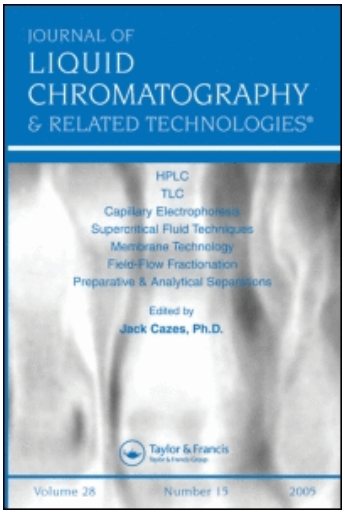

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High-Performance Liquid Chromatography of Fat-Soluble Vitamins. I. Determination of Vitamin A-Acetate in Pharmaceutical Preparations and Blood

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF FAT-SOLUBLE VITAMINS. I. DETERMINATION OF VITAMIN A-ACETATE IN PHARMACEUTICAL PREPARATIONS AND BLOOD

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Summary :

A reversed phase high-performance liquid chromatography (HPLC) has been developed for the quanititive determination of retinol acetate (vitamin A-acetate) in pharmaceutical formulations and biological materials.
The extraction of vitamin A-acetate from capsules and dragees and from blood is performed in a fully automated, electronically controlled extraction apparatus within 3 - 10 minutes. For reversed phase HPLC a column of LiChrosorb RP18 and methanol as eluent were used. Vitamin A-acetate was separated in less than 1 minute. The detection limit was 1-2 ng. The described methods were proved useful for extraction and determination of vitamin A-acetate in pharmaceutical preparations such as capsules and dragees and in blood, and can be well reproduced with a maximum coefficient of variation of $< 4\%$.

Introduction :

Vitamin A is classified as a fat-soluble vitamin (1). The determination of this vitamin in pharmaceutical preparation and especially in multivitamin formulations and biological fluids require extraction and analysis methods that are reliable in the presence of other vitamins and mixtures of excipients and degradation products. In the literature some publications have appeared on the HPLC separation and determination of vitamin A in vitamin preparations and in biological fluids (2 - 13). None of the described techniques completely satisfy the stability - and pharmacokinetic-studies needed for the pharmaceutical preparations containing vitamin A as the sensitivities of such reported methods were inadequate (the lowest limit was more than 25-50 ng of vitamin A (7,10)).
In this paper a simple and efficient extraction method and a rapid, specific and sensitive HPLC quantitative method have been developed for vitamin A-acetate. The time required for extraction and analysis of vit. A-acetate in pharmaceutical preparations and blood was 10 - 20 minutes.

Experimental :

Materials and reagents :

Vitamin A-acetate standard was obtained from Pfizer Pharmaceutical Co. (Cairo, Egypt). The following pharmaceutical preparations were purchased locally :

preparation A : dragees containing 50.000 I.E. = 100 mg Vit. A-acetate per dragee.

Preparation B : Capsules containing 7500 I.E. = 15 mg Vit. A-acetate per capsule.

Heparinized blood : 6 samples of 20 ml heparinized blood with 0.1 mg Vit. A-acetate per sample.

Methanol used for the chromatography was obtained from E. Merck (Darmstadt, GFR).

Procedure :

Preparation of standard solution :

25 mg Vit. A-acetate was accurately weighed to 0.01 mg and dissolved in 100 ml methanol. 1 ml of this solution was pipetted into a 100 ml calibrated flask and diluted to volume with methanol.

Sample preparation :

Pharmaceutical preparations :

Either one dragee or the contents of one capsule was pulverized and transferred accurately into a fully automated extraction apparatus and extracted 3 times with a total volume of 100 ml methanol. The solutions were diluted with methanol so that 1 ml of the solutions contained 2.5 µg Vit. A-acetate.

Heparinized blood :

In order to test the applicability and reproducibility of the methods developed for the pharmacokinetic and bioavailability studies, 6 samples of 20 ml heparinized blood containing 0.1 mg Vit. A-acetate per sample were examined. The blood samples were centrifuged for 5 min. at 1260 g, the plasma decanted from the coagulum and the coagulum again centrifuged for 5 min. at 1260 g after mixing with 5 ml 0.9% aqueous sodium chloride solution. After decanting, the supernatant was transferred into a fully automated extraction apparatus and was treated with 100 ml acetone-methanol (1:1). The deproteinized plasma was filtered and after retransferring into a new interchangeable filter funnelled into the extraction apparatus and the organic solvent mixture was drawn off in vacuum at room temperature. The remaining deprotein-

ized plasma was extracted 3 times with a total volume of 100 ml chloroform-methanol (1:1).

Apparatus :
Electronically controlled extraction apparatus*

The apparatus consists of a glass set for simultaneous extraction of three samples, a thermostat, a cryostat, a computer and a cabinet which houses the valves, a nitrogen pump and a vacuum pump (Fig. 1, and 2). In this apparatus all necessary sequences of operation for extraction such as addition of extracting agent, stirring, warming, cooling and filtering, are fully automatically performed by means of an electronic control system (14). A good reproducibility of the extraction of many active substances from pharmaceutical formulations using this apparatus has been demonstrated (14,15,16). For Vit. A-acetate 3 and 10 minutes were sufficient to quantatively extract it from commercial preparations and also from blood.

High-performance liquid chromatography (HPLC) :

Reversed phase HPLC was carried out using a Kauner compact apparatus with a variable wave-length spectrophotometer detector and a syringe-loaded loop injection valve with an internal volume of 50 µl. A stainless steel column 100 x 4.6 mm i.d. packed with vertex LiChrosorb RP18, 7 µm and methanol as eluent were used. The chromatogramms were recorded on a Knauer recorder with a 100 mv span set.

Conditions :

The following conditions were maintained: detection wavelength = 320 nm; pressure = 80 bar; flow rate = 2 ml. min^{-1};

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* produced by W. Krannich K. G., 3400 Göttingen, Ellihausen Weg 17, W. Germany.

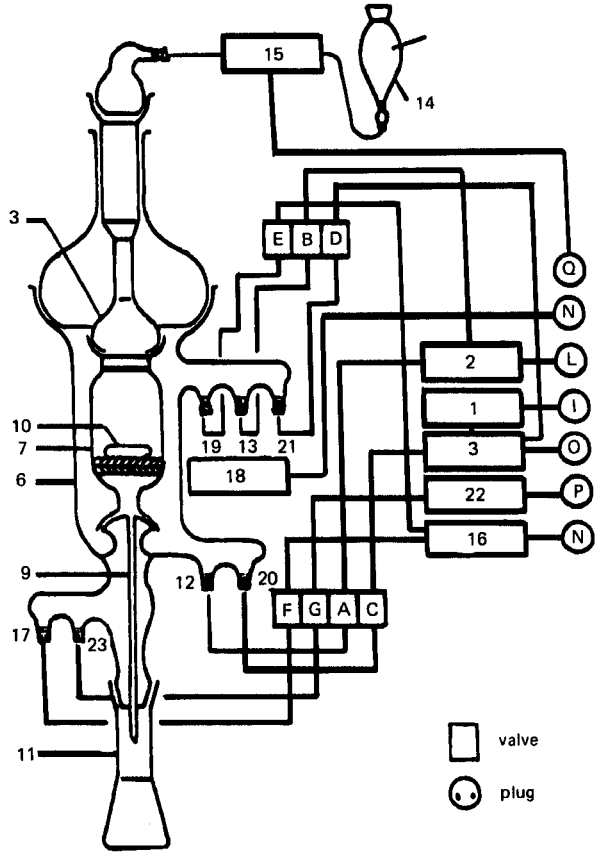

Fig. 1 Schematic diagram of the extraction apparatus.
The numbers in the figure were described previously (15).

temperature = 22 - 25°C; injection volume = 5 - 50 µl; chart speed = 1 cm. min^{-1} and detector sensitivity = 0.04 AUFS.

Calculation :

The amount of recovery of Vit. A in preparations A, B and in blood can be calculated by using either the calibration

Fig. 2 Electronically-controlled extraction apparatus according to Amin and Korbakis.

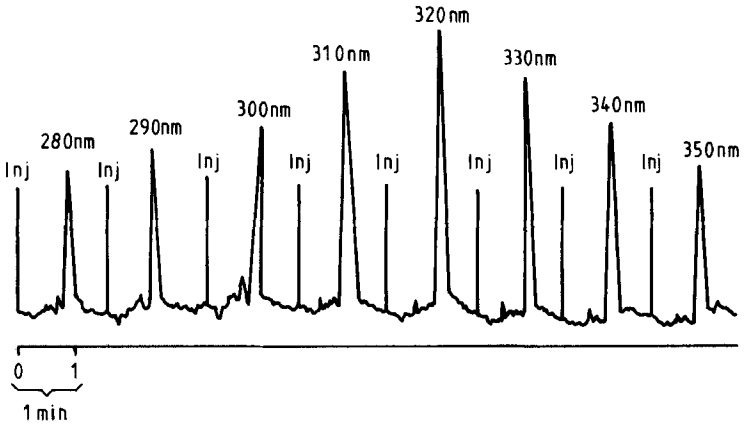

Fig. 3 Absorption spectrum of Vit. A-acetate
condition: LiChrosorb RP18, 7 µm, 100 x 4.6 mm i.d.; eluent: methanol; flow rate: 2 ml · min-1; pressure: 80 bar; detector sensitivity: 0.04 AUFS; volume injected = 10 µl ~25 ng

line method or the external standard method according to the following equation :

$$\text{Amount of recovery of vitamin (\%)} = \frac{C_R \cdot P_S}{C_S \cdot P_R} \times 100$$

Where C_R is the amount of reference compound (ng), P_S is the peak area for the sample substance (cm^2), C_S is the amount of the test compound (ng) (calculated from the stated amount of the preparation) and P_R is the peak area for the reference substance (cm^2).

Results :

Figure 3 shows the absorption spectrum and Figure 4 presents the calibration line of Vit. A-acetate by HPLC. Figure 5 shows the HPLC separation of Vit. A-acetate from preparations A and B. Figure 6 presents the HPLC separation of Vit. A-acetate from heparinized blood. The arithmetic mean of six Vit. A determinations, the stan-

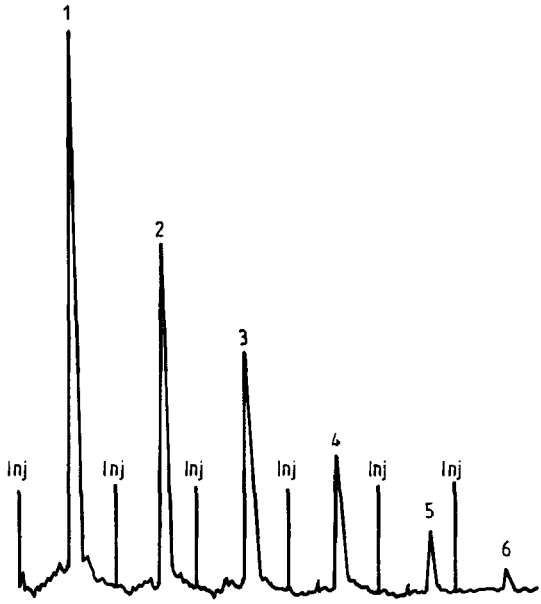

Fig. 4 Calibration line of Vit. A-acetate by HPLC
condition: same as in Fig. 1.
detection wavelength: 320 nm
detector sensitivity: 0.04 AUFS
1 = 50 ng; 2 = 25 ng; 3 = 12.5 ng;
4 = 7.5 ng; 5 = 2.5 ng; 6 = 0.75 ng.

dard deviation of single value and the coefficient of variation are summarized in Table 1. The results of HPLC determination of Vit. A-acetate in preparations A, B and heparinized blood are shown in Table 2.

Discussion :

The present procedure, including extraction step and HPLC measurement, was found to be rapid as compared to the previously published methods. The time needed, for the assay of Vitamin A-acetate as the pure standard drug or in its pharmaceutical preparations such as dragees and capsules

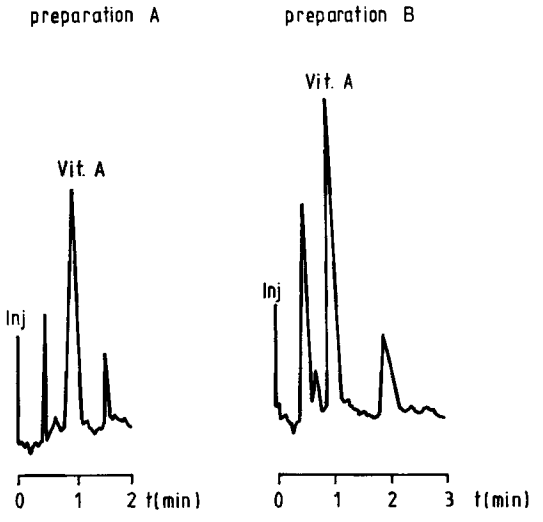

Fig. 5 Separation of Vit. A-acetate from preparation A (left side) and from preparation B (right side) by HPLC
condition: same as in Fig. 1
volume injected: preparation A: 5 µl = 12.5 ng
preparation B: 10 µl = 25 ng

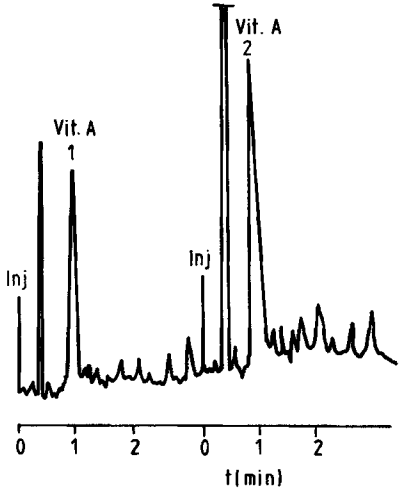

Fig. 6 Separation of Vit. A-acetate from heparinized blood
conditions: same as in Fig. 1
volume injected: 1 = 15 µl = 10 ng
2 = 20 µl = 20 ng

Table 1 : Reproducibility of HPLC determination of Vit. A-acetate carried out with pure substance.
(The results given are the means of 6 determinations)

	Vit. A-acetate
Amount injected (ng)	50
Arithmetic mean $\bar{X}$ (peak area cm^2)	0.81
Standard deviation of single value (cm^2) S. D.	0.029
Coefficient of variation (%) VK	3.58

Table 2 : Quantitative HPLC determination of Vit. A-acetate in preparations A, B and in heparinized blood.

(The results given are the means of 6 determinations)

Preparation	Vit.A-acetate present in preparation	Amount injected (ng)	Amount of Vit. A-acetate found $\bar{X}$ (ng)	S.D. (ng)	V.K. (ng)
A	100 mg/dragee	12.5	12.80	0.36	2.80
B	15 mg/capsule	25	25.65	0.68	2.65
heparinized blood	0.1 mg/20 ml	10	9.4	0.27	2.87

A linear relationship between the peak areas and the concentrations for Vit. A-acetate was found to be in the range of 5 - 25 ng, 150 ng and 150 - 600 ng.

as well as in blood, was found to be within 10 - 20 minutes while the reported methods needed more than 50 minutes for the determination of Vit. A-acetate (8,9).
In addition to the accuracy of the present procedure it has high sensitivity as compared to the previously reported methods. The lowest limit for estmating Vitamin A-acetate in the present work was found to be 1-2 ng with a coefficient of variation of $\sim$ 3,5% while the lowest reported limit was more than 25-50 ng (7, 10).
The HPLC methods without prior extraction of the active ingredient would load the column with extraneous matters from the excipients or biological materials which would decrease its separation efficiency. However, in the present work the use of the described extraction apparatus enables us to extract the desired ingredient without or with minimum amounts of excipients or biological materials which would add the advantage of prolonging the life time of the HPLC column.

In conclusion, the method, described in this paper, is specific and highly sensitive for the assay of Vitamin A-acetate and thus would be suitable for the pharmacokinetic and stability studies.

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