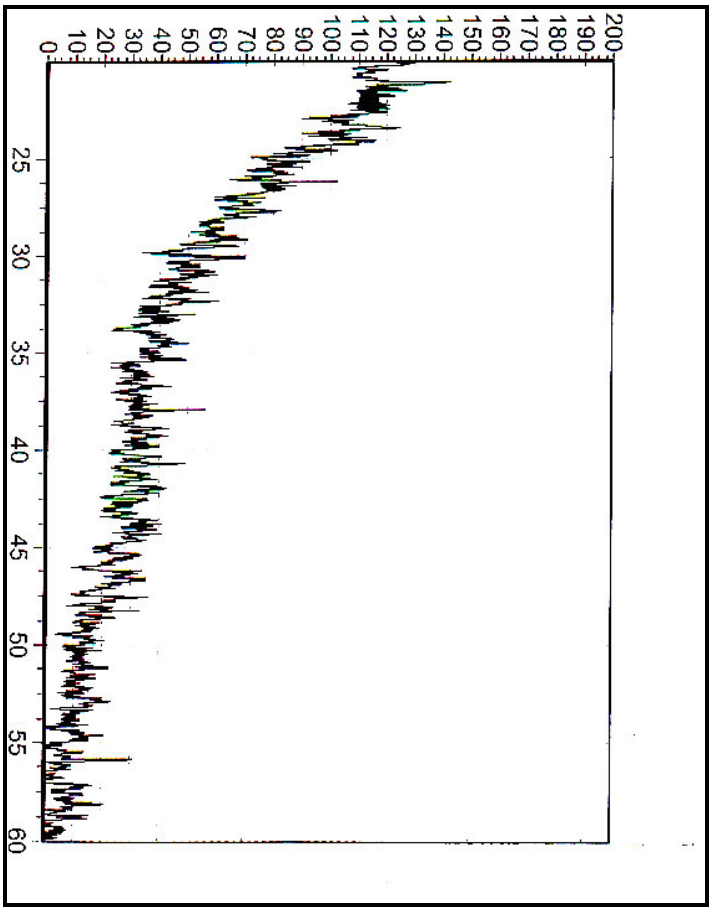


Figure 2
X-ray diffraction of the copolymer triethanolamine-glycerol-maleate



The vitamins, *vitamin A and β-carotene* were analyzed by using the copolymer triethanolamine-glycerol-maleate column (25 x 0.4 cm). The effect of the pH and phosphate buffer concentration of these drugs was studied. The results of pH showed that at pH <2 and at higher pH > 9 the drug cannot be detected. The capacity factor Ḱ using ethanol and phosphate buffer as eluent at different pH were calculated and shown in figure 3. These variations in capacity factors and separation factors of these analytes may indicate that the pH 4.5 is the best pH buffer that can use for separating. The variation of the capacity factor and separation factor for *vitamin A and β-carotene* with different percentage of phosphate buffer (percentage in ethanol) ranged from 5% to 50% are listed in table 1. The results indicate that a good competitive interaction of these drugs with the stationary phase and the best mobile phase of ethanol at 25% phosphate buffer.

Figure 3
Plot of Capacity factor, versus pH, using triethanolamine-glycerol-maleate column, flow rate 1.0ml/min, detection wavelength 230nm and 2ppm vitamin A and β-carotene sample

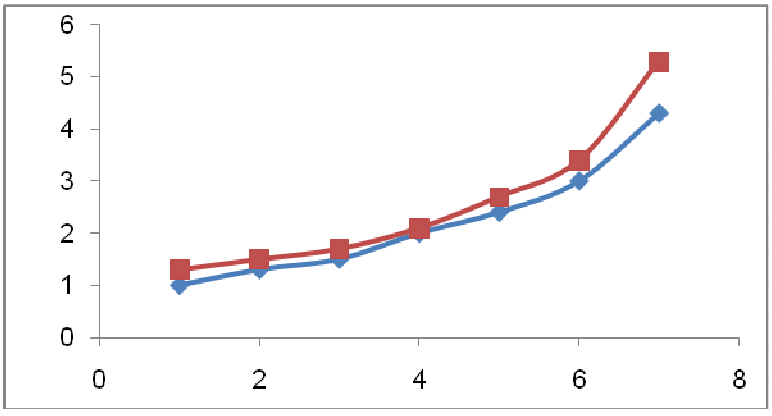


Table 1
Capacity Ḱ and separation α factors variation with changing of composition of mobile phase for drugs using triethanolamine-glycerol column

Compounds	Percentage of phosphate buffer in mobile phase									
	5%(0.01M) of buffer		15%(0.04M) of buffer		25%(0.06M) of buffer		35%(0.08M) of buffer		50%(0.1M) of buffer	
	Ḱ	A	Ḱ	A	Ḱ	A	Ḱ	A	Ḱ	A
Vitamin A	3.12		2.19		1.61		2.56		--	
B-carotene	5.43	1.74	2.87	1.31	1.83	1.14	3.15	1.23	--	--

So to improve this separation of a programmer of gradient elution has been designed based as changing both mobile phase component with time. The best program was achieved by using the results listed in Table (2).

Table 2
Time programming of gradient elution of Vitamins A and β-Carotene used triethanolamine-glycerol-maleate column (25×0.4 cm (id))

Time duration, min	% flow rate of pump B (buffer phosphate 60mM pH 2.5)	% flow rate of pump A(acetonitrile)
0	25	75
3	35	65
6	45	55
9	65	35
12	75	25

In figure 4 a chromatogram of the separation of two vitamins; 0.5µg/ml vitamin A, and 1.0µg/ml β-carotene, using gradient elution of 75% of acetonitrile and 25% of buffer phosphate (60mM) pH=4.5, flow rate of 1.0 ml/min and wavelength at 230nm. As shown vitamin A eluted first one according to its polarity compared with β-carotene. The retention time and other parameters for separation of the drugs are listed in table 3.

Figure 4
Chromatogram of vitamin A and β-carotene using gradient mobile phase Acetonitrile: buffer phosphate (60mM) pH=4.5 (75:25),triethanolamine-glycerol-maleate column (25×0.4 cm (id)),flow rate 1.0ml/min and detection wavelength 230nm

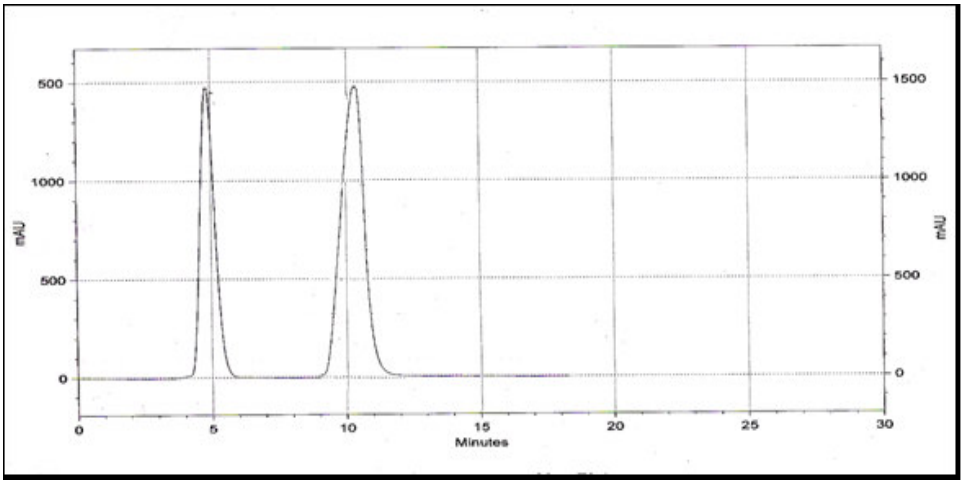


Table 3
Retention time t_R, capacity factor Ḱ, separation factor α, resolution, peak asymmetry, number of theoretical plates N and height to a theoretical plate HETP for vitamins A and β-carotene using triethanolamine-glycerol-maleate column(25×0.4 cm (id))

Compounds	t_R	Ḱ	A	R_S	A_S	N	HETP
Vitamin A	4.64	2.01			1.04	2356	0.142
β-carotene	10.37	2.45	1.22	1.56	1.05	2387	0.156

The column triethanolamine-glycerol-maleate column was compared with commercial column ODS-C18. Vitamin A and β-carotene vitamins were chromatogram on C-18 with flow rate of 1.0 ml/min. The mobile phase for elution the vitamins as follows: vitamin A was consisted of Methanol / 20mmol/l Phosphate buffer(pH2.5) 90/10, and wavelength 350nm gives retention time of 10.5min[19], for β-carotene was acetonitrile :methanol :ethyl acetate (v/v) in a gradient, from 95:5:0 to 60:20:20 in 20 min, the latter proportion being maintained until the end of the run. Flow rate was set at 0.5 mL min^{-1} and injection volume was 10µL. Acetonitrile contained 0.05 % of triethylamine to improve carotenoid recovery from the chromatographic column. A UV–VIS was used, and detection being at the wavelengths of maximum

absorption λ = 450 nm a retention time of 13.35 min[20]. The separation of a mixture cannot be performed because of the different in the composition of the mobile phases and have a long run time compare with triethanolamine-glycerol-maleate column. Therefore, ODS-C18 was very good column for determination of each vitamins individually. Quantitative analysis was studied from the construction of calibration curves for vitamin A and β-carotene. The linear calibration curves for these compounds are shown in figure 5. The slopes for the linear calibration curves using triethanolamine-glycerol-maleate column ranged from 2011.6–6430.1 depends upon the kind of vitamins. The correlation coefficients ranged from 0.9997–0.9998 with detection limit ranged from 0.05 to 0.10µg/ml as listed in table 4.

Figure 5
Calibration curves of vitamins A and β-carotene separation used triethanolamine-glycerol-maleate column (25×0.4 cm (id))

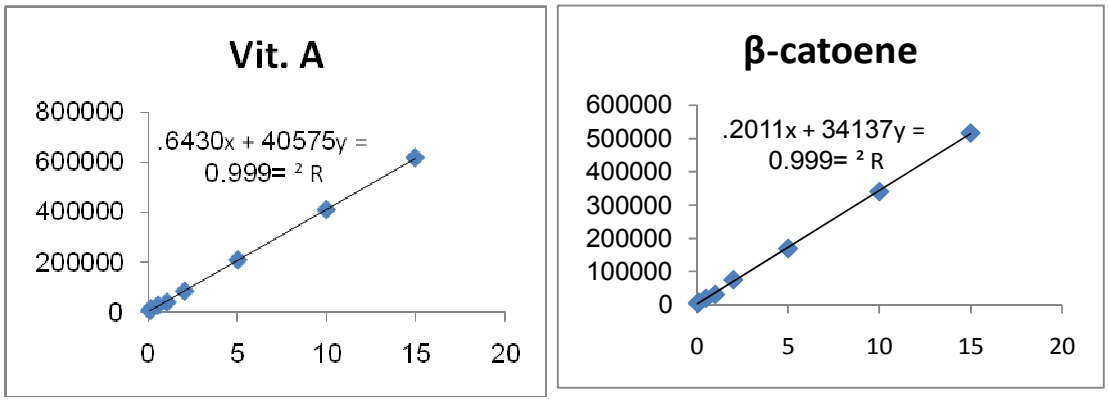


Table 4
Linear equation, correlation coefficients R and detection limit; of vitamins A and β-carotene using triethanolamine-glycerol-maleate column (25×0.4 cm (id))

Compounds	Linear Equation Y*=mx*+b	Conc. range	R	Detection Limit (µg/ml)
Vitamin A	Y=40757x+6430.1	0.05-15	0.9998	0.05
β-carotene	Y=34137x+2011.6	0.05-15	0.9997	0.1

y represent peak area, * x represent concentration

Table 5
Recovery and percentage relative error; of vitamins A and β-carotene, using triethanolamine-glycerol-maleate column (25×0.4 cm (id)). Flow rate flow rate 1.0ml/min and detection wavelength 230nm

Compounds	Conc. Intended (µg/ml)	Conc. calculated* (µg/ml)	Recovery **%	Relative error***%
Vitamin A	5.00	4.91	98.20	1.80
β-carotene	5.00	4.86	96.80	2.80

**** using the linear equation for each vitamins A and β-carotene***
*****calculated by (concentration measured/concentration intended)*100***
******calculated by ((concentration measured-concentration intended)/ concentration intended)*100.***

Standard solutions were injected for at least three times under the same condition for both columns triethanolamine-glycerol-maleate. The RSD% are; 0.43% and 1.91%, recovery% ranged from 96.8% to 98.2% and relative errors% ranged from 1.80% to 2.88% for vitamin A and β-carotene respectively the results are listed in table 5. Also recovered concentrations of vitamins were calculated without intended concentration and with 2µg/ml and 5µg/ml as listed in table 6.

Table 6
Recovered concentration of vitamins A and β-carotene

Compounds	Serum pool	Intended conc.(µg/ml) mean±SD	Measured conc.(µg/ml) mean±SD	Recovered conc.(µg/ml)
Vitamin A	A	0	0.145±0.0124	-
	B	2	2.243±0.0167	1.122
	C	5	5.317±0.0191	1.064
β-carotene	A	0	0.098±0.0043	-
	B	2	2.141±0.0078	1.071
	C	5	5.167±0.0057	1.033

Also the vitamins were determined in serum under the same column. The value of reproducibility in human sera of vitamin A 1.74% and of β-carotene 0.97% as listed in table 7.

Table 7
Reproducibility of vitamin A and β-carotene in human sera

Samples	Vitamin A(µg/ml) (Mean±SD)	CVs%	β-carotene (µg/ml) (Mean±SD)	CVs%
1	0.136±0.003	2.42	0.411±0.006	1.02
2	0.158±0.002	2.11	0.358±0.002	0.95
3	0.134±0.002	1.53	0.376±0.002	0.81
4	0.123±0.004	1.94	0.343±0.004	1.08
5	0.146±0.003	1.87	0.291±0.005	0.89
6	0.147±0.003	1.56	0.253±0.003	0.93
7	0.125±0.002	1.42	0.344±0.003	1.13
8	0.137±0.004	1.62	0.282±0.004	1.17
9	0.148±0.003	1.49	0.388±0.002	0.83
10	0.141±0.002	1.41	0.378±0.002	0.86
RSD%		**1.74**		**0.97**

CONCLUSION

A new method of reversed phase high performance chromatography has been developed for separation and quantification of two water-soluble vitamins vitamin A and β-carotene in human blood serum.

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