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DETERMINATION OF THE CAROTENOID PHYTOENE IN BLOOD BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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SUMMARY

A sensitive and specific high-pressure liquid chromatographic assay was developed for the determination of phytoene in blood with an overall recovery of 86 ± 6.0% and a limit of detection of 50–100 ng per ml of blood. This method provides for rapid and simple quantitation of phytoene using 1 ml or less of blood.

The assay was used in the determination of phytoene blood levels in the dog following intravenous and oral administration of 10-mg/kg doses.

INTRODUCTION

Phytoene, a naturally occurring carotenoid[1], is an endogenous precursor in the biosynthesis of β-carotene[2], and is reported to be under investigation as an oral sunscreen[3]. β-Carotene has been used successfully in patients with erythropoietic protoporphyria to reduce the photosensitivity associated with this disease[4,5]. Studies with β-carotene to determine its effects on a normal individual's response to sunburn radiation (UV-B, 280–315 nm) indicate it is somewhat effective against sunburn radiation. These findings prompted the investigation of phytoene as an oral sunscreen[6].

The major structural differences between phytoene and β-carotene are the presence of three conjugated double bonds in phytoene compared to the nine conjugated double bonds of β-carotene and the cyclization of the terminal ends of phytoene to form the β-ionone rings in β-carotene (Fig. 1). The biosynthesis of Vitamin A from β-carotene is known to take place mainly in the intestinal mucosa during absorption[7] in an *in vivo* reaction sequence involving (a) cleavage of β-carotene into retinal (aldehyde), followed by (b) reduction of retinal to retinol (alcohol). Retinoic acid is formed by oxidation of the aldehyde (Fig. 1). These conversions can be effected either by symmetric or asymmetric fission or by terminal oxidation[8].

In order to assess the pharmacokinetics and oral absorption characteristics of phytoene, it was necessary to develop an assay capable of determining phytoene in the presence of β-carotene in blood. The classical column chromatographic–spectrophotometric assays for the determination of carotenoids are time-consuming[7], while

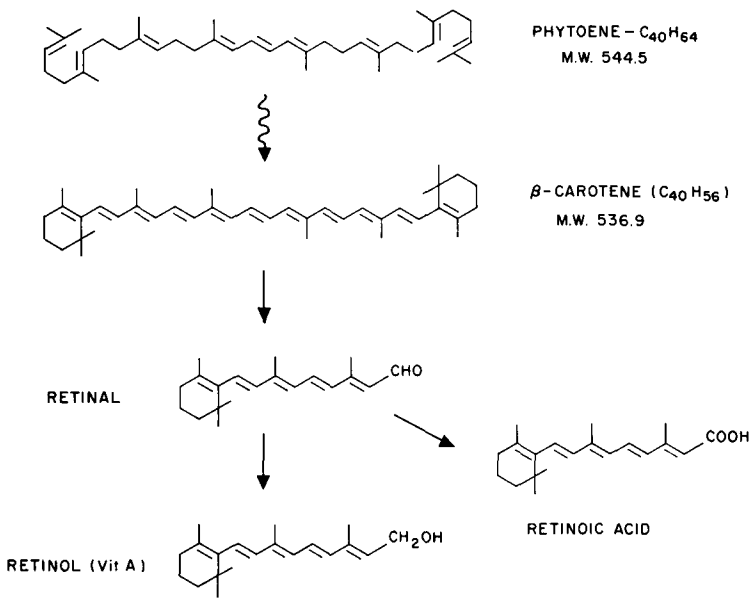

Fig. 1. Biosynthetic pathway for the conversion of phytoene to retinol (Vitamin A).

the spectrofluorometric methods are non-specific and prone to errors due to endogenous interferences[9–11]. Gas chromatographic analysis has been used following derivatization with silylating reagents, but this method is prone to error due to thermal instability of the derivatives[12,13]. High-pressure liquid chromatography (HPLC) appeared to be the method of choice, since these compounds can be analyzed at ambient temperatures without chemical derivatization and exploiting their differential spectrophotometric properties for quantitation. This technique has been successfully used in the analysis of vitamin A in bulk chemical products and pharmaceutical formulations[12].

The method presented here quantitates phytoene using its ultraviolet (UV) absorbance at 280 nm to advantage, since neither β-carotene ($\lambda_{max.}$ = 445 nm) nor vitamin A ($\lambda_{max.}$ = 325 nm) absorb significantly at 280 nm. Furthermore, they are all chromatographically resolved, thus imparting further specificity to the assay.

EXPERIMENTAL

HPLC analysis of phytoene in blood

Column. The column used was a 0.5 m × 2 mm I.D. stainless-steel column containing 1% ODS Permaphase chemically bonded on Zipax (DuPont, Wilmington, Del., U.S.A.).

Instrumental parameters. A DuPont Model 830 high-pressure liquid chromatograph equipped with a Model 836 multi-wavelength UV and fluorescence detector (operated in the UV mode at 280 nm) was used. The isocratic mobile phase used was a mixture of water–methanol (5:95) at a head pressure of 500 p.s.i. and a flow-rate of 0.8 ml/min. The column was operated at ambient temperature. Under these con-

ditions the retention time of phytoene was 2.5 min and that of β-carotene was 4.9 min. Typical chromatograms are shown in Fig. 2. The detector sensitivity was 4×10^{-2} a.u.f.s., and the chart speed on the 1.0-mV Honeywell recorder (Model No. 194) was 30 in./h. Under these conditions 100 ng of phytoene per 10 μl injected gives nearly full-scale pen response. The minimum detectable amount of phytoene is 50–100 ng/ml of blood.

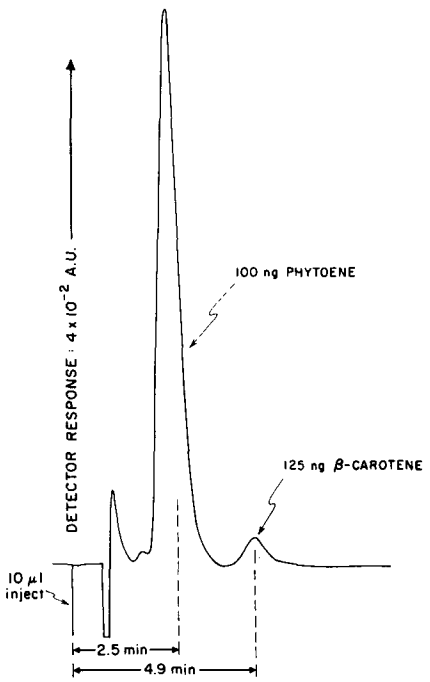

Fig. 2. Relative sensitivity of phytoene and β-carotene at 280 nm.

Calibration of phytoene by HPLC. A calibration (external standard) curve of the peak area of phytoene *versus* concentration in the range of 25–100 ng per 10 μl of standard solution is prepared. The linear range of quantitation is from 10–1000 ng of compound. A fresh calibration curve of the external standards and of the recovered internal standards are prepared for each day of analysis to establish the reproducibility of the HPLC system.

Assay in blood

Preparation of standard solutions. Weigh out 10 mg of phytoene and dissolve in 10 ml of isopropanol to yield a stock solution (A) containing 1 mg/ml. Make serial dilutions of solution A to yield working solutions B_1–B_4 containing the following concentrations of phytoene (per 0.1 ml of isopropanol): B_1, 250 ng; B_2, 500 ng; B_3, 750 ng and B_4, 1000 ng. Aliquots (10 μl) of these solutions are injected as external standards for establishing the liquid chromatographic parameters. Aliquots (0.1 ml) of the same solutions are added to blood as the internal standard calibration curve

for the determination of the concentration in the unknowns and for the determination of percent recovery.

Reagents. All reagents must be of analytical reagent grade (> 99% purity). Normal saline is prepared by dissolving 0.9 g of NaCl in 100 ml of deionized distilled water (0.9% solution). Absolute ethanol is used to deproteinize the blood, and a mixture of *n*-hexane (Fisher, H-301) and isopropanol (ACS grade) (95:5) is used as the solvent for extraction. A water–methanol (5:95) mixture is used as the mobile phase for HPLC analysis.

Procedure. The flow diagram of the extraction procedure is shown in Fig. 3.

Into a 50-ml centrifuge tube (PTFE No. 16, stoppered), place 1.0 ml of whole blood, 2.0 ml of normal saline, and mix well on a vortex mixer. Add 2.5 ml of absolute ethanol, mix occasionally, and wait for complete deproteinization (approx. 5 min). Extract the entire mixture with 10 ml of *n*-hexane–isopropanol (95:5) by shaking for 10 min on a reciprocating shaker (Eberbach, Ann Arbor, Mich., U.S.A.)

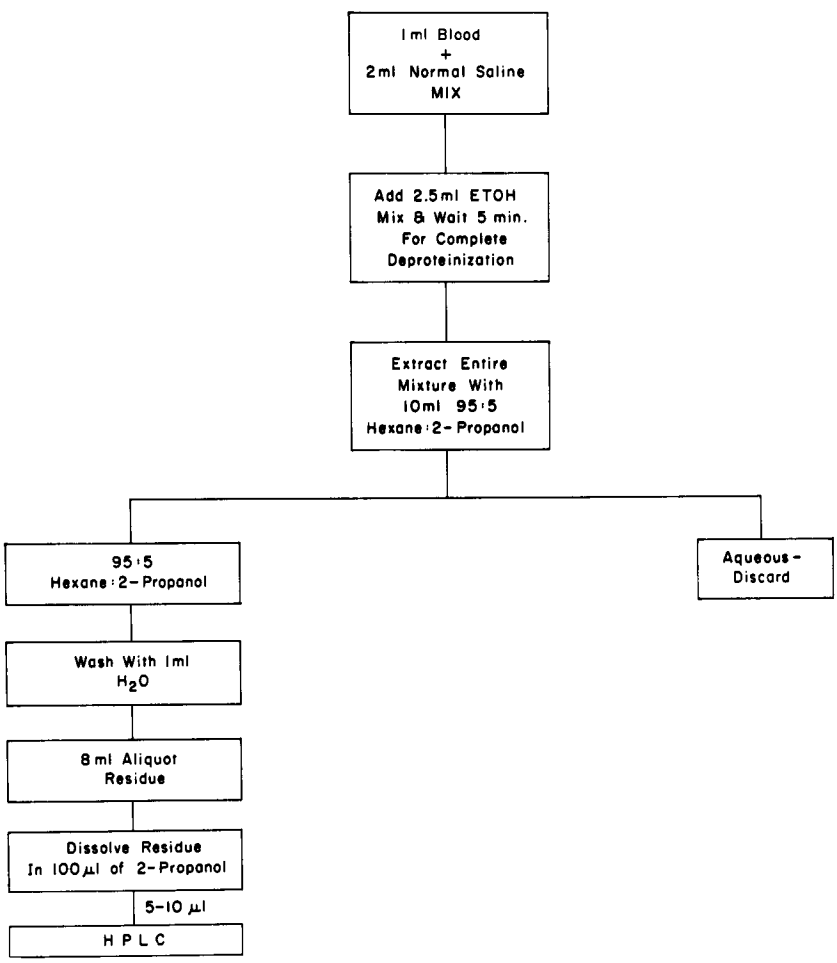

Fig. 3. Flow diagram of extraction procedure.

at 80–100 strokes/min. Along with the samples run a specimen of control blood and four 1.0-ml specimens containing 0.1 ml of standard solutions B_1, B_2, B_3, or B_4. Centrifuge the samples at 2500 rpm (1500 *g*) in a refrigerated centrifuge (Model PR-J, rotor No. 253; Damon/IEC, Needham, Mass., U.S.A.) at 5° and transfer a 9.0-ml aliquot into a 15-ml conical centrifuge tube. Wash this aliquot with 1.0 ml of deionized, distilled water by shaking on a reciprocating shaker for 5 min and centrifuging at 2500 rpm (1500 *g*) in a refrigerated centrifuge (5°) for 5 min. Remove and discard the lower aqueous layer with a hypodermic syringe fitted with a 20-gauge 6-in. cannula (Becton Dickinson, Rutherford, N.J., U.S.A.). If a heavy lipid layer remains at the interphase, a second 1.0-ml water wash is necessary. Transfer an 8.0-ml aliquot into a 15-ml conical centrifuge tube and evaporate to dryness at 60° in a

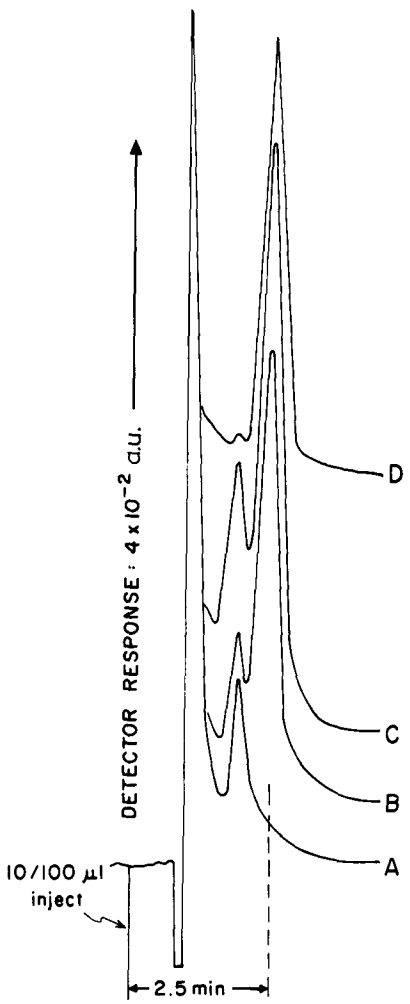

Fig. 4. Chromatograms of HPLC analysis of dog blood hexane–2-propanol (95:5) extracts of (A) control blood, (B) control blood containing added authentic standard, (C) dog blood post 10-mg/kg oral dose of phytoene, and (D) authentic standard.

N-EVAP evaporator (Organomation Assoc., Worcester, Mass., U.S.A.) under a stream of clean, dry nitrogen. Dissolve the residues in 100 μl of isopropanol and inject a 5–10-μl aliquot. Typical chromatograms of blood extracts are shown in Fig. 4.

Calculations. The concentration of phytoene in the unknowns is determined by interpolation from the calibration curve of the internal standards processed along with the unknowns, using the direct calibration (peak area *versus* concentration) technique. The percent recovery of the internal standards is determined by comparing the slope value (cm^2 peak area per ng of compound) of the internal standard curve to that of the external standard curve.

RESULTS AND DISCUSSION

A sensitive and specific HPLC assay was developed for the determination of phytoene in blood using the principle of reverse-phase partition chromatography. The extraction procedure used is a modification of a previously published assay for phytofluene and retinol determined by spectrofluorometry[9], and provides for rapid

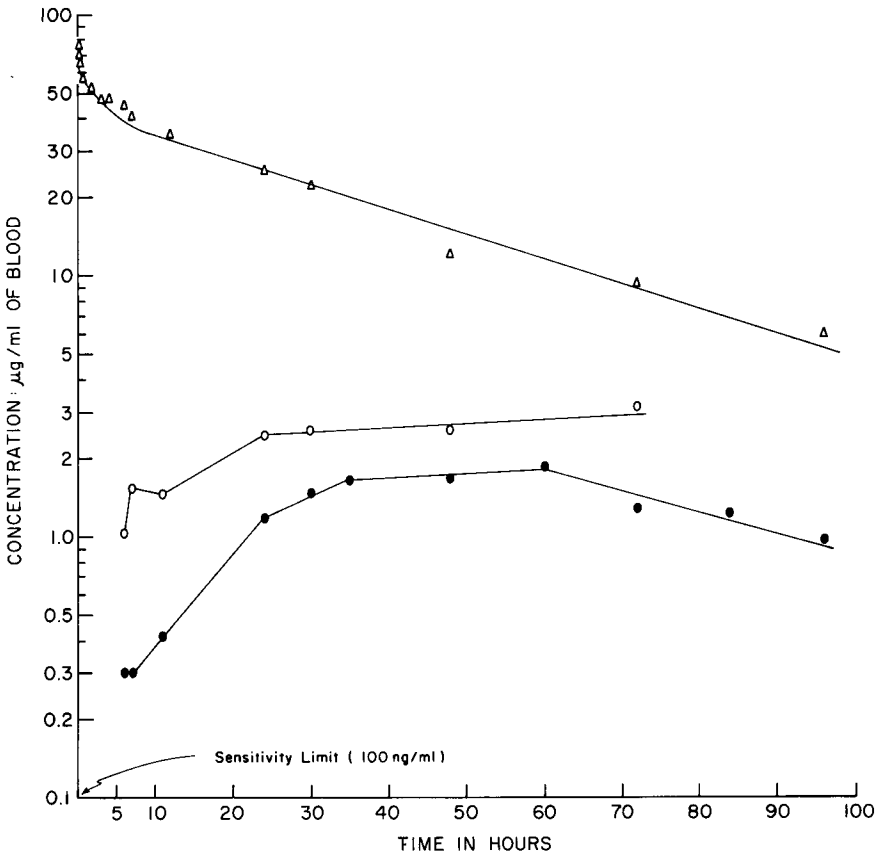

Fig. 5. Blood levels of phytoene in a dog following 10-mg/kg doses by intravenous (△) and oral (○, encapsulation; ●, suspended) routes.

and simple quantitation of phytoene using 1 ml or less of blood. A typical chromatogram of phytoene in dog blood after a 10-mg/kg oral dose is shown in Fig. 4 and a typical chromatogram of authentic phytoene and β-carotene (Fig. 2) shows baseline resolution between the two compounds. The difference in sensitivity at 280 nm between the two is approximately 20:1. Therefore, β-carotene will not interfere in the quantitation of phytoene, even if present in very large amounts. Under these conditions Vitamin A ($\lambda_{max.} = 325$ nm) will not be detected at all, thus imparting further specificity to the assay. Studies are in progress to investigate the use of multi-wavelength detection systems and differential extraction procedures for the selective determination of β-carotene and retinol produced endogenously following the administration of phytoene as a biosynthetic precursor[14]. No endogenous phytoene is measurable in control blood (Fig. 4A).

Recovery and sensitivity limits of HPLC assay

The overall recovery of phytoene is of the order of 86 $\pm$ 6.0% (S.D.) from blood. The sensitivity limits of detection are 50–100 ng per ml of blood. These limits can be increased by either extracting larger volumes of blood or by increasing the detector sensitivity. If greater sensitivity is required, extra solvent washes must be carried out in order to effect a better cleanup of the sample so that biological impurities are minimized.

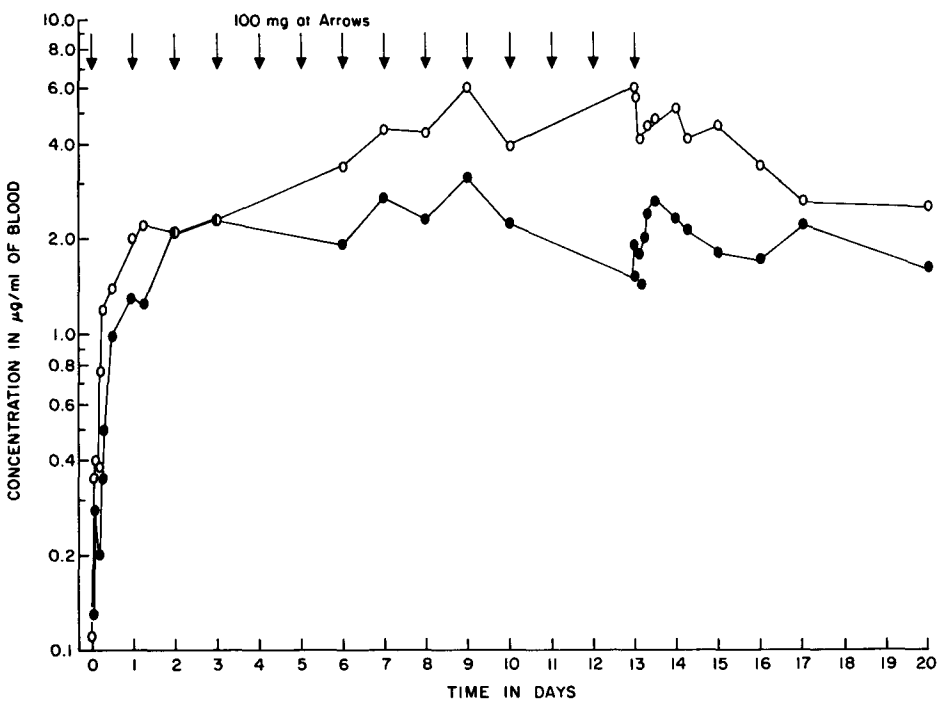

Fig. 6. Blood levels in dogs following multiple oral administration of 100-mg doses (at arrows) of an experimental formulation of phytoene. ○, Dog No. 1; ●, dog No. 2.

Application of the method to biological specimens

Blood levels of phytoene were determined in a pilot study in a single dog following the administration of 10-mg/kg doses by both intravenous and oral routes. Following the intravenous dose, a biphasic blood level curve was observed over the 96-h sampling interval (Fig. 5). The blood level measured at the 96-h time point (6 μg/ml) was about 2 orders of magnitude over the sensitivity limit of the assay, indicating sufficient assay sensitivity to measure blood levels over another 100-h interval. Following two different oral doses, the blood level curves indicated slow and prolonged absorption.

Studies following chronic oral dosing were carried out in which two dogs received 100 mg orally per day for 14 days of an experimental formulation. Blood samples were collected daily for 20 days. The resultant blood level curves (Fig. 6) suggest that the drug appears to have reached a plateau ranging between 2 and 4 μg/ml. These studies confirm oral absorption of phytoene and allow for the investigation for its intended clinical application as an oral sunscreen[6].

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