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High-performance liquid chromatographic method for routine determination of vitamins A and E and β-carotene in plasma

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ABSTRACT

A simple and reliable reversed-phase high-performance liquid chromatographic (HPLC) method for the routine determination of vitamins A and E and β-carotene in plasma (or serum) with wavelength-programmed ultraviolet–visible absorbance detection is described. A 200-μl aliquot of serum or plasma sample, after deproteinization with ethanol, and containing tocopherol acetate as internal standard, was extracted with butanol–ethyl acetate. Sodium sulphate was added for dehydration. Analytes of extracted samples were found to be stable for at least four days. A 10-μl aliquot of this organic extract was used for HPLC analysis. The mobile phase was methanol–butanol–water (89.5:5:5.5, v/v) and the flow-rate was set at 1.5 ml/min. The analytes of interest were well separated from other plasma constituents within 22 min at 45°C. The lowest detection limits of vitamins A and E and β-carotene were 0.02, 0.5 and 0.1 μg/ml, respectively. The recovery and reproducibility of the present method were around 90%. The method is sensitive, specific and can be used for epidemiological studies and for routine determination of vitamin deficiency. Several important factors that may affect the analysis are also discussed in this paper.

INTRODUCTION

Recently, a number of laboratories have begun studying the cancer chemopreventive properties of vitaminA (all-*trans*-retinol, VA), vitamin E (α-tocopherol, VE) and pro-vitamin A (all-*trans*-β-carotene, BC) [1–7]. Assessments of the human nutritional status of these fat-soluble vitamins are normally carried out by measuring their level in serum or plasma. Clinical assays of these analytes using extraction followed by colorimetry are no longer considered reliable, as they are subject to non-specific interference from other constituents and unresolved isomers of the analytes [8,9]. Many high-performance liquid chromatographic (HPLC) methods for single [4,9–11] or simultaneous multiple [5,12–18] determinations of the three micronutrients using either ultraviolet–visible absorbance or electrochemical detection have also been published. Fluorimetry is also a commonly used method for routine determination of VE in serum; however, this method, like the colorimetric method, suffers from interferences. Most of the published methods involve the use of either double-instead of single-column separation [4], gradient instead of isocratic elution [14–16], two or three separate HPLC lines connected to different detectors [5,15,18] or even three to four types of solvents for the mobile

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phase [5,17,19,20]. The complexity of these HPLC conditions no doubt effectively improves the selectivity and sensitivity of VA, VE and BC determinations, nevertheless, they are considered complicated and costly for routine analysis.

For the purpose of fast screening and routine assessments of vitamin status for community or epidemiological study, we have developed a simpler and more reliable HPLC method. The analytes, after butanol–ethyl acetate extraction, are very stable and can be directly injected into the HPLC system. HPLC separation of VA, VE and BC was performed in a single C_{18} column and detected at different wavelenths with a variable-wavelength and programmable spectrophotometer. The use of methanol–butanol–water (89.5:5:5.5, v/v) as the mobile phase with a flow-rate 1.5 ml/min enables the baseline separation of the three analytes free from interferences with isocratic elution at 45°C. The analysis time was 22 min per injection. It is possible to carry out 24-h continuous analysis for up to five working days if an autoinjector is available. The proposed method is considered most convenient and cost-effective for routine simultaneous measurement of VA, VE and BC in human serum or plasma.

EXPERIMENTAL

Reagents and chemicals

VA, VE, BC and tocopherol acetate (EA) were purchased from Sigma (St. Louis, MO, USA). Methanol, *n*-butanol, ethyl acetate and acetonitrile were HPLC grade. Absolute ethanol and disodium sulphate were obtained from Merck (Darmstadt, Germany). Distilled and deionized water was used for the preparation of all solutions.

Sample collection and storage

Blood samples were collected in brown polypropylene tubes containing heparin as anticoagulant. For serum samples, blood was collected in foil-wrapped glass tubes without heparin. Samples were stored in an ice box prior to centrifugation at 1000 *g* for 10 min at 4°C. A 200-μl aliquot of serum or plasma was then transferred into a foiled-wrapped polypropylene tube. Serum and plasma samples were either used for extraction immediately or stored in the dark at −70°C for stability studies.

Standards preparation and storage

Individual stock standards were prepared in appropriate solvents (VA in ethanol and *n*-hexane, VE in acetonitrile and BC in *n*-hexane) and stored at −20°C. Mixed working standards of various concentrations were prepared in butanol–ethyl acetate (1:1, v/v), protected from light and kept at −20°C when not in use. Tocopherol acetate (EA, 25 μg/ml) was used as an internal standard (I.S.) and prepared in ethanol weekly from stock solution of 1 mg/ml (acetonitrile) concentration.

Sample preparation

A 200-μl aliquot of ethanol containing I.S. (EA, 25 μg/ml) was added to 200 μl of serum or plasma in a polypropylene microfuge tube wrapped in aluminium foil. After vortex-mixing, the specimen was extracted with 400 μl of butanol–ethyl acetate (1:1, v/v) and further mixed for 1 min. Approximately 20 mg of sodium sulphate were added. After vortex-mixing for another 1 min, sample was allowed to stand at −20°C for 20 min before centrifugation at 15 000 *g* for 2 min (Eppendorf microcentrifuge, Model 5414S, Hamburg, Germany). The organic upper layer was transferred into a sealed amber sample vial and stored at −20°C until HPLC analysis.

HPLC apparatus and conditions

HPLC analysis was performed by isocratic elution. A Gilson Model 305 solvent delivery system (Villiers-le-Bel, France) was set at a flow-rate at 1.5 ml/min. The mobile phase, consisting of methanol–butanol–water (89.5:5:5.5, v/v), was premixed and vacuum-filtered through a 0.45-μm polypropylene membrane filter (Whatman, Clifton, NJ, USA) before use. A disposable Whatman IFD filter of 0.2 μm pore size was used as an on-line filter and degassing device. Autoinjection of 10 μl of organic extract was performed by a Gilson autoinjector (Model 231-401). The ana-

lytical column used was a replaceable Partisphere 5 C_{18} cartridge (110 mm × 4.7 mm I.D., 5 μm particle size, Whatman), protected by a guard cartridge (C_{18}, 5 μm) system and maintained at 45°C. VA, VE, EA (I.S.) and BC were detected by a UV–visible spectrophotometer (Hewlett Packard, Model 1050, Palo Alto, CA, USA) at different wavelengths programmed for analysis as follows: at 0 min, 340 nm; 3 min, 290 nm; 4.5 min, 280 nm; and 15–22 min, 450 nm. Analytes were identified by retention time and quantitated by peak height with a Shimadzu Model CR-5A integrator (Kyoto, Japan).

RESULTS AND DISCUSSION

Mobile phase and chromatographic separation

VA, VE and BC are insoluble in water. Therefore, the primary constituent of the mobile phase should be a weak organic solvent with low viscosity. These criteria limit the choices to methanol and acetonitrile. Methanol has been recommended in several recent reports [2,14,21] as it yields higher recoveries of carotenoids than acetonitrile. In order to achieve the desired retention and increase solubility and selectivity, stronger organic modifiers such as tetrahydrofuran, ethyl acetate, chloroform and methylene chloride have been used in several recent publications [9,16–20]. In order to reduce the complexity of analytical conditions for continuous measurement, we decided to use methanol-based solvent as the mobile phase with butanol and water as modifiers.

The present method avoids the use of gradient elution as well as on-line solvent-mixing techniques. The use of a gradient system combined with on-line mixing for vitamin determination often leads to poor reproducibility. Owing to the sensitivity of vitamins to the modifiers, slight changes in the mobile phase composition would lead to a change in retention time. Furthermore, the differences in solvent refractive index cause an unstable chromatographic baseline. On-line mixing of water and methanol also leads to outgassing at the detector flow cell and increases baseline noise.

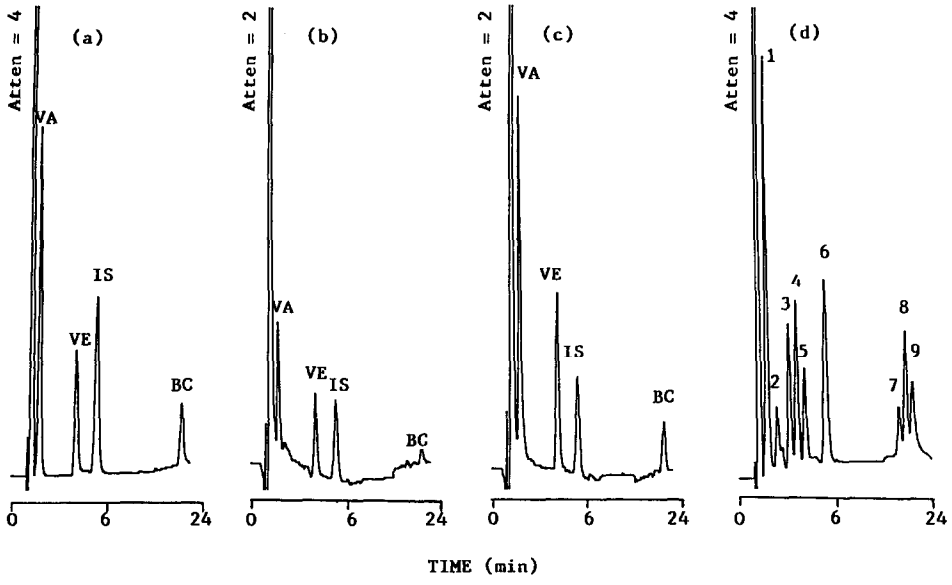

Fig. 1. Chromatograms of (a) pure standards containing VA, VE, EA (I.S.) and BC at a concentration of 1, 10, 25 and 1 μg/ml, respectively, (b) a blank plasma sample, (c) the same plasma sample spiked with known concentrations of analytes as in (a) and (d) a manipulated standard mixture of nine vitamins. Peaks: 1 = retinol (VA); 2 = xanthophyll; 3 = δ-tocopherol; 4 = γ-tocoperol; 5 = α-tocoperol (VE); 6 = tocopherol acetate (EA, I.S.); 7 = lycopene; 8 = α-carotene; 9 = β-carotene. Chart speed: 0–6 min, 3 mm/min; 6–24 min, 1 mm/min.

Using the present isocratic elution method, the analyte solubilities were improved by 5% butanol and the selectivities were affected by 5.5% water added to the mobile phase. The retention time of carotene was dramatically reduced by maintaining the column temperature at 45°C. The use of an on-line filter and degassing device enhanced the stability of chromatographic conditions throughout the whole course of the analysis. Fig. 1a is a chromatogram of pure aqueous standards containing VA, VE, EA (I.S.) and BC at concentrations of 1, 10, 25 and 1 μg/ml and eluted at 1.7, 4.0, 5.3 and 20.1 min, respectively. As shown in Fig. 1b and c, the retention time of the three micronutrients in a blank and a spiked serum sample were identical to those of known standards (Fig. 1a). The analytes of interests were free from other components such as xanthophyll, δ- and γ-tocopherol, lycopene and α-carotene, which were detected and identified at different retention times, as shown in Fig. 1d. Thus, the specificity of the present HPLC analysis of VA, VE and BC in serum is reliable and compatible with other established methods using more complicated techniques.

Standard preparation and stability

Chlorinated solvents such as chloroform and methylene chloride have been widely used in carotenoid analysis [5,16–20]. VA and BC are very soluble in these solvents compared with other solvents used. However, it was found that VA and BC standards prepared with these solvents were rapidly degraded by traces of hydrochloride in these solvents.

On the other hand, in the present investigation, VA and BC were found to be very stable in *n*-hexane. Unfortunately, VA is insoluble in *n*-hexane. However, it is very soluble but unstable in ethanol. Thus the alternative for VA standard preparation was to dissolve VA with one volume of ethanol and maintain its stability with three volumes of *n*-hexane. Unlike VA and BC, VE and EA (I.S.) were easily dissolved in ethanol, methanol or acetonitrile and stable in acetonitrile. The stock standards of different analytes prepared with their respective solvents were all found to be stable at −20°C in the dark for more than one month. Working standards prepared in butanol–ethyl acetate (1:1, v/v) were also stable for more than a week. The calibrations of analytes based on the internal standard method were linear from 0.1 to 2 μg/ml for VA and BC, and from 1 to 20 μg/ml for VE, as shown in Table I. Calibration curve slopes and correlation coefficients of four days' calibration are presented in Table I. The lowest detection limits were 0.02 μg/ml for VA, 0.5 μg/ml for VE and 0.1 μg/ml for BC with an injection volume of 10 μl.

Standard addition and recovery

The standard addition method has been used to determine extraction efficiencies in many studies. It is known that VA, VE and BC are stable in serum because they are transported by lipoproteins. However, it has been reported that "unreal-

TABLE I

LINEARITY AND DAY-TO-DAY VARIATION (n = 4)

LR = Linear regression; y = concentration (μg/ml); x = peak-height ratio (analyte/I.S.); r = coefficient of correlation; C.V. = coefficient of variation.

Compound	Concentration range (μg/ml)	Mean LR	Mean r	C.V. (%)	
				LR	r
VA	0.1–2	$y = 0.4735x + 0.06$	0.99	0.4	0.03
VE	1–20	$y = 13.1975x + 0.75$	0.99	0.9	0.01
BC	0.1–2	$y = 2.5817x + 0.05$	0.99	3.3	0.05

TABLE II

RESULTS OF RECOVERY STUDIES

Original levels: VA, 0.46 μg/ml; VE, 10.44 μg/ml; BC, 0.35 μg/ml; all samples were processed as described in the Experimental section.

Compound	Added (μg/ml)	Mean recovery (n = 3) (%)
VA	0.5	106
	1.0	106
Mean		106
VE	5.0	104
	10.0	109
Mean		107
BC	0.5	101
	1.0	105
Mean		103

istic recovery" may be obtained if free analytes dissolved in solvents are added to serum without the addition of protein denaturant. This is because of the low kinetics of uptake of analytes by the lipoproteins of serum [14]. Therefore, for recovery study, the analytes of known concentrations were added to serum or plasma samples with protein denaturant. Since VA and BC are insoluble in acetonitrile and VE is not so stable in methanol, acetonitrile and methanol are not recommended for sample treatment. On the other hand, it was observed that ethanol yielded higher recovery for all analytes. The recoveries of standards spiked in serum samples were calcualted and are shown in Table II. Recoveries for all three analytes were generally around 100% using the proposed method.

Sample preparation and reproducibility

The proposed extraction procedure was a modification of the method established by Nierenberg and Lester [2]. This procedure is simple and rapid compared with others [14–17]. The final extract with butanol–ethyl acetate permits direct injection into the HPLC system. It eliminates the tedious procedures of drying and reconstitution. However, a disadvantage of this method is that the protein was found to reprecipitate in stored extracted sample and is harmful to the HPLC column.

The modified procedure described here enables complete deproteinization, enhances efficiency and maintains analyte staiblity. One volume of serum or plasma sample was deproteinized with an equal volume of ethanol instead of one fifth

TABLE III

PRECISION OF THE ASSAY (n = 3)

Compound	Sample	Within-day assay		Between-day assay	
		Mean (μg/ml)	C.V. (%)	Mean (μg/ml)	C.V. (%)
VA	a	0.37	1.6	0.38	3.0
	b	0.52	6.7	0.52	5.1
	c	0.63	2.7	0.64	1.8
Mean			3.7		3.3
VE	a	11.94	2.2	11.72	2.8
	b	18.50	1.6	17.47	3.4
	c	14.78	4.7	14.98	3.6
Mean			2.8		3.3
BC	a	0.19	0	0.17	15.1
	b	0.68	12.3	0.63	2.7
	c	0.49	8.9	0.44	14.7
Mean			7.1		10.8

TABLE IV

COEFFICIENTS OF VARIATION OF TEN EXTRACTED SAMPLES FOR FOUR CONSECUTIVE DAYS

VA		VE		BC	
Mean (μg/ml)	C.V. (%)	Mean (μg/ml)	C.V. (%)	Mean (μg/ml)	C.V. (%)
0.52	5.1	12.36	2.3	0.37	9.4
0.49	1.7	14.21	2.5	0.55	13.0
0.39	3.6	11.46	3.1	0.16	27.2
0.68	2.2	10.95	2.7	0.19	7.7
0.52	4.2	17.65	1.9	0.62	20.1
0.43	2.9	8.34	4.0	0.18	37.2
0.55	3.3	10.84	3.3	0.36	11.1
0.70	1.1	8.41	3.3	0.40	23.5
0.68	1.4	18.43	2.7	0.34	29.0
0.65	1.3	14.68	4.2	0.45	11.0
Mean	2.7		3.0		18.9

volume of acetonitrile as proposed by Nierenberg and Lester [2]. Two volumes of butanol–ethyl acetate instead of half were used for extraction. Sodium sulphate instead of dipotassium hydrogenmonophosphate was introudced into the extracted sample for dehydration. The reproducibility of the present method was assessed by extracting and analysing three plasma samples with different concentrations of vitamins, three times a day for three consecutive days. The results are shown in Table III. The average coefficients of variation (C.V.) of within-day and between-day assays were 3.7 and 3.3% for VA, 2.8 and 3.3% for VE and 7.1 and 10.8% for BC analysis.

Vitamin stability in extraction solvent

VA, VE and BC are very unstable in water and are lost instantly in aqueous matrices. On the other hand, VA has been reported to be stable in ethyl acetate for 4 h at room temperature and for 24 h at −20°C [4]. Craft *et al.* [5] reported that there was no significant alteration in the concentrations of VA and VE in alcohol or carotenoids in acetonitrile–methylene chloride–methanol (70:20:10, v/v) after 18 h at room temperature in the dark. The loss of analyte stability within 24 h may be the result of the presence of water in the extracted sample. In the present studies, the extracted analytes were found to be very stable in butanol–ethyl acetate (1:1, v/v) after dehydration with sodium sulphate.

The stability study was carried out by reanalysing ten extracted samples by HPLC for four consecutive days. As shown in Table IV, the C.V.s were generally less than 3.0% for VA and VE. β-Carotene appeared to be less stable, with a C.V. close to 19%. The results show that the analytes in the final extract were stable for at least four days when stored at room temperature in the dark. The stability of analytes allows a large number of samples to be extracted and stored for later HPLC analysis.

Stability of vitamins in plasma/serum

It was noted that VA, VE and BC are very sensitive to light and oxidation. Special care has to be taken for sample collection and storage [1,4,5,22,23]. Plasma samples for VA, VE and BC measurements can be stored up to one to two years at −20°C or −70°C without loss of stability, as reported by Peng *et al.* [4] and Craft *et al.* [5]. Others have reported significant losses [1] and increases [23] in the concentrations of analytes when stored at −20°C. To study the effect of storage on the stability of analytes in plasma/serum, 200 μl of each of the serum extracts in foiled-wrapped polypropylene tubes and tubes containing larger volume samples were stored at

−70°C in the dark until extraction. It was noted that there was significant degradation of VE and BC if stored samples were thawed and refrozen. In contrast, there was no significant loss of small aliquots of serum/plasma were stored at −70°C for up to 30 days. This finding is in good agreement with those reported previously [4,5]. It is recommended that plasma/serum samples be stored in small aliquots in small polypropylene tubes at −70°C in the dark.

Reference values

Both plasma and serum samples collected from twenty male subjects with no known medical disease had been previously analysed by using the present method. The mean values of the three micronutrients in plasma (serum) of the twenty subjects were detected and calculated as: VA, 0.49 ± 0.09 (0.52 ± 0.10); VE, 11.59 ± 3.20 (12.11 ± 3.48); BC, 0.43 ± 0.13 (0.42 ± 0.16) mg/ml. These results are compatible with recent reports [6,9,12–14,18,25]. No significant differences in vitamin concentrations were observed between serum and plasma.

CONCLUSION

The present method can be considered a relatively simple, convenient and cost-effective procedure for routine determinations of VA, VE and BC in serum and plasma. It involves a single-step extraction and direct injection of samples. The extracted samples were found to be stable. The analytical method avoids the complication of gradient separation systems and the use of multiple detectors. The sample preparation and analytical time is relatively short and thus can be used for epidemiological study of these three micronutrients.

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