THIN-LAYER CHROMATOGRAPHY OF VITAMIN A METABOLITES IN HUMAN SERUM AND LIVER TISSUE

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A very sensitive method of determining vitamin A in blood based on extraction with cyclohexane–aqueous ethanol and fluorimetry has been recently described[1]. The method seems to be more specific than previously recommended procedures and interference from various blood components or fluorescent drugs could not be demonstrated. Studies reported here were designed to characterize further the components extracted by cyclohexane–aqueous ethanol, as well as to compare blood and liver tissue in this respect.

A number of studies have previously been published on the nature of the vitamin A metabolites in blood. For the separation of the compounds several chromatographic methods have been used such as partition between immiscible solvents[2–5], column chromatography[6–11], and reverse phase chromatography on impregnated paper[10,12–14].

Recently, thin-layer chromatography has been successfully applied to the detection of stereoisomers and different derivatives of the vitamin A_1 and A_2 series[15–23]. An attempt was therefore made to use this technique to characterize the fluorescent components extracted from human serum or liver tissue by cyclohexane–aqueous ethanol.

MATERIALS AND METHODS

Chemicals

The chemicals used in this study have been described previously[1]. In addition, crystalline vitamin A_1 palmitate was obtained from the Nutritional Biochemical Corp., Cleveland, Ohio. *Anhydro*vitamin A_1 was prepared from crystalline all-*trans* vitamin A_1 alcohol by the reaction with *p*-toluene-sulfonic acid according to SHANTZ[24]. *Retro*-vitamin A_1 acetate was prepared from crystalline all-*trans* vitamin A_1 acetate by treatment with aqueous hydrobromic acid as described by BEUTEL, HINKLEY AND POLLAK[25]. *Retro*-vitamin A_1 alcohol was prepared by saponification of *retro*-vitamin A_1 acetate.

Samples

Blood was collected by venipuncture from healthy volunteers (members of the hospital staff) before and 5 h after the ingestion of a single test dose of 350,000 I.U. of vitamin A palmitate (Arovit®, F. Hoffmann-La Roche & Co. Ltd., Basle, Switzerland).

Post mortem liver specimens were obtained from suddenly deceased persons without history of liver disease or metabolic disorders.

Extraction

40–100 ml serum or 10–20 g liver tissue, the latter being minced in a Turmix Blender with an equal volume of distilled water, were mixed with 5 volumes of ethanol and 1 volume of cyclohexane. The mixture was shaken in a mechanical shaker for a few minutes. The cyclohexane layer was separated by centrifugation and concentrated to a few ml on a water bath at 60° under reduced pressure. When saponification was included in the extraction procedure, the ethanol contained 5 % (w/v) potassium hydroxide and the extraction was completed by heating the mixture in a water bath at 60° for 30 min.

Thin-layer chromatography

The plates were prepared by standard techniques using Silica Gel G (for thin-layer chromatography according to STAHL, E. Merck A.G., Darmstadt, Germany). The plates were dried at 105° for 30 min. Plates used for reverse phase chromatography were immersed in 10 % (v/v) paraffin oil (infrared spectroscopy grade, Merck, Darmstadt) in petroleum ether for 2 min, and dried in an oven at 120° for 10 min. The reference standards, 5–20 μg compound in 5–20 μl ethanol or cyclohexane, were applied to the cooled plates with a micropipette. Application of the samples was made under minimum light conditions; the chromatography chambers were also protected against light.

The chromatograms were developed with one of the following solvent systems: (1) 1 % (v/v) ethanol in chloroform; (2) 3 % (v/v) ethanol in cyclohexane; (3) petroleum ether–ethyl ether–acetic acid (90:10:1); and for reverse phase chromatography, (4) methanol saturated with paraffin oil. The solvent front was allowed to move 18 cm from the origin (requiring 25–45 min) before the plates were removed from the chromatography chamber. Reverse phase chromatography required 6–8 h.

The spots were detected by spraying with one of the following reagents: (1) 20 % (v/v) antimony pentachloride in carbon tetrachloride; (2) 20 % (w/v) antimony trichloride in chloroform; (3) 2 % (w/v) phosphomolybdic acid in ethanol; (4) 5 % (w/v) potassium dichromate in 40 % (v/v) sulphuric acid. The sprayed plates were heated in an oven at 90–120°, or, for potassium dichromate, at 180–220°, for 5–10 min.

Alternatively, the spots were located under ultraviolet light, scraped out and eluted with chloroform. The further characterisation of the spots was carried out by recording absorption, excitation and fluorescence spectra, as well as absorption spectra for the reaction products with antimony trichloride. In addition the nature of the compounds was tested by conversion to *anhydro*vitamin A by treatment with ethanolic hydrochloric acid after preceding removal of the solvent and hydrolysis with ethanolic potassium hydroxide.

Methods

The procedures used for the determination of vitamin A have been described previously[1].

RESULTS

Pure compounds

The resolving capacity of the developing systems was tested by applying the method to different reference compounds. The R_F values of some reference compounds in different solvent systems are given in Table I.

TABLE I

R_F VALUES OF VITAMIN A REFERENCE COMPOUNDS IN DIFFERENT SOLVENT SYSTEMS

Values are the means of 8 determinations using different plates.

Compound	*Developing system*		
	Petroleum ether–ethyl ether–acetic acid (90:10:1)	*Cyclohexane–ethanol (97:3)*	*Chloroform–ethanol (99:1)*
Vitamin A_1 alcohol	0.09	0.10	0.25
Retro-vitamin A_1 alcohol	0.08	0.09	0.33
Vitamin A_1 acetate	0.42	0.48	0.72
Retro-vitamin A_1 acetate	0.40	0.45	0.72
Vitamin A_1 palmitate	0.73	0.78	0.87
*Anhydro*vitamin A_1	0.90	0.87	0.94

As shown in Fig. 1 (A, B, C), the chromatograms for the pure compounds did not only show intense spots with the R_F values recorded in Table I, but also additional faint spots detectable when 5 μg or more of the compounds were applied. These additional spots were interpreted as decomposition products regarding the fact that the spots increased on exposure of the compounds to light. The degree of formation of decomposition products showed some variation with the solvent used. Petroleum ether and acetone promoted such formation both when these solvents were used for chromatography and for elution.

The spectral properties of the decomposition products were also studied. Products migrating slower than the compound absorbed chiefly in the 240 and 280 mμ regions, whereas those migrating faster than the compound absorbed chiefly in the 350–390 mμ region. The main spots for the various compounds also contained some decomposition products to judge from the appearance of small absorption peaks at 350, 375 and 395 mμ in addition to the main band of the compound at 326 mμ.

The formation of the decomposition products described could not be prevented by using the reverse phase chromatographic technique.

Serum extracts

Cyclohexane extracts of normal human serum collected 5 h after vitamin A ingestion showed three distinct fluorescent spots in all solvent systems (Fig. 1E). The R_F values of the spots in the different systems are given in Table II. As seen from Tables I and II, the slow moving fluorescent spot had the same R_F value in all the systems as all-*trans* vitamin A_1 alcohol.

The absorption spectrum of the slowest fluorescent spot (R_F = 0.08–0.24) showed a triple peak in the visible region and a subsidiary maximum at 326 mμ

TABLE II

R_F VALUES AND SPECTRAL PROPERTIES OF THE FLUORESCENT SPOTS OF SERUM EXTRACTS

Serum collected from four healthy persons 5 h after ingestion of 350,000 I.U. vitamin A palmitate. The results given are the mean values.

Chromatographic fraction	*Developing system*			λ_{max}* (*mμ*)
	Petroleum ether–ethyl ether–acetic acid (90:10:1)	*Cyclohexane–ethanol* (97:3)	*Chloroform–ethanol* (99:1)	
Slow moving	0.08	0.08	0.24	420 450 480 (326) 245
Medium moving	0.65	0.37	0.77	326 326 350 S
Fast moving	0.85	0.85	0.86	375 S 390 S (245)

* S = shoulder; ( ) = subsidiary max.

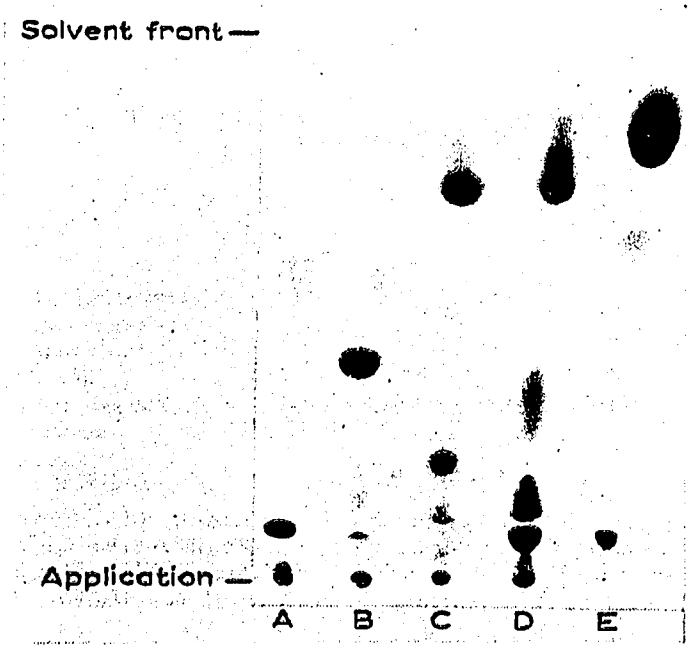

Fig. 1. Thin-layer chromatogram of serum and liver extracts. Experimental conditions: adsorbent: Kieselgel G; developing solvent system: petroleum ether–ethyl ether–acetic acid (90:10:1); detection of the spots with antimony pentachloride spray. A = Vitamin A alcohol; B = vitamin A acetate; C = vitamin A palmitate; D = liver extract; E = serum extract (5 h after vitamin A ingestion).

(Table II). The second spot (R_F = 0.37–0.77) absorbed chiefly at 245 and 326 mμ. The third fluorescent spot (R_F = 0.85–0.86) had an absorption maximum at 326 mμ, shoulders at 350, 375 and 390 mμ, and a subsidiary maximum at 245 mμ.

The fluorescence characteristics of all spots were found to be identical with those of vitamin A. The spots gave the same chromatographic colour reactions as vitamin A. The absorption spectra of the chromogens with antimony trichloride were also identical. Further, the eluted medium and fast moving fluorescent spots could be converted to *anhydro*vitamin A by treatment with ethanolic hydrochloric acid.

Thin-layer chromatograms of cyclohexane extracts of normal human serum collected before, 2, and 5 h after ingestion of 350,000 I.U. vitamin A palmitate are shown in Fig. 2 (A, B, C). The fast moving fluorescent spot is seen to increase successively following ingestion. However, the chromatographic pattern of the fast moving serum fraction is different from that of the vitamin A palmitate ingested (Fig. 2E). Extracts of serum to which vitamin A palmitate had been added *in vitro* also produced a pattern different from that found in serum after vitamin A ingestion (Fig. 2D).

Fig. 3 shows that the fast moving fluorescent compound found in serum after

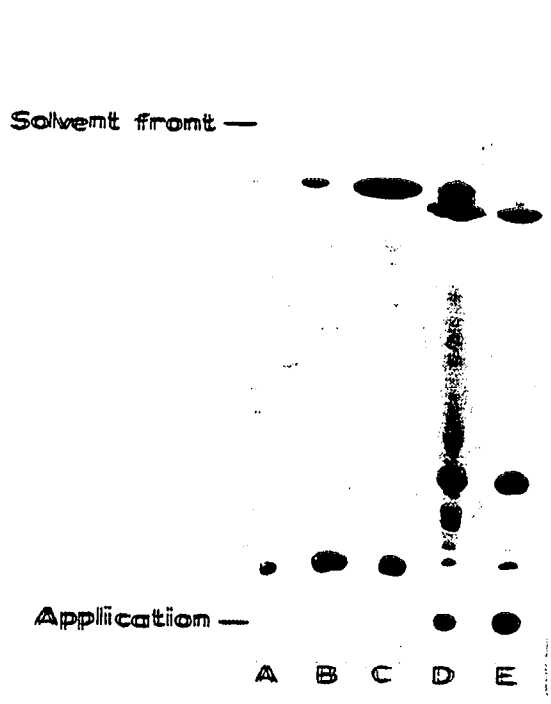

Fig. 2.

Fig. 3.

Fig. 2. Thin-layer chromatographic pattern of serum extracts. Experimental conditions: adsorbent: Kieselgel G; developing solvent system: 3% (v/v) ethanol in cyclohexane; detection of the spots with antimony pentachloride spray. A = Fasting serum; B = serum 2 h after vitamin A ingestion; C = serum 5 h after vitamin A ingestion; D = vitamin A palmitate + serum; E = vitamin A palmitate.

Fig. 3. Thin-layer chromatogram of serum extract before and after treatment with potassium hydroxide. Experimental conditions: Blood was collected 5 h after vitamin A ingestion. Adsorbent: Kieselgel G; developing solvent system: 1% (v/v) ethanol in chloroform; detection of the spots with antimony pentachloride spray. (A) Before treatment with KOH; (B) after treatment with KOH.

vitamin A ingestion may easily be hydrolyzed by treatment with ethanolic potassium hydroxide with the formation of vitamin A alcohol and an additional product.

Table III shows the distribution of vitamin A in the fluorescent thin-layer chromatographic fractions of serum extracts 5 h after vitamin A ingestion. Three different developing systems were used in these experiments, and the vitamin A content of the eluted spots was estimated by three different methods. As seen, the main bulk of the vitamin is, with all systems and methods, found in the fast moving fraction. The percentage distribution was approximately the same for all systems using fluorimetry or U.V. absorption. The results with the antimony trichloride reaction were slightly different. The mean recovery was of the order of 80–90 % of the vitamin content of the samples before chromatography.

Liver tissue extracts

Cyclohexane extracts of human liver tissue showed a number of fluorescent spots in the thin-layer chromatograms. (Fig. 1D). Three of the spots were found to have the same R_F values in all systems as all-*trans* vitamin A_1 alcohol, acetate and palmitate. All fluorescent spots were vitamin A derivatives to judge from absorption spectra, fluorescence characteristics, colour reactions, antimony trichloride colour-test

TABLE III

DISTRIBUTION OF VITAMIN A IN THE FLUORESCENT THIN-LAYER CHROMATOGRAPHIC FRACTIONS OF SERUM EXTRACTS AFTER VITAMIN A INGESTION

Serum was collected from four healthy persons 5 h after ingestion of 350,000 I.U. vitamin A palmitate. After extraction and thin-layer chromatography the distribution of fluorescence, U.V. absorption, or colour with the antimony trichloride reaction was determined for the three distinct major spots and the remaining minor diffuse fractions. The results given are the mean values. Recoveries are calculated as per cent of the measured vitamin A content of the samples before chromatography.

Developing system	*Method of determination*	*Percentage distribution*				*Recovery (%)*
		Slow moving spot	*Medium moving spot*	*Fast moving spot*	*Additional minor fractions*	
Petroleum ether–ethyl ether–acetic acid (90:10:1)	Fluorimetry	4.8	3.3	81.5	10.4	84.6
Chloroform–ethanol (99:1)	Fluorimetry	0.9	3.9	73.0	22.2	89.8
Cyclohexane–ethanol (97:3)	Fluorimetry	1.6	4.3	80.6	13.5	87.0
Cyclohexane–ethanol (97:3)	U.V. absorption	1.9	6.4	75.8	15.9	88.5
Cyclohexane–ethanol (97:3)	Antimony trichloride colour-test	6.7	16.9	61.7	14.7	82.3
	Mean	3.1	6.8	74.7	15.4	86.4

absorption maxima and convertibility to *anhydro*vitamin A. The proportion between slow, medium and fast moving fractions was approximately 1:2:7 with fluorimetry or U.V. absorption and 1:3:6 with the antimony trichloride reaction.

COMMENTS

The thin-layer chromatographic technique described is simple and possesses great resolving capacity. The method is also very rapid which reduces the formation of decomposition products known to occur during chromatography of the extremely labile vitamin A compounds[10,20,21,26,27]. The nature of the decomposition products appears to be similar with this method as described for other techniques[28–30].

Serum obtained from fasting normal individuals shows with this method a slow moving fluorescent fraction with an R_F value identical with that of all-*trans* vitamin A_1 alcohol (Tables I and II). This observation is in agreement with earlier reports[3,4,6] that the alcohol form of vitamin A predominates in human serum in the fasting state.

In serum collected after vitamin A palmitate ingestion most of the fluorescence is found in a fast moving fraction which, however, has an R_F value different from that of the vitamin A palmitate (Fig. 2). The fast moving fraction represents a vitamin A ester to judge from results obtained after hydrolysis with ethanolic potassium hydroxide (Fig. 3). Sera obtained after vitamin A ingestion also show a minor medium fraction which so far is unidentified.

Ingestion of vitamin A has been reported to result in an increase in serum in the ester fraction only [4–6,12,13]. Experiments in the rat[14,31], and recently also in humans[23] indicate that vitamin A is esterified with long chain fatty acids during absorption. In the post-absorptive rat lymph[21], the saturated esters predominate (palmitate and stearate in a ratio of approximately 2:1) with small amounts of oleate and linoleate.

Liver extracts, with this technique, show three fluorescent spots with R_F values identical with those of all-*trans* vitamin A_1 alcohol, acetate and palmitate (Fig. 1). In addition, several other fluorescent spots were found in the chromatograms, all representing compounds of vitamin A nature. The results are in good agreement with previous reports[2,10,12,18,32] that long chain fatty acid esters of vitamin A, mainly palmitate, predominate in the liver with small amounts of the alcohol always being present.

Slow moving fractions, both of serum and liver extracts, contain components of carotenoid nature to judge from spectral characteristics and colour reactions with antimony trichloride. Due to the presence of carotenoids, slow fractions show a higher apparent vitamin A content with the antimony trichloride reaction than with fluorimetry or U.V.-absorption (Table III).

SUMMARY

A simple and rapid thin-layer chromatographic method of good resolving capacity is described for the separation of compounds of vitamin A nature extracted from human serum and liver tissue by aqueous ethanol–cyclohexane.

In extracts of sera collected in the fasting state only a slow moving fraction was found which contained both all-*trans* vitamin A_1 alcohol and carotenoids.

In extracts of sera collected after vitamin A palmitate ingestion about 75 % of

the vitamin was found in a fast moving fraction which was identified as a vitamin A ester different from that ingested.

In liver extracts several spots were found with this technique, all representing compounds of vitamin A nature. Three of the spots were identified as all-*trans* vitamin A_1 alcohol, acetate and palmitate.

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