Simultaneous Determination of Retinol Acetate, Retinol Palmitate, Cholecalciferol, α-Tocopherol Acetate and Alphacalcidol in Capsules by Non-Aqueous Reversed-Phase HPLC and Column Backflushing

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Key Words

Column liquid chromatography
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Fat-soluble vitamins in capsules
Retinol palmitate and related compounds
Alphacalcidol

Summary

A very simple non-aqueous reversed-phase HPLC method has been developed for analysis of retinol acetate, retinol palmitate, cholecalciferol, α-tocopherol acetate and alphacalcidol in capsules without the need for saponification. A reversed-phase (LiChrospher C8, 4.6 mm i.d.) column is used with acetonitrile–methanol, 95:5 (*v/v*) as mobile phase at flow rate of 1 mL min^{-1}. Sample treatment consists only in dilution of the capsule contents with *n*-hexane and methanol. This method is suitable for routine quantification in the industrial quality-assurance laboratory.

Introduction

High-performance liquid chromatography (HPLC) has been successfully applied to the separation of fat-soluble vitamins in pure standard solution [1], pharmaceutical formulations [2], human serum [3, 4] and food samples [5], by either gradient elution or isocratic separation techniques.

Traditional methods for assay of vitamins A, D, E and alphacalcidol in multivitamin preparations entail separate analysis of each vitamin by different biological or physicochemical techniques [6–9]. Many of these methods include very sophisticated and time-consuming procedures for sample preparation and purification. Usually the methods also lack selectivity, high precision and accuracy because of interference from other ingredients in the formulation. As a result separate chromatographic procedures have been developed for the quantitative determination of each of these fat-soluble vitamins. HPLC analyses of vitamins D_2 and D_3 using both normal- and reversed-phase separations have been published [10, 11]. Williams et al. have also reported simultaneous determination of all the fat-soluble vitamins (except alphacalcidol) by HPLC [12], although they could not achieve baseline separation. In a more recent publication, Barnett and Tricke described a reversed-phase HPLC system for the simultaneous determination of vitamins A and E [13].

The general procedure used for vitamin analysis involves preparation of the sample extract then HPLC, and during the last decade a standard procedure for the preparation of samples for HPLC analysis of oil-soluble vitamins has been developed [14, 15]. This usually involves hydrolysis with ethanolic potassium hydroxide and extraction of the samples with a variety of solvents (the most efficient being a mixture of light petroleum and diethyl ether).

Saponification followed by solvent extraction is the technique most widely employed to prepare samples for reversed-phase separation. Direct extraction without saponification has also been used, although to a lesser extent [16]. Results obtained from compendial methods are dependent on the manipulative skills of the individual analyst, and generally lack precision within and between laboratories. The results are not in accord with GLP rules.

Unfortunately, none of these manual HPLC procedures completely satisfies quality-control objectives, particularly those in which cost effectiveness is important, because of the time taken for sample preparation.

This paper describes a reversed-phase HPLC and column-backflushing procedure for the simultaneous determination of vitamin A (retinol acetate, palmitate), vitamin D_3 (cholecalciferol), vitamin E (α-tocopherol acetate) and alphacalcidol in capsules from a single sample extract. The procedure eliminates saponification, lengthy extractions and sample clean-up, is specific for the compounds of interest in the presence of

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interference, and has very good internal precision compared with current compendial methods. This technique provides the high resolution, high reproducibility and quantitative accuracy required in the industrial quality-assurance laboratory. Increased use of such a new method will greatly reduce the imprecision and inaccuracy associated with vitamin analysis.

Experimental

Chemicals and Reagents

Vitamin A (all-*trans*-retinol acetate, all-*trans*-retinol palmitate) was purchased from Promochem (USP, BP-standard), vitamins D_3 (cholecalciferol) and E (DL-α-tocoferol acetate) from Sigma, and alphacalcidol from Infarm Poland. All other chemicals, such as *n*-hexane, acetonitrile and methanol are commercially available (Merck, Baker).

Instrumentation and Equipment

Liquid chromatography was performed with Hewlett–Packard equipment–an HP1100 pump module, an HP1100 photodiode-array detector, an HP1100 autosampler, a degasser and a chromatography data station (Chemstation rev. 5.01).

Standard Preparation

An accuracy weighed sample, estimated to contain 30 000 UI retinol palmitate and acetate, 30 000 UI cholecalciferol and alphacalcidol and 30 UI tocopherol acetate were dissolved in *n*-hexane (10 mL) and diluted to 100 mL with methanol. The standard was sonicated for 15 min at 30 °C.

Air should be avoided and excluded, by working in an atmosphere of pure nitrogen, to avoid oxidative degradation.

Sample Solution Preparation

A multivitamin capsule (containing all capsule components) was formulated to enable determination of recovery efficiency. The contents of one capsule were transferred quantitatively to a graduated flask (100 mL), dissolved in *n*-hexane (10 mL) and diluted to volume with methanol. The sample was then placed in an ultrasonic bath for 10 min at 30 °C.

Chromatographic Conditions

The HPLC separation was performed on a 25 cm × 4.6 mm i.d. stainless-steel column packed with an octyl stationary phase; isocratic elution was performed with acetonitrile–methanol, 95:5 (*v/v*) at a flow rate of 1.0 mL min^{-1}. Sample injections (10 μL) were made by use of a loop injection-valve and autosampler. All data were obtained as chromascan, from which spectra and chromatograms could be taken by use of a Chemstation Data Station.

Developed Method

Eight different chromatographic separation conditions (combinations of normal and chemically bonded stationary phases and different mobile phases) were evaluated and selected because of their different separation characteristics with regard to the vitamin compounds. It was expected that one of these phases would give good resolution of fat-soluble vitamins. The packing materials and mobile phase used are summarized in Table I.

The reversed-phase mode was eventually selected for chromatographic separation of the vitamins because of their relatively non-polar nature. The reversed-phase system also provides stability of operation and reproducibility. Separation is based on the partition of non-polar vitamin molecules between a polar mobile phase and a non-polar stationary phase. The mixed vitamin standard

Table I. The packing materials and mobile phases investigated.

Packing material	Mobile phase (mL min^{-1})	Flow-rate volume (μL)	Injection	Note
LiChrosorb Si–60 250 mm × 4.6 mm	*n*-Hexane–CH_2Cl_2–C_2H_5OH 49.5:50:0.5 (*v/v*)	1	5	Asymmetric peak for vitamin A; poor separation of D_3 and alphacalcidol
LiChrosorb Si–60 250 mm × 4.6 mm	*n*-Hexane–2-propadiol 97.5:2.5 (*v/v*)	1	5	Poor separation of vitamin D_3 and alphacalcidol
LiChrosorb RP–18 250 mm × 4.6 mm	MeOH–CH_3CN gradient from 0 to 100 %	1.5	10	Poor separation of vitamin A acetate and vitamin D_3
LiChrosorb RP–18 250 mm × 4.6 mm	MeOH–H_2O 50:50 (*v/v*)	2	10	Asymmetric peak for vitamin A acetate and alphacalcidol
LiChrosorb RP–8 250 mm × 4.6 mm	MeOH–H_2O 60:40 (*v/v*)	1.5	25	Poor *k* values for all vitamins
LiChrosorb RP–8 250 mm × 4.6 mm	CH_3CN–CH_2Cl_2 80:20 (*v/v*)	2	25	Tailing problem for vitamins A acetate and E acetate
LiChrosorb RP–8 250 mm × 4.6 mm	CH_3CN–MeOH 75:25 (*v/v*)	2	25	Good resolution of vitamins A acetate, D_3, and E acetate
LiChrosorb RP–8 250 mm × 4.6 mm	CH_3CN–MeOH 95:5 (*v/v*)	1	25	Good resolution for all vitamins

solution was chromatographed under a variety of isocratic and gradient conditions.

The chromatographic conditions were then modified to improve the resolution of the system. Improved separation of complex multicomponent samples can be achieved by varying the parameters that affect k–mobile phase composition, stationary phase and temperature.

Comparison of the results obtained from the different chromatographic systems indicates that an acetonitrile–methanol mixture would provide favourable separation in reasonable time. The chromatogram presented in Figure 1 shows the separation of the vitamins on the C_8 column with detection at 285 nm.

Column backflushing was used to remove strongly adsorbed components from the column inlet (i.e. the end at which the sample was injected) after the fat-soluble vitamins had been analysed. After elution of the solutes of interest the switching valves were used to reverse the mobile phase flow, enabling flow of the mobile phase into the column outlet and out of the inlet to waste. This quickly removed strongly held components from the top of column, thus extending its life.

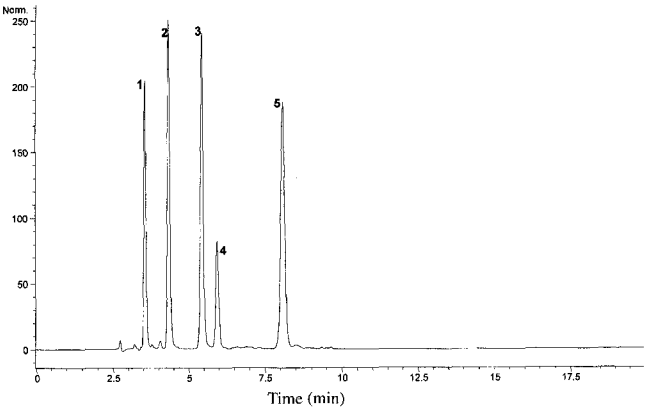

Figure 1
Standard chromatogram of retinol acetate (1), cholecalciferol (2), alphacalcidol (3), α-tocopherol acetate (4) and retinol palmitate (5) on a 250 mm × 4.6 mm i.d. 5 μm LiChrosorb RP–8 column with detection at 285 nm.

Results and Discussion

Blank Studies

Placebo multivitamin capsule formulations containing all the normal ingredients except the vitamins were prepared for this study. These were treated in the same manner as the normal samples, and chromatograms were inspected for interferences from the fat-soluble vitamins or from any of the other ingredients in the capsule formulation.

Linearity Studies

The linearity of the response ratios of the vitamins was determined with standard preparations containing the vitamins at concentrations ranging from 0 to 150 % of the working level. The percentage recoveries was determined by comparing the peak areas obtained from the standard and from the test sample. Recoveries of the vitamin were calculated and results are shown in Table II.

Precision and Accuracy

The precision and accuracy of the method was assessed by measurement of the average, standard deviation and relative standard deviation. The intra-assay precision was tested by repeated ($n = 6$) analyses of the same stock sample solution. The inter-assay variability was evaluated by repeated ($n = 6$) analysis of independent samples with different instruments, reagents and personnel. The results obtained from the assessment of precision and accuracy are summarized in Table III.

With the instrumentation used the sensitivity of this technique is approximately 0.9 UI for all vitamins. Determination of the signal-to-noise ratio (10:1) was performed by comparing measured signals from samples containing known low concentrations of the analytes with those of blank samples to establish the minimum concentration at which the analyte can be reliably detected. The limits of detection obtained were from 7.5 to 30 $\mu g\ mL^{-1}$.

Samples of final dosage form were then re-assayed. The results for all the vitamins agreed with the theoretical, or known, concentrations. Table IV summarizes the results obtained for all the vitamins present in the capsules.

Conclusion

The separation of fat-soluble vitamins under different chromatographic conditions has been investigated. The best separation was achieved on an octyl-bonded phase and methanol–acetonitrile mobile phase coupled with column backflushing technique.

The validation criteria, which have been examined and discussed, show the procedure to be reliable for the intended application.

The method can be considered relatively simple, convenient and cost-effective for routine determinations of fat-soluble vitamins in capsules. It involves single-step dilution and direct injection of the sample. The analytical method avoids the complication of gradient separation, multi-step extraction and the use of multiple detectors. Sample preparation is very simple and, because the analysis time is short, the method can be used in the industrial quality-assurance laboratory.

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Table II. Recoveries of vitamins at concentrations of 50, 100 and 150 % of the working level.

Level (%)	Recovery (%) Retinol palmitate	Retinol acetate	Cholecalciferol acetate	Alphacalcidol	Tocopherol
50	98.9	98.5	95.5	98.2	97.9
100	99.6	99.5	98.1	102.2	99.6
150	102.1	103.5	99.7	103.2	99.2

Table III. The results obtained from assessment of precision and accuracy.

Parameter	Retinol palmitate	Retinol acetate	Cholecalciferol	Alphacalcidol	Tocopherol acetate
Average (UI)*	29 875	29 756	301 571	309 676	30.9
Declaration (UI)*	31 050	30 300	296 000	300 400	30.4
Standard deviation*	245.11	178.87	1265.12	1312.14	0.4532
Relative standard deviation (%)*	0.82	0.6	0.42	0.42	1.07
Relative standard deviation (%)**	1.01	0.87	0.82	0.80	1.09

*Intra-assay analyses; **Inter-assay analyses

Table IV. Analysis of multivitamin capsules.

Analysis number	Retinol palmitate	Retinol acetate	Cholecalciferol	Alphacalcidol acetate	Tocopherol
1	30 750	30 300	296 000	300 400	30.45
2	29 870	30 245	295 467	294 532	30.62
3	29 950	29 885	297 635	303 235	29.82
4	30 052	29 061	306 457	296 541	29.73
5	30 325	30 288	291 192	299 876	30.28
6	29 870	29 970	297 251	302 305	30.16
Theoretical [UI]	30 000	30 000	300 000	300 000	30
Average [UI]	30 136	29 958	297 333.7	299 481	30.18
Standard deviation	345.22	472.93	5026.25	3352.74	0.35
Relative standard deviation [%]	1.15	1.58	1.69	1.12	1.16

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