J. Pharm. Pharmacol., 1965, **17**, 474–479 Received March 2, 1965

Occurrence of anhydrovitamin A and *retro*-vitamin A in pharmaceuticals; their biological potency and effect on the assay of vitamin A

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Analyses of commercial samples of liquid multivitamin preparations revealed that many contained anhydrovitamin A, *retro*-vitamin A ether, vitamin A ether, and *retro*-vitamin A alcohol in varying proportions. Anhydrovitamin A and *retro*-vitamin A ether were present in greater amounts than the other vitamin A-derivatives. Anhydrovitamin A and *retro*-vitamin A ether had no measurable potency in the liver storage and vaginal smear assays, whereas *retro*-vitamin A acetate was 12% as potent as vitamin A. These compounds interfered with the assay of vitamin A by the United States Pharmacopeia XVI and British Pharmacopoeia 1963 methods but the interference could be eliminated by chromatography.

ANHYDROVITAMIN A (Fig. 1) was first prepared by Edisbury, Gillam, Heilbron & Morton (1932) and later characterised by Shantz, Cawley & Embree (1943) and Meunier, Dulou & Vinet (1943). It showed λ_{max} at 351, 371 and 392 mμ and with antimony trichloride gave a blue colour of the same intensity and wavelength as did vitamin A. Its

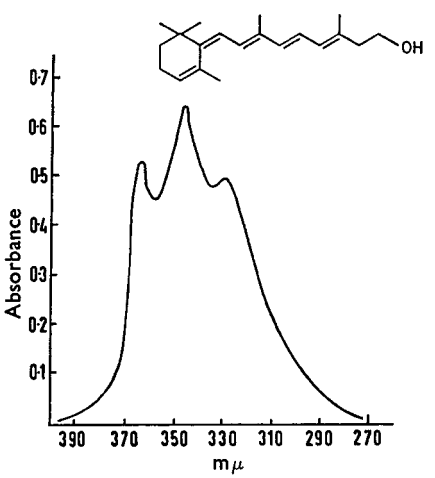

FIG. 1. Absorbance curve of Anhydrovitamin A.

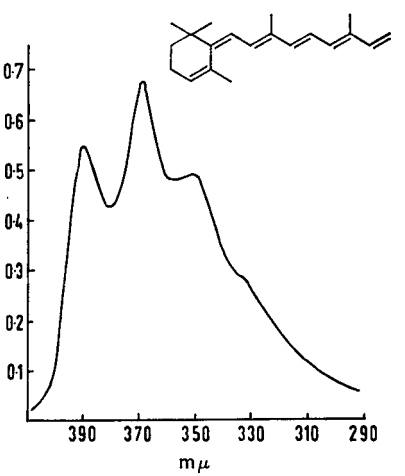

FIG. 2. Absorbance curve of *Retro*-vitamin A.

biological potency, measured by rat growth assay, was about 0·4% that of vitamin A (Shantz & others, 1943). Higuchi & Reinstein (1959) studied the kinetics of formation of anhydrovitamin A from vitamin A alcohol and acetate and noted its possible formation in pharmaceutical preparations.

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Retro-vitamin A, shown in Fig. 2, was prepared by Beutel, Hinkley & Pollak (1955) by reacting vitamin A acetate with aqueous hydrobromic acid. Varma & Murray (1963) fed *retro*-vitamin A to vitamin A-deficient rats and reported that about 10% of the dose was stored in the liver, 8% as vitamin A, 2% as *retro*-vitamin A. With antimony trichloride *retro*-vitamin A produced a blue colour with λ_{max} at 575 mμ.

The presence in pharmaceutical preparations of appreciable amounts of anhydro- and *retro*-vitamin A would pose an analytical problem. Their reported biological activity is negligible but they would contribute to absorbance in the ultraviolet and to the colour produced with antimony trichloride. Murray, Pelletier & Campbell (1963) examined one multivitamin preparation in which the vitamin A was largely replaced by *retro*-vitamin A, possibly in the ether form, but there is no evidence in the literature that this is a problem of any magnitude.

We now report the incidence of anhydro- and *retro*-vitamin A in two types of pharmaceutical preparations, and studies on the biological potencies of these compounds.

Experimental

MATERIALS AND METHODS

Forty-one different liquid* vitamin preparations were obtained from commercial sources. They ranged in complexity from mixtures of vitamins A and D, to multivitamin preparations with minerals and amino-acids. Several samples of some products were examined. In addition, twelve different vitamin tablets were assayed.

The samples were saponified and extracted by the USP XVI method (1960). Vitamin A was separated from interfering compounds by partition chromatography on a celite-polyethylene glycol column (Murray, 1962a) and measured by the USP XVI method. For identification of the vitamin A derivatives it was necessary to separate them on alumina. Woelm alumina (neutral, activity grade 1) was weakened by the addition of 6% water. The sample was introduced onto the column in light petroleum (B.P. 40°–60°) and eluted successively with 2, 4, 8, 12, 20 and 40% (v/v) diethyl ether in light petroleum. The composition of fractions from the chromatographic column and the purity of the preparations used in biological studies was checked by thin-layer chromatography (Varma, Panalaks & Murray, 1964).

The anhydrovitamin A isolated showed λ_{max} at 350, 368 and 390 mμ rather than at 351, 371 and 392 mμ shown by the crystalline material (Shantz, 1950). Estimation of this compound was made on the basis of $E(1\%, 1\text{ cm}) = 3000$ at 390 mμ. *Retro*-vitamin A alcohol and ether were estimated on the basis of $E(1\%, 1\text{ cm}) = 2200$ at 366 mμ, the value for the ether being expressed in terms of the alcohol.

For biological studies, anhydrovitamin A was prepared from crystalline all-*trans* vitamin A alcohol by the reaction with anhydrous methanolic

* These were almost all water dispersions, none were oil solutions.

hydrogen chloride (Shantz, 1950). It was purified by repeated chromatography on alumina until the purest fractions gave $E(1\%, 1\text{ cm})$ values of 2340, 3130 and 2600 at 350, 368 and 390 mμ respectively. *Retro*-vitamin A acetate was prepared as described by Beutel, Hinkley & Pollak (1955) and purified by chromatography on alumina. It had $E(1\%, 1\text{cm})$ values of 1500, 2030, and 1910 (equivalent to 1730, 2340 and 2200 for the alcohol form) at 332, 348 and 366 mμ respectively. *Retro*-vitamin A ether was purified by chromatography from a liquid multivitamin preparation and had E (1%, 1 cm) values of 587, 653 and 594 at 332, 348 and 366 mμ respectively.

Vaginal smear assays were made according to Pugsley, Wills & Crandall (1944) and liver-storage assays by the method of Ames & Harris (1956), except that liver stores were not estimated by the usual antimony trichloride reaction but spectrophotometrically after saponification and separation of the components on alumina. The relative proportions of vitamin A alcohol and *retro*-vitamin A alcohol were estimated as described by Varma & Murray (1963). For both kinds of biological assays the doses were given orally in corn oil and subcutaneously in water dispersions.

Results and discussion

CHEMICAL

Table 1 contains information on the properties of the various vitamin A derivatives found in the liquid pharmaceutical preparations. Vitamin A ether and *retro*-vitamin A alcohol were found in several products but only in small amounts. No quantitative measurements were made of these derivations.

TABLE 1. PHYSICO-CHEMICAL PROPERTIES OF VITAMIN A ALCOHOL AND RELATED COMPOUNDS ISOLATED FROM THE LIQUID MULTIVITAMIN SAMPLES

Compund	Eluting solvent % ether in light petroleum*	Distribution ratio**	max (mμ)	
			Ultraviolet	$SbCl_3$ colour test
Anhydrovitamin A	0	98 : 2	350,368,390	618
Retro-vitamin A ether ..	0 to 2	95 : 5	332,348,366	575
Vitamin A ether	2 to 4	95 : 5	324	618
Retro-vitamin A alcohol ..	15 to 20	48 : 52	332,348,366	575
Vitamin A alcohol	18 to 22	50 : 50	324	618

*From Alumina.
**Between light petroleum and 83% EtOH–H_2O mixture.

Almost all products contained anhydrovitamin A and *retro*-vitamin A ether but only six contained appreciable amounts of these derivatives. The data on these six products are summarised in Table 2.

The data do not indicate the cause or mechanism of formation of the vitamin A derivatives. The products which contained appreciable amounts of these derivatives were complex mixtures but in many similar mixtures the vitamin A had not deteriorated. All samples tested were acidic, but differences in pH were not related to the presence or absence of vitamin A derivatives. Age did not appear to be an important factor

except when the deterioration of a single product was considered. Samples F_1 to F_6 were the same product assayed at various times after manufacture and the results illustrate the rapid changes that may take place in water dispersions of vitamin A. Detectable amounts of anhydrovitamin A and *retro*-vitamin A ether were present within one month of manufacture and within a year the destruction of vitamin A was almost complete. The loss of vitamin A could not fully be accounted for by the appearance of anhydrovitamin A and *retro*-vitamin A ether, and the small amount of vitamin A ether and *retro*-vitamin A alcohol (not shown in Table 2). It is likely that much of the vitamin A was destroyed by oxidative processes.

Measurable amounts of anhydro- or *retro*-vitamin A were not found in the multivitamin tablets examined.

TABLE 2. VITAMIN A, ANHYDROVITAMIN A AND *retro*-VITAMIN A ETHER IN LIQUID MULTIVITAMIN SAMPLES

Product	Age (months)	Vitamin A*	Anhydrovitamin A*	*Retro*-vitamin A ether*
A	17	60	28	3
B_1	?	140	7	4
B_2	18	120	2	2
C_1	?	61	5	4
C_2	?	63	5	3
D	9	30	30	22
E	13	29	33	18
F	6	5	0	78
F_1	1	70	12	3
F_2	2	62	18	9
F_3	3	20	28	14
F_4	8	8	30	16
F_5	10	6	20	22
F_6	13	7	20	21

*Expressed as % total vitamin A claimed on label.

BIOLOGICAL STUDIES

Anhydrovitamin A will support growth if given in sufficient amounts; thus Shantz & others (1943) found the biological potency to be about 0·4% that of vitamin A in the rat-growth assay. An attempt was made to confirm this finding by liver storage and vaginal smear assays. When anhydrovitamin A was administered to vitamin A-deficient rats orally or by subcutaneous injection, no vitamin A was found in the liver. In view of this, the validity of a liver-storage assay of anhydrovitamin A was questionable. The nature of the compounds stored in the liver has been reported by Varma, Erdody & Murray (1965). Furthermore, a potency value for anhydrovitamin A could not be obtained from the vaginal smear assays. The smears were changed from those typical of a vitamin-deficiency to normal by an oral dose of 375 μg anhydrovitamin A but within four days the rats were again deficient. A fourfold increase in the size of the dose did not increase the number of days between dosing and depletion. Similar results were obtained when the doses were administered subcutaneously. This response was reminiscent of that observed by Murray (1962b) when vitamin A acid was fed to vitamin A-deficient rats, except that anhydrovitamin A gave rise to liver storage of related compounds (Varma & others, 1965). Two explanations

seem possible. The compounds stored in the liver after the administration of anhydrovitamin A may not be converted to vitamin A but may be capable of performing some of its functions (growth) although not all (maintenance of a normal vaginal smear). Alternatively, the compounds stored in the liver may be converted to vitamin A (or some "active form") at a very slow rate, sufficient for growth, but not to maintain a normal vaginal smear. The metabolism of vitamin A derivatives is being further examined, but it is safe to conclude that anhydrovitamin A has, at best, very low biological potency.

The potency of orally administered *retro*-vitamin A was 11% ± 10% that of vitamin A by the liver storage assay and 12% ± 10% by the vaginal smear assay. A 94:6 mixture of vitamin A and *retro*-vitamin A was found in the liver in contrast to the 80:20 mixture previously reported by Varma & Murray (1963). The higher ratio of vitamin A to *retro*-vitamin in the present experiment might be due to the longer period (3 instead of 2 days) between dosing and killing the rats.

When the doses for the vaginal smear assay were given subcutaneously in aqueous dispersion, the biological potency of *retro*-vitamin A was only 4% ± 12% that of vitamin A and no vitamin A or *retro*-vitamin A was found in the liver indicating rapid destruction and little or no conversion of the derivative to vitamin A. It is likely, therefore, that the major site of conversion of *retro*-vitamin A to vitamin A is the gastrointestinal tract.

The biological potency of chemically prepared *retro*-vitamin A was similar to that of the "rehydrovitamin A" isolated by Shantz (1950) from the livers of rats fed anhydrovitamin A although the two compounds are not identical. "Rehydrovitamin A" had ultraviolet absorbance maxima very close to those shown by *retro*-vitamin A prepared from vitamin A by the aqueous hydrobromic acid reaction but the former had λ_{max} near 618 mμ and the latter near 575 mμ in the antimony trichloride reaction. The *retro*-vitamin A alcohol and ether isolated from pharmaceutical products reacted with antimony trichloride in the same manner as did the chemically prepared compound.

Retro-vitamin A ether isolated from pharmaceutical products was biologically inactive.

EFFECT ON VITAMIN A ASSAY

Anhydro- and *retro*-vitamin A were mixed in various proportions with pure vitamin A alcohol and the vitamin A of the mixtures estimated by the USP XVI method. By this means it was found that if the absorbance ratio 350 mμ/325 mμ exceeded 0·640, chromatographic purification was necessary.

The USP XVI method makes use of a Morton & Stubbs correction based on absorbance readings at 310, 325 and 334 mμ without regard to the shape of the absorbance curve. The B.P. 1963 states that if, after saponification, maximum absorbance lies outside the wavelengths 323–327 mμ, or if the extinction at 310 mμ relative to that at 325 mμ exceeds 0·730, the sample must be chromatographed.

It is suggested that, in addition to the safeguards of the B.P. method, the absorbance be measured at 350 mμ and the condition added that chromatography is necessary if the absorbance ratio 350 mμ/325 mμ exceeds 0·640.

The antimony trichloride reaction is commonly used for the determination of vitamin A when the ultraviolet absorbance curve is distorted and, in fact, this reaction has been suggested (Ames, Swanson & Lehman, 1960) as a means of estimating the isomer content of water-dispersed vitamin A. This method would result in serious overestimation of potency if anhydrovitamin A were present. *Retro*-vitamin A ether and alcohol found in the non-saponifiable fractions of the pharmaceutical products have λ_{max} near 575 mμ in the antimony trichloride test and interfere only to a limited extent with the colorimetric determination of vitamin A.

Separation of vitamin A from any of the common types of interference can be accomplished by the partition system described by Murray (1962a) or on alumina. The former system is easier to use and has the added advantage that the column may be re-used many times. For separation of the individual vitamin A derivatives alumina is to be preferred. Neither system will separate vitamin A alcohol from *retro*-vitamin A alcohol but in our experience the latter occurs in pharmaceutical products in very small amounts.

It was concluded that anhydrovitamin A and *retro*-vitamin A may occur in pharmaceutical products in amounts that interfere with the assay of vitamin A by the USP XVI and B.P. methods. Analysts should be aware of the errors caused by these compounds and should check for their presence by use of the absorbance ratio 350 mμ/325 mμ.

Acknowledgements. The authors wish to acknowledge the technical assistance of Mr. C. Desloges.

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