Journal of Chromatography, 273 (1983) 410—414
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1572

Note

High-performance liquid chromatography of the provitamin A β-carotene in plasma

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(First received August 10th, 1982; revised manuscript received October 25th, 1982)

Vitamin A, its naturally occurring provitamins (i.e., α- and β-carotene, Fig. 1), and synthetic analogues have become important drugs in ongoing chemoprevention and treatment trials of human cancers. These compounds have been shown to prevent or cure several chemically induced benign and neoplastic animal tumors [1—5]. In humans, the retinoids have caused regressions of premalignant lesions such as actinic keratosis [6, 7] and leukoplakia [8]. Recent epidemeologic studies have shown that the risk of developing epithelial cancers is inversely related to consumption of retinol and the provitamin, β-carotene [9, 10]. On these bases, large clinical trials of β-carotene are now being organized to evaluate its chemopreventive activity against a variety of epithelial cancers. Unfortunately, little data exist concerning the plasma pharmacokinetics of β-carotene after long-term dosing in humans.

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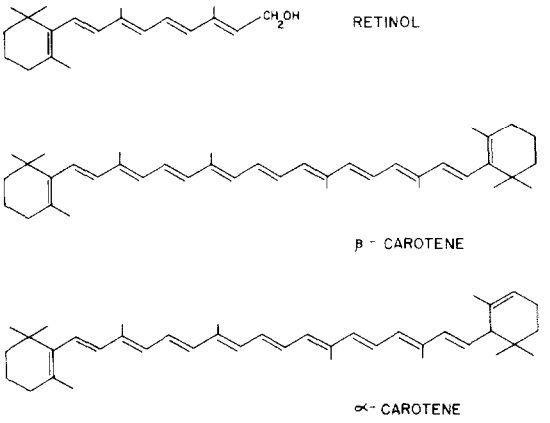

Fig. 1. Chemical structures of α- and β-carotene.

Published analytical methods for provitamin A have included colorimetry [11], open column chromatography [12] and thin-layer chromatography [13]. These have proven tedious, imprecise and fail to resolve β-carotene from its geometrical isomers (i.e. α-carotene and other carotenoids). In addition, gas chromatography has proven impractical because of the thermal instability of carotenoids [14]. Recently, Zakaria et al. [15] described a high-performance liquid chromatographic (HPLC) method for the measurement of carotene in tomatoes. The extraction method is tedious, time consuming, and not suitable for the assay of β-carotene in plasma and other biological fluids.

We have developed a rapid, simple, selective, and sensitive sample cleanup procedure and HPLC assay for the measurement of β-carotene in human plasma. Using this method we have studied the in vitro plasma stability of β-carotene and have shown that low concentrations can be assayed following oral dosing in humans.

EXPERIMENTAL

Materials

α- and β-carotene, obtained from Sigma (St. Louis, MO, U.S.A.), were dissolved in tetrahydrofuran (THF) and stored at −80°C in brown glass vials. All organic solvents were distilled in glass (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) and filtered through an 0.45-μm fluoropore filter (Millipore, Bedford, MA, U.S.A.) prior to use. Aqueous solvents for HPLC were filtered through an 0.45-μm cellulose acetate filter prior to use. Ammonium acetate (HPLC grade) was obtained from Fisher Scientific (Fair Lawn, NJ, U.S.A.), and perchloric acid was obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.).

Sample cleanup procedure

To an 0.5-ml aliquot of plasma in a microcentrifuge tube were added 100 μl of 5% perchloric acid and mixed rapidly. An 0.5-ml aliquot of THF—ethyl acetate (1:1, v/v) mixture was added and the samples again mixed for 60 sec and

centrifuged with a microcentrifuge (Fisher Scientific) at 13,000 *g* for 1 min. A 50-μl aliquot of the resulting organic layer was analyzed by HPLC.

HPLC analysis

HPLC analysis was performed using two Model M45 solvent delivery systems, a Model 710B WISP autoinjector, a Model 441 UV detector, a Model 730 data module and a Model 720 system controller (Waters Assoc., Milford, MA, U.S.A.). A Beckman (Berkeley, CA, U.S.A.) Ultrasphere-ODS 5-μm (25 cm × 4.6 mm I.D.) reversed-phase column preceded by a guard column (Waters Assoc.) packed with CO:Pell ODS (Whatman, Clifton, NJ, U.S.A.) was used for all analyses. The mobile phase consisted of 88% acetonitrile—THF (3:1, v/v) and 12% of methanol—ammonium acetate (1%) (3:2, v/v) at a flow-rate of 3.0 ml/min. Carotenes were detected at 436 nm. Quantitation was done by the external standard method.

Peak identification

Initial peak identification was based on retention times and comparison with the standards as well as co-chromatography with the standards. Identifications were additionally confirmed by mass spectrometry using a Finnigan Model 3300 mass spectrometer coupled to an Incos Model 2061 data system (Finnigan Instruments, Sunnyvale, CA, U.S.A.). Complete spectra were obtained for β-carotene isolated from the HPLC columns and compared to reference β-carotene spectra.

Recovery studies

Various amounts of standard β-carotene were added to an 0.5-ml aliquot of human plasma at room temperature. The plasma samples were prepared immediately for HPLC analysis as described above. Recovery was calculated by comparing the peak areas of the spiked samples to that of the standards. All experiments were carried out in triplicate on different days.

Stability studies

Stability studies of β-carotene were carried out in THF, THF—ethyl acetate (1:1, v/v) and human plasma. Samples stored at 25°C, 37°C, and -80°C were assayed for β-carotene concentration at time intervals.

RESULTS AND DISCUSSIONS

Fig. 2 shows representative HPLC chromatograms of α- and β-carotene extracted from human plasma: (a) endogenous α- and β-carotene, and (b) 1000 ng/ml standard β-carotene added to plasma. The precision and recovery data for the assay are shown in Table I. The average recovery of β-carotene from plasma was 106 ± 2.65% with a coefficient of variation (C.V.) of less than 5% (Table I). Recovery was linear from 50 to 2000 ng/ml. The assay had a detection limit of 10 ng/ml, determined on the basis of a signal equal to twice the noise level.

β-Carotene was stable in human plasma for more than 24 h at 25°C and 37°C in laboratory light. Reference standard stored in THF at −80°C was stable for

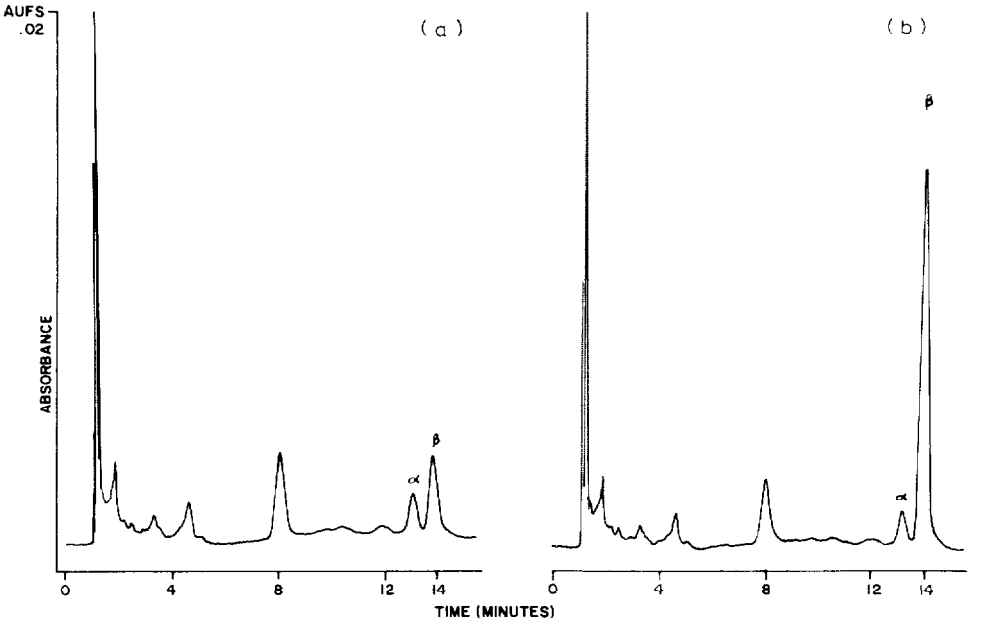

Fig. 2. HPLC chromatograms of α- and β-carotene extracted from human plasma. (a) Endogenous α- and β-carotene; (b) sample spiked with 1000 ng/ml standard β-carotene.

TABLE I

RECOVERY OF β-CAROTENE FROM PLASMA

Recovery of β-carotene at 25°C. All experiments were carried out in triplicate on different days.

β-Carotene plasma concentration (ng/ml)	Recovery (%) ($\bar{x}$ ± S.D.)	C.V. (%)*
1000	105 ± 3.15	3.0
500	109 ± 4.58	4.2
100	104 ± 4.35	4.2
Average	106 ± 2.65	2.5

*C.V. = S.D./$\bar{x}$, where S.D. is the standard deviation and $\bar{x}$ is the mean of three analyses on different days.

at least three months; however, β-carotene was stable for only 1 h in the extraction solvent (THF–ethyl acetate, 1:1, v/v) at 25°C. Thus, HPLC analysis should be performed within 1 h after extraction of β-carotene from plasma samples.

Our assay differs significantly from that of Zakaria et al. [15]. Saponification of the sample and evaporation of the extraction solvent are not required. These two procedures, used by Zakaria et al., are tedious, time consuming and tend to decrease drug recovery and assay precision. Additionally, we have im-

proved chromatographic separation by using 88% acetonitrile—THF (3:1, v/v) and 12% methanol—ammonium acetate (1%) (3:2, v/v). We believe that the present assay will prove useful for monitoring β-carotene concentrations in biological samples, and will help us study its pharmacokinetics and metabolism in cancer patients and normal subjects entered into chemoprevention trials.

ACKNOWLEDGEMENTS

This work was supported by Grants CA 27052 and CA 23074 from the National Institutes of Health, U.S. Public Health Service, Bethesda, MD, U.S.A.

The authors wish to thank Janine Einspahr for graphic illustrations.

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