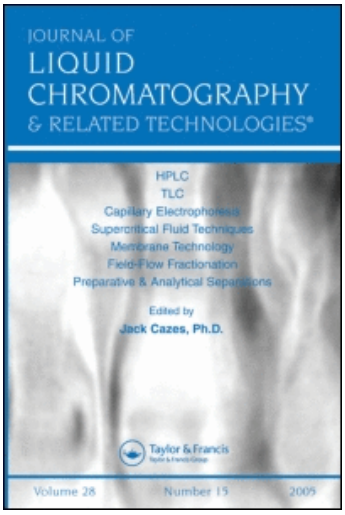

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHODS FOR THE QUANTITATION OF VITAMIN A PALMITATE IN LIQUID MULTIVITAMIN FORMULATIONS

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ABSTRACT

Several normal-phase high performance liquid chromatographic methods for the quantitation of Vitamin A palmitate in liquid multivitamin formulations using both radially-compressed and conventional silica columns are described. The methods are reproducible, require minimal sample preparation and resolve the predominant cis-trans isomers of vitamin A (13-cis, all-trans, 9-cis and 9,13-dicis). Good agreement is obtained between HPLC and USP assay for samples containing a small amount (6% or less) of the vitamin A as the 13-cis isomer.

INTRODUCTION

The current compendial method (1) for the determination of vitamin A esters - acetates, palmitates and propionates - involves saponification, extraction and subsequent spectrophotometric quantitation as the alcohol, using the Morton-Stubbs technique (2,3). The method is time consuming, operator dependent, does

not account for the difference in extinction coefficients, nor for the difference in biological activities of the predominant cis-trans isomers of vitamin A (4-6).

Recently several high-performance liquid chromatographic methods for the determination of vitamin A, either separately (7-12) or simultaneously (13-18) with vitamins D and E, have been reported. The simultaneous methods, although much faster than the compendial techniques, still do not separate the predominant cis-trans isomers of vitamin A, and therefore may overestimate the available potency. Most of the single determination methods have achieved separation of the predominant cis-trans isomers of vitamin A. However, these methods, like the simultaneous determination techniques, are concerned only with vitamin A acetate. The two reported HPLC vitamin A palmitate methods (10,13) have not been applied to liquid multivitamin formulations, and only one (13) is capable of resolving the major isomers.

This report describes several normal-phase HPLC methods suitable for the quantitation of vitamin A palmitate in liquid multivitamin formulations. The methods require minimal sample preparation, are reproducible and can utilize both conventional steel and radially-compressed silica columns. Most importantly, the methods resolve the four predominant sterically unhindered (19) isomers of vitamin A (all-trans, 13-cis, 9-cis and 9,13-dicis) and anhydro vitamin A.

EXPERIMENTAL

REAGENTS - Hexane and methyl t-butyl ether were obtained from Burdick and Jackson. 1,4-Dioxane was obtained from Fisher Scientific and fluoranthene from Matheson, Coleman and Bell. All other reagents were analytical reagent grade. The standard is a vitamin A palmitate dispersed in oil, the potency of which is

about one million International Units (IU) per gram. It was characterized by the USP assay, by the direct spectrophotometric British Pharmacopeia (20) assay and by HPLC against a six-times recrystallized vitamin A palmitate reference standard. The standard, which has an all-trans to 13-cis ratio of about 95 to 5, was overlayed with nitrogen and stored under refrigeration.

INSTRUMENTATION - Two chromatographic systems were used throughout. One system consisted of a Perkin-Elmer Series 2/2 pump, a Rheodyne Model 7120-loop injector and a Perkin-Elmer Model LC-75 variable wavelength detector set at 325 nm.

The other system consisted of a Waters Associates Model M45 pump, a Model 710 automatic injector and a Model 440 UV detector set at 313 nm.

COLUMNS AND MOBILE PHASES - System I - several columns were used. These were: a Waters Associates μPorasil (30 cm x 3.9 mm), an E. S. Industries Chromegasorb 100R Silica, 10 μ (30 cm x 4.6 mm) and an E. M. Industries Lichrosorb SI-60, 5 μ (25 cm x 4.6 mm). The mobile phase was a 1% methyl t-butyl ether solution in hexane. The flow rate can be varied from 1.4 to 2.0 ml/min.

System II - A Waters Associates Radial Compression Module, RCM-100, fitted with a Radial-Pak B silica cartridge, 8 mm ID, was also used. The mobile phase, pumped at 1.4 ml/min, was a hexane: 1,4-dioxane: triethylamine mixture (997:3:0.02).

PROCEDURE - Low actinic glassware was used throughout. Extractions and dilutions were done with a 0.1% solution of butylated hydroxytoluene (BHT) in hexane.

Standard Preparation - Approximately 50 mg of accurately weighed vitamin A standard was dissolved and diluted to volume in a 50-ml volumetric flask (Standard Solution). Approximately 75.0 mg of fluoranthene were accurately weighed, dissolved and diluted to volume in a 50-ml volumetric flask (Internal Standard).

A 2.0 ml aliquot of the Standard Solution and 2.0 ml of Internal Standard were pipetted into a 50-ml volumetric flask and diluted to volume (Working Standard).

Sample Preparation - Water dispersed multivitamin formulations - A 2.0 ml sample aliquot was transferred into a 50-ml centrifuge tube. The pipet or volumetric flask was rinsed twice with 2 ml portions of dimethylsulfoxide (DMSO). The combined sample-DMSO mixture was extracted with 3 x 25 ml portions of 0.1% BHT in hexane with the aid of a mechanical shaker (approximately 5 minutes shaking time) and centrifuged. The clear supernatants were combined and diluted to 100 ml in a volumetric flask (Method I). Ten ml of this solution and 2.0 ml of the Internal Standard were pipetted into a 50-ml volumetric flask and diluted to volume (Sample Solution).

An alternate preparation (Method II) was also used. To the DMSO-sample mixture 25.0 ml of the BHT/hexane solution was pipetted. The mixture was shaken for 5 minutes on a mechanical shaker and centrifuged. Two ml of the supernatant and 2.0 ml of the Internal Standard were pipetted into a 50-ml volumetric flask and diluted to volume (Sample Solution).

Sample Preparation - Oil dispersed multivitamin formulations - Oil-dispersed formulations were treated in the same manner as the vitamin A standard. The sample solutions to be injected, however, were prepared to contain about 35 IU/ml of vitamin A palmitate.

System Suitability - A solution was prepared containing about 40 IU/ml of isomerized vitamin A palmitate (about 30% of the vitamin A exists as the 13-cis isomer) and 20 µl were injected. The system was considered suitable if baseline separation was obtained between the 13-cis and 9-cis vitamin A peaks. Alternately, the resolution factor between the all-trans and 13-cis peaks

should be greater than 3.2 if system suitability requirements are to be satisfied.

Quantitation - The vitamin A palmitate content was calculated using internal standard peak-area techniques and expressed in terms of IU per volume or weight. The total area of the vitamin A was expressed as the sum of the areas of the 13-cis and all-trans peaks. The extinction coefficients and biopotencies of both isomers were assumed to be equal. The validity of this assumption will be discussed in a later section. Twenty microliter injection volumes were made throughout.

RESULTS

An accurate analysis of vitamin A is a fairly difficult task. The instability of the molecule (5, 8, 9, 21, 22), the possible presence of several isomers and the lower relative biological activity (4-6) of these isomers all contribute to the difficulty.

The present methods minimize the above problems. The addition of BHT to the extracting solvent prevents both air oxidation (21) and possible column degradation/isomerization (8) of vitamin A palmitate. In addition by separating the most probable cis-trans isomers and utilizing the published extinction coefficients (6, 7), the calculation of sample potencies with increased accuracy will be possible once reference standard materials and reliable potencies become available.

Chromatograms of the working standard, and some sample solutions, obtained with system I, are shown in Figures 1-3. A chromatogram of a system suitability solution, obtained with system I, is similar to the one shown in Figure 3. A chromatogram of this same solution, obtained with system II, is shown in Figure 4. In all cases the 13-cis and all-trans vitamin A peaks are separated.

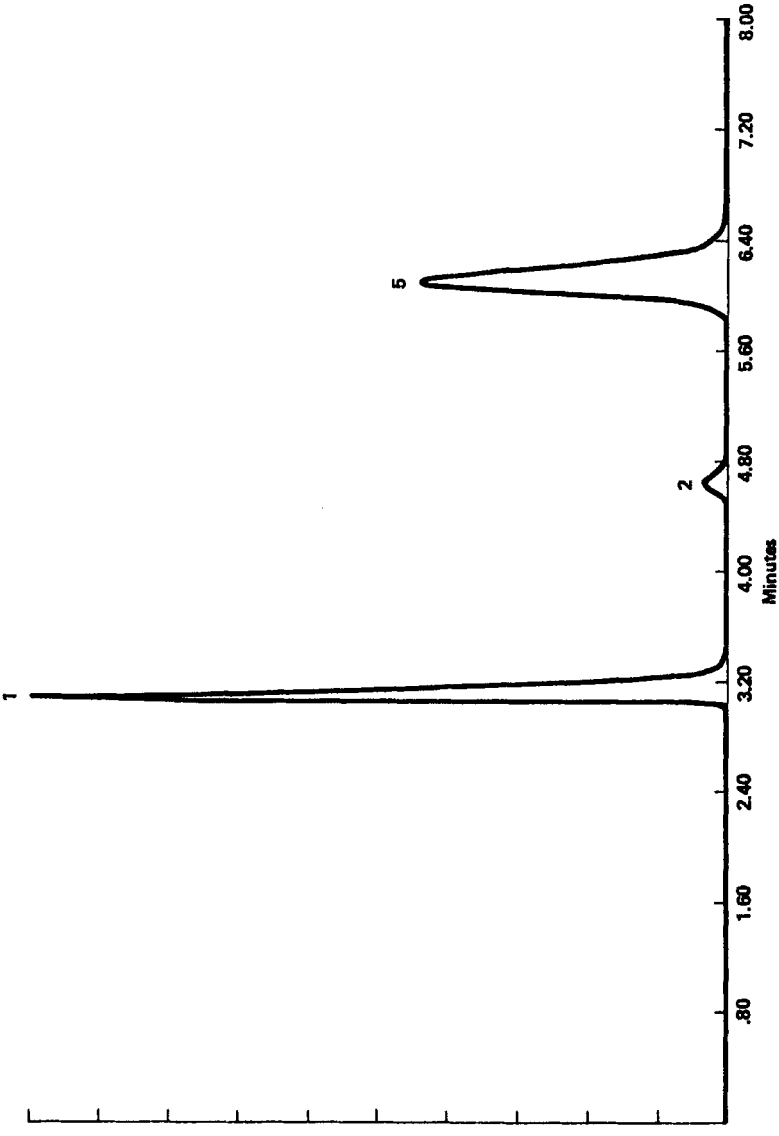

FIGURE 1. Chromatogram of the Working Standard at 313 nm. Column: Chromegasorb 100R. Mobile Phase: 1% Methyl-t-butyl ether in hexane. Flow rate: 2.0 ml/min. (1) Fluoranthene (Internal Standard); (2) 13-cis vitamin A palmitate; (5) all-trans vitamin A palmitate.

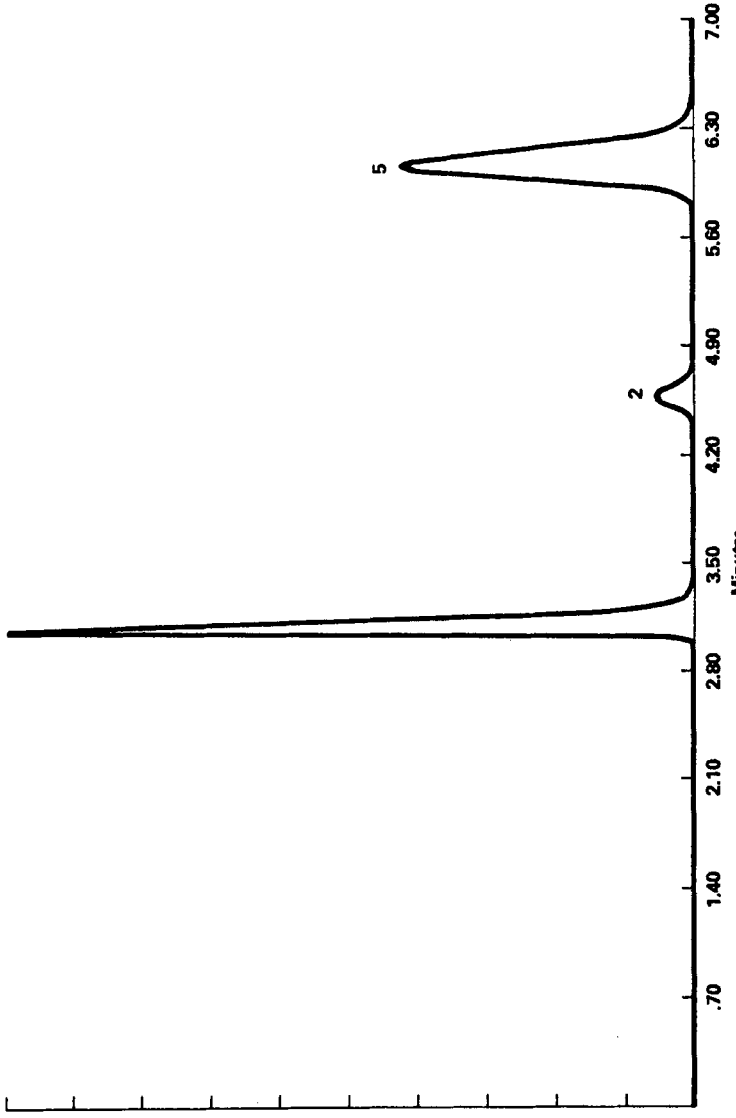

FIGURE 2. Chromatogram of a sample containing about 5% of the vitamin A as the 13-cis isomer. Conditions and explanation as in Fig. 1.

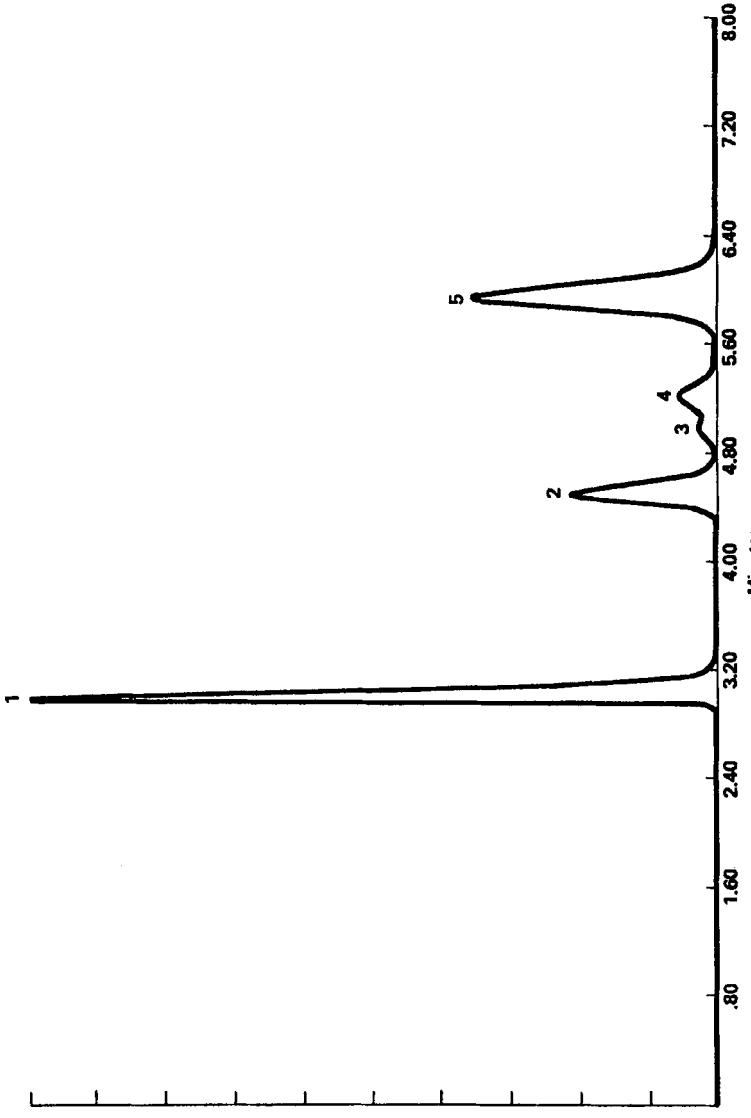

FIGURE 3. Chromatogram of a sample containing about 30% of the vitamin A as the 13-cis isomer. Conditions and explanation as in Fig. 1. (3) 9,13-dicis vitamin A palmitate; (4) 9-cis vitamin A palmitate.

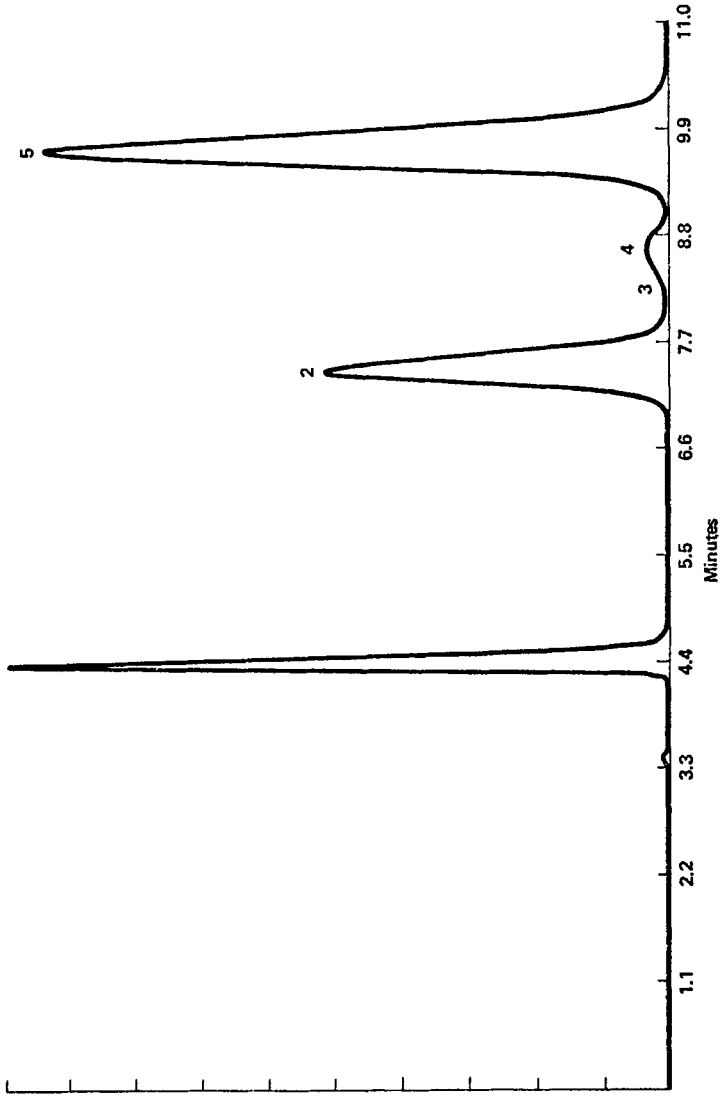

FIGURE 4. Chromatogram of a System Suitability solution at 325 nm containing internal standard (1). Column: Radial-Pak B (8 mm I.D.). Mobile phase: hexane-dioxane-triethylamine (997:3:0:0.02). Flow Rate: 1.4 ml/min. (2) 13-cis vitamin A palmitate; (3) 9,13-dicis vitamin A palmitate; (4) 9-cis vitamin A palmitate; (5) all-trans vitamin A palmitate.

Two other peaks corresponding to the 9-cis and 9,13-dicis isomers, are also separated from the main peaks. Both systems are capable of resolving the 13-cis and all-trans isomers from the low-potency 9-cis and 9,13-dicis isomers. System I, however, gives better separation between the 9-cis and 9,13-dicis isomers. The best separation between the isomers was obtained on the Lichrosorb 5 μ column.

Quantitation of vitamin A in the various samples was accomplished by adding the areas of the 13-cis and all-trans peaks. This method assumes equal extinction coefficients and biopotencies for these two isomers. A calculation based on published extinction coefficients (6, 7) and some available biopotencies (4-6) shows that this method of quantitation may overestimate by 4% the true potency of a sample containing 30% of the vitamin A as the 13-cis isomer (Figure 3). However, there is no overestimation in samples containing 4-6% of the vitamin as the 13-cis isomer (Figure 2) since these samples are similar to the standard. The areas of the low-potency 9-cis and 9,13-dicis isomers were not considered, even though they could amount to as much as 15% of the total vitamin A area in some very old samples (Figure 3). It should be noted that the USP method does not correct (6, 12) for the low biological activity of these isomers even though they do contribute to the measured absorbance.

Results of vitamin A palmitate HPLC assays in oil and water-dispersed multivitamin formulations are shown in Tables I and II.

Table I also shows the agreement between USP and HPLC assay results. Table II shows the HPLC assay results obtained under different experimental conditions: 325 nm versus 313 nm, single extraction versus triple extraction and system I versus system II. Identical results were obtained using the Lichrosorb and Chromegasorb columns.

Table I.

Comparison of HPLC and USP Vitamin A Assays of Various Formulations

Sample Description	Lot No.	% Label Claim USP	% Label Claim HPLC
Vitamin A and β-Carotene in Oil	1	110	106
	2	106	107
	3	110	107
Vitamin A, D_2 and β-Carotene in Oil	1	110	107
	2	107	109
	3	103	104
Water-dispersed vitamin A	1	110	107[a]
Water-dispersed vitamins A, D_2 and E	1	100	101[a]
Water-dispersed vitamin A and D_2	1	110	108[a]

[a]Single extraction, 313 nm

Table II.

HPLC Results of Vitamin A Assays on Water-dispersed Pharmaceutical Dosage Forms

Product	Lot	Months Shelf-Life	Months Age	Potency, % Claim System I	Potency, % Claim System II	
A	1	36	78	107[c]	105[a]	104[b]
A	2		60	120	116	116
A	3		40	123	121	
A	4		27	121	119	120
B	1	24	37	84	86	
B	2		11	108	114	114
C	1	24	36	99	96	
C	2		16	129	124	
D	1	36	43	127	129	
D	2		27	126	121	123

[a]Triple extraction technique, 325 nm
[b]Single extraction technique, 325 nm
[c]Triple extraction technique, 313 nm

Recovery studies were conducted by adding a solution of the standard vitamin A to previously assayed water-dispersed formulations and assaying as previously described. Recovery rates were well within the range of 100 ± 4%. Sample matrix interferences were found to be non-existent by stopped-flow spectral comparison and absorbance ratioing techniques.

The linearity of the chromatographic methods was determined between 15% and 230% of the working range by injecting vitamin A palmitate solutions containing a constant amount of internal standard. The relative standard deviation of the peak-area response ratio was less than 1.8% over the entire range.

The reproducibility of the chromatographic systems was determined by making six injections of the working standard and computing the peak-area response ratio. The relative standard deviation of the response ratio ranged from 0.3 to 1.6%.

Table III.

Results of Replicate Assays from Two lots of Water-dispersed Multivitamin Dosage Form

	% of Claim	
	System I[a]	System II[b]
	106	121
	107	126
	106	122
	106	122
	108	128
	108	124
Average	107	124
Standard Deviation	0.98	2.7
Rel. Std. Deviation	0.92%	2.2%

[a]Sample A, lot 1
[b]Sample C, lot 2

The reproducibility of the methods was determined by conducting six replicate assays of a water-dispersed multivitamin sample. The results and the relative standard deviations are shown in Table III.

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