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An isocratic LC method for the simultaneous determination of vitamins A, C, E and β-carotene

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Abstract

An isocratic liquid chromatographic method for the separation and simultaneous determination of retinyl acetate, propionate or palmitate (esters of vitamin A), β-carotene (pro-vitamin A), ascorbic acid (vitamin C) and DL-α-tocopherol (vitamin E) is described. Samples are analysed by means of a reversed-phase column (LiChrospher 100 RP-18), using methanol as mobile phase. The UV–Vis detector used was set at a wavelength of 300 nm and switched to 450 nm at 17 min, allowing the determination of β-carotene. These vitamins were separated within 25 min and the detection limits ranged from 7 (β-carotene) to 65 ng ml^{-1} (ascorbic acid). © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Liquid chromatography; Retinyl acetate; Retinyl palmitate; Retinyl propionate; β-Carotene; Ascorbic acid; α-Tocopherol

1. Introduction

Free radicals that are produced through biological processes in response to exogenous stimuli in normal conditions are controlled by various enzymes and antioxidants in the body. Laboratory evidence suggests that oxidative stress, which occurs when free radical formation exceeds the ability to protect against them, may form the biological basis of several acute medical problems, including tissue injury after trauma, and chronic conditions, such as atherosclerosis and cancer [1]. The potential role for the antioxidant micronutrients (e.g. vitamin C, vitamin E and carotenoids) in modifying the risk for conditions that may result from oxidative stress has stimulated intense research efforts, increased interest in micronutrient supplements, and heightened consumer interest in these compounds [1].

The most common method for analysing water-soluble or fat-soluble vitamins in pharmaceutical preparations, foodstuffs, and biological materials is high performance liquid chromatography (HPLC) combined with a UV–Vis detector [2–13]. Several procedures for simultaneous measurement of vitamin A and vitamin E by HPLC have

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been described [2–12]. However, to our knowledge, HPLC methods for the simultaneous determination of these fat-soluble vitamins and the water-soluble vitamin C under isocratic conditions have not been reported so far. The United States Pharmacopoeia [13] only describes individual HPLC methods to quantify these vitamins in pharmaceutical preparations.

The aim of the present study was to develop a rapid, simple and sensitive HPLC isocratic method for the simultaneous determination of vitamins A, C, E and β-carotene in the presence of several commonly used pharmaceutical excipients.

2. Materials and methods

2.1. Apparatus and chromatographic conditions

A Merck-Hitachi liquid chromatograph system (Merck, Darmstadt, Germany) equipped with a model L-6000 pump, a model AS-2000 automatic injector and a model L-4000 variable-wavelength UV–Vis detector were used. The chromatographic column was a LiChrospher 100 RP-18, 125 × 4 mm i.d., particle size 5 μm, (Merck). The mobile phase consisted of methanol delivered at a flow-rate of 1.5 ml min^{-1}. It was filtered through a 0.45 μm membrane and degassed by sonication prior to use. The UV–Vis detector operated at 300 nm until the elution of retinyl palmitate (~ 17 min) and 450 nm for the remaining part of the chromatogram. The sensitivity of the detector was at 0.1 a.u.f.s., and data were processed with a model D-7500 Merck-Hitachi integrator.

2.2. Reagents and standards

Methanol, acetonitrile and chloroform were of HPLC-grade from Merck. Ascorbic acid, DL-α-tocopherol, retinyl acetate, retinyl palmitate and retinyl propionate were a gift from BASF Portuguesa (Lisbon, Portugal) and β-carotene was purchased from Sigma (St. Louis, MO). Individual stock solutions containing 150 I.U. ml^{-1} vitamin A (as retinyl acetate, palmitate, propionate and β-carotene), or 4.5 I.U. ml^{-1} vitamin E (as DL-α-tocopherol) were prepared in chloroform. Another stock solution containing 9 mg ml^{-1} vitamin C was prepared in methanol. The international units (I.U.) provide information on biological activity of vitamins and are defines as follows [14]: 1 I.U. vitamin A = 0.344 μg retinyl acetate, 0.550 μg retinyl palmitate, 0.359 μg retinyl propionate, 0.6 μg β-carotene; 1 I.U. vitamin E = 0.91 mg DL-α-tocopherol. These solutions were further diluted with methanol to give a series of working standards. A standard vitamin mixture was prepared by taking 2 ml of each stock solution to a final volume of 100 ml with methanol. Stock solutions were freshly prepared, and used immediately afterwards.

2.3. Liquid vitamin mixtures

Four liquid preparations with the same composition, differing only in the ester of vitamin A, were prepared. One ml of each liquid mixture contained 150 I.U. of vitamin A (as retinyl acetate, propionate or palmitate or as β-carotene), 9 mg of vitamin C and 4.5 I.U. of vitamin E (as DL-α-tocopherol). To validate the analytical method herein described, a placebo was first prepared, without vitamins, and only containing excipients dissolved in deionised water (propylene glycol 15% v/v, PEG-15 hydroxystearate 15% w/v, PEG-40 glyceryl trihydroxystearate 15% w/v, saccharin 0.1% w/v and orange flavour 0.1% w/v). The placebo was further spiked with known amounts of each vitamin. Two ml of each combination were mixed with 2 ml of chloroform and diluted with methanol to a final volume of 100 ml.

3. Results and discussion

3.1. Chromatography

The development of the analytical method started by the selection of the mobile phase composition in order to separate vitamin A (as retinyl acetate, palmitate or propionate, or β-carotene), vitamin C and vitamin E (as DL-α-tocopherol). Methanol, acetonitrile and water were used as the basis for the different mobile phases preparation.

Various proportions of these solvents were tested (results not shown). However, the best mobile phase for the separation of these vitamins was found to be 100% methanol. The wavelength for the detection of β-carotene was 450 nm. For the other vitamins, 300 nm was chosen after different wavelengths had been tested. Both wavelengths chosen are well above the cut-off for most organic solvents. In fact, upon testing no solvent peak, which might interfere with excipients or ascorbic acid, which is eluted close to the void volume of the chromatograms, can be detected at 300 nm (Figs. 1 and 2(A)). Moreover, since the absorbance maximum for ascorbic acid is 245 nm, the use of a sub-optimal wavelength reduces the detector response to this vitamin, which is advantageous because of the disproportional higher amount of ascorbic acid present in multivitamin preparations as compared to other vitamins due to its recommended daily intake requirements [14].

The optimum flow rate for the mobile phase was found to be 1.5 ml min^{-1}, resulting in a maximum run time of 25 min. Fig. 2 shows a typical chromatogram obtained with a placebo mixture (Fig. 2(A)), together with placebo spiked with a standard mixture of the vitamins (Fig. 2(B)). Retention times and the maximum absorption of the vitamins are shown in Table 1. For the vitamin liquid mixture (placebo plus vitamins) not including as retinyl palmitate or β-carotene, the run time could be reduced to 7 min.

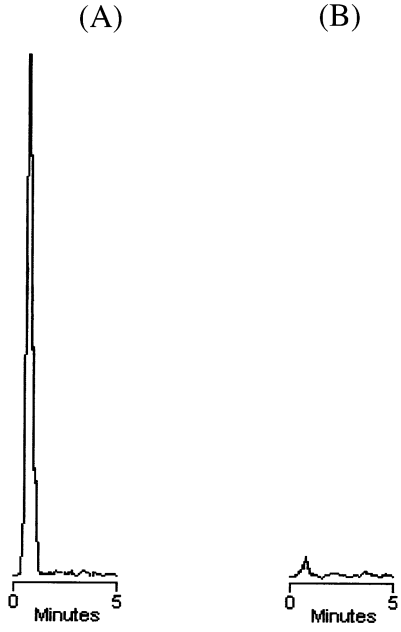

Fig. 1. HPLC chromatogram of methanol at: (A) 280 nm; and (B) 290 nm.

The sample preparation method was simple and consisted in diluting the liquid mixture herein described in chloroform and methanol. These solvents were selected to prepare the standards and sample solutions since vitamin A, E and β-carotene are soluble in chloroform and vitamin C in methanol.

3.2. Linearity

Linearity was checked for each vitamin using five standard solutions with concentrations ranging from ~ 50 to 200% of the theoretical amounts of vitamins in the studied liquid mixture. Peak areas and analyte concentrations were found to be linearly related over this range for all the vitamins (Table 2). Linear regression was used to determine the slope and intercept. The correlation coefficients of the calibration curves were > 0.997 for all vitamins tested.

As previously reported, ascorbic acid is present in concentrations much higher than the other vitamins. This makes quantification difficult since it has been noticed that for high range of concentrations (100.7–402.8 μg ml^{-1}), the intercept is also high, which might be due to any sort of interference, particularly from the solvent or the placebo components, due to its low retention time. However, as shown in Fig. 3, ascorbic acid presents a biphasic linearity pattern over the range of concentrations studied using these experimental procedure, Hence, the chromatographic method may be applied because the low intercept found for the concentration range 5.0–100.7 μg ml^{-1} precludes the possibility of interference from both the solvent or the placebo.

3.3. Precision

The precision of the method was determined by replicate analysis ($n = 5$) of placebo mixtures

Table 1
Retention times, maximum absorption, precision and limits of detection and quantification of the investigated vitamins separated by HPLC

Substance	Retention time (min)	Maximum absorption (nm)[a]	Detection wavelength used (nm)	Precision concentration (ml^{-1})	CV (%)	LOD (ng ml^{-1})	LOQ (μg ml^{-1})
Ascorbic acid	0.9–1.1	245	300	180 μg	2.14	65	1.518
Retinyl acetate	2.3–2.6	324	300	3.0 I.U.	2.45	36	0.240
Retinyl propionate	2.7–3.0	324	300	3.0 I.U.	2.51	22	0.137
DL-α-Tocopherol	4.5–5.0	291	300	0.09 I.U.	1.27	53	0.836
Retinyl palmitate	15.0–16.0	324	300	3.0 I.U.	1.83	15	0.185
β-Carotene	18.0–20.0	450	450	3.0 I.U.	2.09	7	0.037

[a] Determined in previous experiments not reported here

Table 2
Results of linear regression analysis of calibration data

Parameters	Ascorbic acid		β-Carotene	DL-α-Tocopherol	Retinyl acetate	Retinyl palmitate	Retinyl propionate
Range	5.0–100.7 μg ml^{-1}	100.7–402.8 μg ml^{-1}	0.7–8.3 I.U. ml^{-1}	0.04–0.19 I.U. ml^{-1}	1.9–7.7 I.U. ml^{-1}	1.6–6.3 I.U. ml^{-1}	1.7–6.8 I.U. ml^{-1}
Slope (b)	4438.0	2340.3	88101.0	2408.2	9852.9	59762.6	43187.2
Intercept (a)	9599.0	226131.5	34879	−4115.5	−782	−1691.5	753
Standard error of slope (S_b)	76.4	99.8	3026.9	21.9	114.4	911.2	1071.9
Standard error of intercept (S_a)	3645.2	27512.7	1352.2	2366.2	400.9	1152.8	1979.9
Correlation coefficient (r)	0.9994	0.9982	0.9978	0.9999	0.9999	0.9998	0.9994

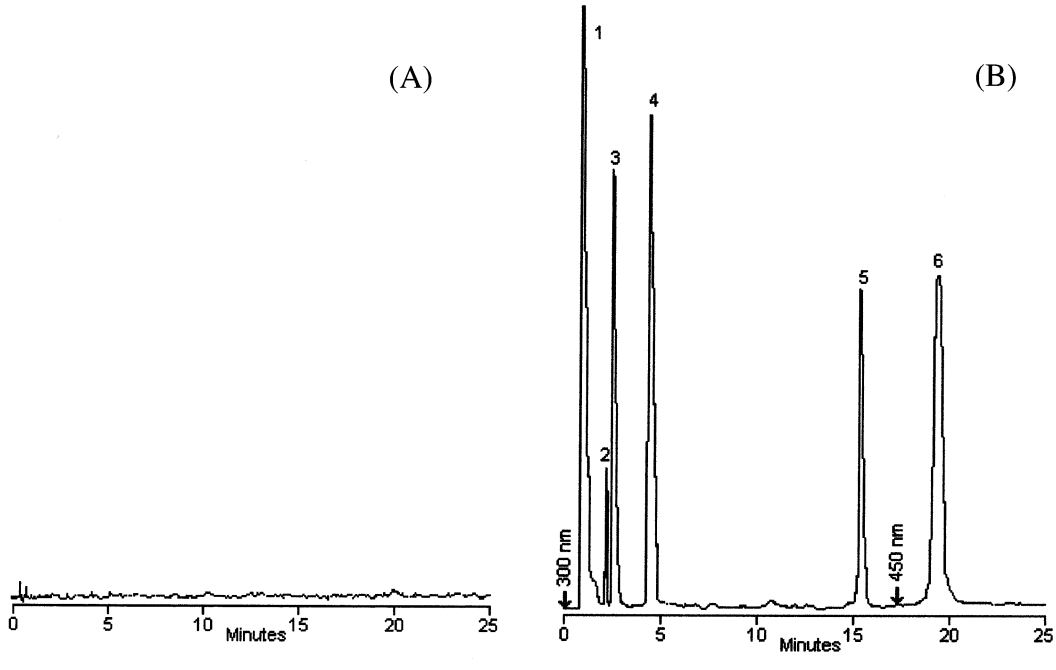

Fig. 2. HPLC chromatogram of preparations: (A) placebo (see composition in text); (B) liquid mixture of placebo and vitamins. Peaks: 1. ascorbic acid; 2. retinyl acetate; 3. retinyl propionate; 4. DL-α-tocopherol; 5. retinyl palmitate; 6. β-carotene.

spiked individually with vitamins (100% of the theoretical amounts used in our preparations). The coefficients of variation (CV) ranged from 1.27 to 2.51% (Table 1).

3.4. Recovery

The mean relative recovery was calculated comparing the concentrations obtained for the vitamin spiked placebo with three nominal concentrations used for the calibration curve.

For the vitamins in placebo (Table 3), mean relative recovery ranged from 96.5 (β-carotene) to 101.4% (retinyl acetate).

3.5. Limits of detection (LOD) and quantification (LOQ)

Table 1 shows the LOD and LOQ values for the vitamins tested. The limit of detection was taken as the amount of vitamin giving a signal to noise ratio greater than 3. The LOD determined ranged between 7 (β-carotene) and 65 ng ml^{-1} (ascorbic acid). Furthermore, the LOQ was taken as the minimum quantifiable concentration presenting a CV value $\leq 2.5\%$ ($n = 10$). For the vitamins tested, the LOQ ranged between 1.518 (ascorbic acid) and 0.037 μg ml^{-1} (β-carotene).

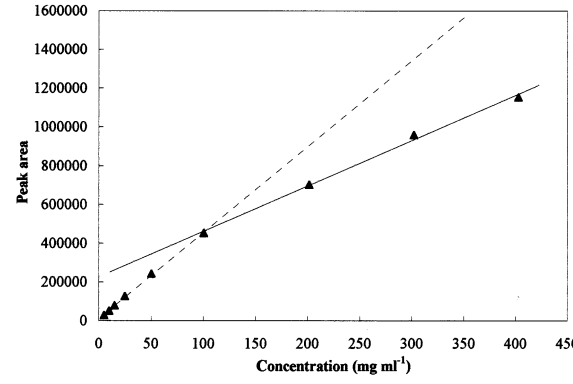

Fig. 3. Standard curves showing the concentration dependence of linearity for ascorbic acid quantification. Concentration ranges: 5.0–100.7 μg ml^{-1} (– – –); 100.7–402.8 μg ml^{-1} (—).

Table 3
Mean relative recovery of vitamins from placebo

Substance	Spiked concentration range (ml^{-1})	Recovery ($n = 6$)	
		Mean (%)	CV (%)
Ascorbic acid	4.50–18.00 mg	98.4	3.13
β-Carotene	75.00–300.00 I.U.	96.5	0.95
DL-α-Tocopherol	2.25–9.00 I.U.	100.8	1.71
Retinyl acetate	75.00–300.00 I.U.	101.4	1.76
Retinyl palmitate	75.00–300.00 I.U.	96.9	1.55
Retinyl propionate	75.00–300.00 I.U.	100.5	4.25

3.6. Specificity

The specificity of the analytical method was checked by analysing all the following excipients commonly used in multivitamin formulations [14]: ethanol, glycerol, propylene glycol, sorbitol, PEG-15 hydroxystearate, PEG-40 glyceryl trihydroxystearate, polysorbate 80, cyclodextrins, citric acid, glucose, mannitol, saccharin and sucrose. No interfering peaks were detected at the retention times of the vitamins, when the amounts of excipients used were similar to those employed for pharmaceutical purposes (Fig. 2(A)).

4. Conclusions

Under the chromatographic conditions employed, all vitamins were clearly separated from each other. Using an isocratic HPLC method and varying the detection wavelength, ascorbic acid could be successfully separated and quantified in liquid mixtures containing some hydrophobic vitamins. Calibration curves were linear ($r > 0.997$) from 50 to 200% of the theoretical amounts used in the liquid preparation and the precision of the assay is acceptable. Vitamin recovery levels from formulation were high (96.5–101.4%) and practically concentration independent. The detection limit (LOD) was found to be between 7 and 65 ng ml^{-1}; for vitamins A and E the LOD could be lowered if the detection wavelength used was at their maximum absorption. In addition, the LOQ determination shows that vitamin concentrations between 1.518 (ascorbic acid) and 0.037 μg ml^{-1} (β-carotene) could be quantified.

In summary, the method reported here proved to be selective, precise, linear, and sensitive, being adequate for the simultaneous determination of vitamins A, C, E and β-carotene. Experiments for application of this method to commercially available liquid and solid multivitamin formulations are currently underway.

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