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Endogenous levels of β-carotene in human buccal mucosa cells by reversed-phase high-performance liquid chromatography

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SUMMARY

We have developed a reversed-phase high-performance liquid chromatographic assay for the measurement of low nanogram levels of β-carotene in a single sample of human buccal mucosa cells. The method includes a simple sonification step for cell disruption and release of the compounds into the supernatant. The limits of detection were 0.02, 0.02 and 0.07 ng/mg of protein for β-carotene, retinol and retinol palmitate, respectively. Two patient populations were analysed. Average endogenous levels for β-carotene normalized to protein were 0.25 ng/mg of protein (range 0.04–1.9 ng/mg, twelve patients). No evidence of endogenous retinol or retinol palmitate could be detected in the human samples. An oral dosing study of four normal individuals showed a wide variation of β-carotene uptake. This rapid and sensitive method will enable investigators to use the non-invasive technique of buccal mucosa cell harvesting to determine cellular depot levels of β-carotene in various patient populations.

INTRODUCTION

Measurement of β-carotene, retinol and retinol palmitate are considered important for the determination of the disposition of vitamin A in the human population. Extensive studies of retinol have provided an understanding of the uptake, transport and metabolic mechanisms associated with this compound [1]. In addition, findings of antitumor activity have led to further investigations into the potential role of retinol as a chemopreventive agent [2–5] β-Carotene is a precursor to retinol (via 15,15′-β-carotene dioxygenase [6]) and

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has only recently begun to be studied as a potential chemopreventive agent in man [6–10]. It is speculated that one of the functions of β-carotene is to directly interact within cells to suppress cancer-initiating processes such as UV irradiation and lipid peroxidation [6]. Unlike retinol, the chemopreventive function of β-carotene and its metabolism are not fully understood. Retinol palmitate is also a useful compound to measure since it is a long-lived metabolite of retinol and can aid in the elucidation of the pharmacokinetics of both β-carotene and retinol [11].

Initial studies have shown that the uptake and storage of β-carotene, like retinol, in rat tissues is not directly dependent on serum levels [12,13]. In patients experiencing hypovitaminosis A, β-carotene is first converted to retinol until the deficiency is alleviated [3]. During this period of time the concentration of β-carotene in the serum is not reflective of its concentration in tissues. Therefore, an effective monitoring of β-carotene in anticancer trials must include measurement in its human tissue depot form, where it is thought to act. Very few procedures are available for assaying β-carotene in skin [14,15]. These methods involve sampling large amounts of skin and are impractical for large-scale population studies, which are necessary for evaluating the chemopreventive potential of β-carotene and retinol [3]. A far less invasive approach is to measure levels of carotenoids in buccal mucosa cells. These cells are ideal because they are a known depot for β-carotene and their rapid turnover rate is conducive to pharmacokinetic studies [16]. Stich et al. [17] have recently reported a high-performance liquid chromatographic (HPLC) method to measure levels of β-carotene in buccal mucosa cells. However, the method is not sensitive and requires extensive and destructive sample preparation [17].

We have developed a simple, precise and rapid reversed-phase HPLC method which measures β-carotene at sub-nanogram levels. We have also employed a new technique of sonification for the tissue disruption and release of β-carotene which avoids many of the hazards of the saponification technique used in other protocols [13,17,18]. In addition, we have used this newly developed method to monitor β-carotene levels in buccal mucosa cells of control patients and patients given high doses of β-carotene over a two-week period.

EXPERIMENTAL

Chemicals

Crystalline β-carotene (Type IV), all-*trans*-retinol and all-*trans*-retinol palmitate (Type IV) (Sigma, St. Louis, MO, U.S.A.) were used as analytical standards. The solvents used in the analysis were acetonitrile, hexane (UV grade), methanol, tetrahydrofuran [non-spectro with 250 ppm butylated hydroxytoluene (BHT)], toluene (Burdick & Jackson Labs., Muskegon, MI, U.S.A) and ethanol (Midwest Grain Products, Atchison, KS, U.S.A.). Analytical reagent

grade chemicals included BHT and ammonium acetate (Sigma). BHT was added to all filtered, degassed solvents at 0.1% (w/v), unless otherwise noted.

Patient dosing

Four patients were given 60 mg of capsulated β-carotene (Roche Labs., Nutley, NJ, U.S.A.) each day for fourteen days. A cell collection was made prior to the dosing for a control and every several days following an initiation period of six or eight days.

Buccal mucosa cell collection

A refined procedure similar to that of Stich et al. [17] was used to collect the buccal mucosa cells. Briefly, individuals were asked to thoroughly rinse his or her mouth with tap water, brush each cheek firmly twenty times (an up and down stroke counts as two strokes) with a medium-hard toothbrush, rinse with 20 ml of double-deionized (DDI) water and deposit the rinse into a 50 ml centrifuge tube. Following a second 20-ml rinse, a 10-ml wash of the toothbrush was added to the tube, making it 50 ml total. To concentrate the cells, the tubes were centrifuged for 15 min at 2900 *g* in a Beckman GPR centrifuge (Fullerton, CA, U.S.A.). After removing the supernatants, the pellets were transferred to 2.0-ml micro-centrifuge tubes (Sarstedt, F.R.G) and stored in a freezer at −35 °C until analysis. For the endogeneous level study, cells were harvested twice and pooled. In the pharmacokinetic study, cells were harvested four times in two weeks. This collection procedure was initiated six to eight days following the commencement of the β-carotene dosing.

Cell disruption

Each thawed pellet was diluted to 500 μl with methanol. Sonication was chosen over saponification to lyse and release β-carotene, retinol and retinol palmitate. This disruption technique is clean, simple and fast. As much as 25 mg of cells can be lysed in 15 min. To protect against thermal degradation, the tubes were placed on ice during sonication and periods of rest were incorporated. For individual samples a Branson sonifier 450 was used for 6 min at 50% setting 1.5, with 2 min rest periods in between the three 2-min sonication periods. For pooled samples the cells were sonicated for eight 2-min periods at 100% setting 2.0 with 2-min rest periods. A two-staged micro-tip was used with the sonicator. Verification of cell disruption was accomplished by viewing the cells under a microscope.

Sample extraction

After sonication, 50 μl of DDI water were added to each tube followed by 15 s of vortex-mixing. Next, 600 μl of hexane were added, vortexed for 15 s and centrifuged at 12 000 *g* for 60 s in a Beckman microfuge. The hexane layer was removed and placed into a clean amber-colored microcentrifuge tube to be

evaporated using nitrogen. A second 600-μl aliquot of hexane was added to the sample and the extraction repeated. Once the hexane was evaporated, the tubes were purged with nitrogen and stored at $-35\,^{\circ}C$ until analysis by reversed-phase HPLC.

Carotenoid and retinoid binding

It had been reported earlier [19] that neither β-carotene nor retinol binds to polypropylene tubes. However, we found significant deviations in recovery percentages among five polypropylene microcentrifuge tubes from two companies (West Coast Scientific, Emeryville, CA, U.S.A. and Sarstedt, F.R.G.). Recovery ranged from 30% for a clear plastic screw-top polypropylene tube up to 80% for an amber-colored polypropylene tube (West Coast Scientific).

Reversed-phase HPLC analysis

Samples were kept on ice after removal from the freezer until reversed-phase HPLC analysis. Each sample was reconstituted in 150 μl of mobile phase A immediately prior to its injection onto the column. After vortexing for 10 s, 100 μl were injected.

The HPLC system consisted of a Model 6000A pump incorporating the high-sensitivity accessory, a Model 440 fixed-wavelength UV detector with 436- and 340-nm filters (Waters Assoc., Milford, MA, U.S.A.), a Model 7125 manual injector (Rheodyne, Cotati, CA, U.S.A.), a Model 5880A recording integrator (Hewlett Packard, Palo Alto, CA, U.S.A.) and a strip chart recorder (Linear Instruments, Berkeley, CA, U.S.A.).

The separations were accomplished using a two-step gradient with a Beckman Ultrasphere-ODS, 250 mm $\times$ 4.6, 5 μm particle size, column. Both steps of the gradient contained acetonitrile, tetrahydrofuran, methanol, 1% ammonium acetate (in water) and BHT (0.025%, w/v). Step one utilized mobile phase A with volume percents of 71:15:6:8 and ran for 5 min. Step two utilized mobile phase B with volume percents of 67:25:6:2 and ran for 20 min. Equilibration was achieved by running mobile phase A for 10 min. Flow-rates were maintained at 1.0 ml/min at ambient temperature.

The maximum absorbance (λ_{max}) of β-carotene occurs at 456 nm in ethanol, while both retinol and retinol palmitate maximally absorb at shorter wavelengths of 325 and 328 nm, respectively [20]. Therefore a dual-wavelength detector monitored the eluent for β-carotene (436 nm at 0.005 a.u.f.s) and the retinoids (340 nm at 0.01 a.u.f.s.).

Preparation of analytical standards

Working standards of β-carotene, retinol and retinol palmitate were prepared no fewer than two months prior to use. All three compounds showed slight signs of degradation over a period of three months (shoulder peaks; loss

of analyte peak height). However, none of the losses in peak heights were greater than 6% over three months. Both β-carotene and retinol had to be purified before use. Each standard was made up separately in ethanol (0.1% BHT added) and placed in amber borosilicate glass vials. The concentration of each was determined using a Beckman Model 25 UV–VIS spectrophotometer and extinction coefficients of 2560, 1850 [20] and 975 [21] for β-carotene, retinol and retinol palmitate, respectively. A single standard solution mix with concentrations of 0.1 ng/μl β-carotene and 0.2 ng/μl retinol and retinol palmitate was used for all calibration curves.

Peak indentification

Identification of sample peaks was based on comparison of retention times of the unknown peaks with standard peaks of the same size (concentration). Simultaneous co-elution of endogenous β-carotene with added quantities of the standard mix also verified the identity of unknowns in patient samples.

Quantitative analysis

Levels of β-carotene were determined using the external standard method of quantitation. Approximately $20 \cdot 10^6$ buccal mucosa cells were collected, pooled and sonicated to provide a homogeneous matrix. The standards were added to 500-μl aliquots of this matrix. β-Carotene was added in amounts of 0, 1.5, 3.0 and 6.0 ng, while retinol and retinol palmitate were added in amounts of 0, 3.0, 6.0 and 12.0 ng. To make all volumes equal, an appropriate volume of ethanol was added to each tube. A standard curve was calculated by performing least-squares linear regression of the resulting peak heights on the known concentrations. The peak heights of any endogenous peaks were subtracted out. All concentration levels were done in triplicate. β-Carotene levels were normalized to protein concentration as determined by the Folin–Lowry assay [22]. Normalization to cell number was not used due to inconsistencies in counting large buccal mucosa cells in a hemocytometer. Application of the method to the analysis of retinol and retinol palmitate did not result in the detection of these compounds in buccal mucosa cells.

RESULTS AND DISCUSSION

Reversed-phase HPLC

The separation of nanogram quantities of pure standards of β-carotene, α-carotene, retinol and retinol palmitate is presented in Fig. 1A. β-Carotene is a non-polar hydrocarbon and must be separated from α-carotene, a geometric isomer. The mobile phase consisting of acetonitrile, tetrahydrofuran, methanol, ammonium acetate and BHT provided 'baseline' separation with a resolution factor (R_s) of 1.6 for the two isomers. Even though β-carotene and retinol palmitate were monitored on two different wavelengths, the absorbance

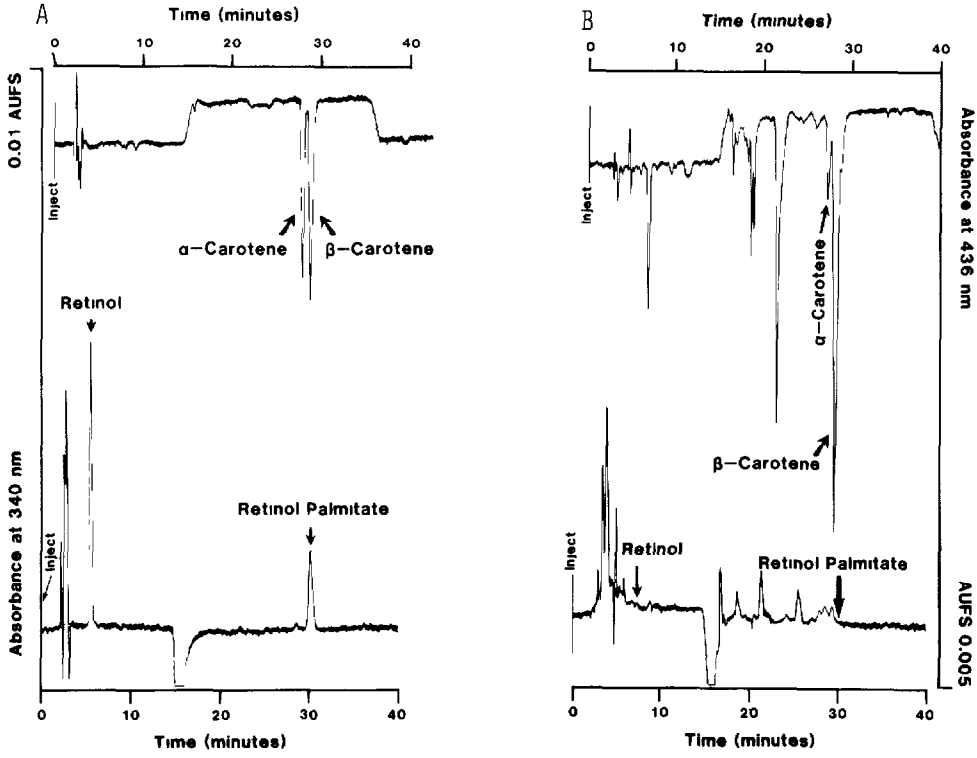

Fig. 1. (A) Reversed-phase HPLC separation of β-carotene, α-carotene, retinol and retinol palmitate. A two-step gradient consisting of acetonitrile–tetrahydrofuran–methanol–1% ammonium acetate–BHT run on a 5-μm Beckman Ultrasphere-ODS column was employed. Solvent A with volume percents of 71·15 6 8:0.025 (w/v) ran for 5 min, followed by solvent B (67:25 6 2 0.025, w/v) for 20 min. The column was equilibrated for 10 min with solvent A prior to the next injection. The flow-rate was maintained at 1.0 ml/min at ambient temperature. Detection of the retinoids was at 340 nm and carotenoids at 436 nm. The standard injection (10 μl) represents 4.0 ng β-carotene, 3.1 ng α-carotene and 8.0 ng retinol and renitol palmitate. (B) Reversed-phase HPLC separation of β-carotene extracted from human buccal mucosa cells. The cells were harvested from a patient dosed with 60 mg of β-carotene for six days. The HPLC conditions were identical to (A). Retinol and retinol palmitate were not detected.

of β-carotene at 340 nm is one eighth its absorbance at 436 nm, making it necessary to resolve the two. Our method yielded an R_s of 2.8. Retinol, a polar molecule with its terminal hydroxyl group, required a more polar mobile phase to separate it from the solvent front. A two-step gradient was incorporated using an initial mobile phase with a greater percentage of water to facilitate the retinol separation. Chromatographic peaks of endogenous β-carotene from dosed patients are presented in Fig. 1B. Application of the method to the analysis of retinol and retinol palmitate did not result in the detection of these compounds in dosed patients.

Linearity and detection limits

The linearity of the correlation between peak height and concentration of standard β-carotene, retinol and retinol palmitate was confirmed by the correlation coefficients. A criterion was established rejecting all calibration curves with a correlation coefficient less than 0.990. We defined the detection limit of the analyte as a signal-to-noise ratio greater than 3. This resulted in detection limits of 0.28, 0.35 and 1.43 ng for β-carotene, retinol and retinol palmitate, respectively, in pure standards or 0.022, 0.022 and 0.075 ng/mg of protein in fortified human samples.

Precision and recovery

Retention times and recovery percentages were used to determine the precision of the assay. Table I shows the average of the retention times of β-carotene, retinol and retinol palmitate for eleven independent runs over a three-month period. The coefficient of variation was no greater than 4% for all three analytes. Each average represents six independent trials performed over a period of three months (Table I). The precision of recovery is shown in Table II. Standards were added to the buccal mucosa cell matrix, extracted and analyzed by reversed-phase HPLC. Recovery percentages were calculated by dividing the peak height of extracted standards by the peak height of standards injected directly onto the column. The result was multiplied by 100. Any endogenous peak heights were subtracted from the extracted peak heights (Table II).

Binding of β-carotene and retinol palmitate

It became evident that the recovery percentages for β-carotene and retinol palmitate were decreasing with increasing concentrations of buccal mucosa cells in 500-μl aliquots. Fig. 2 illustrates the dependence of the recovery percentages on the concentration of buccal mucosa cells analyzed. The recovery percentage was calculated by normalizing the peak height from each experi-

TABLE I

PRECISION OF RETENTION TIMES

Retention times were measured over a three-month period; $n = 11$.

Compound	Retention time (mean ± S.D.) (min)	Coefficient of variation (%)
β-Carotene	24.4 ± 0.9	3.1
Retinol	5.3 ± 0.1	2.4
Retinol palmitate	27.7 ± 0.8	2.7

TABLE II

PRECISION OF RECOVERY FOR β-CAROTENE, RETINOL AND RETINOL PALMITATE

β-Carotene		Retinol		Retinol palmitate	
Amount[a] (ng)	Recovery (%)	Amount[a] (ng)	Recovery (%)	Amount[a] (ng)	Recovery (%)
1.0	58.41	2 0	79.82	2.0	81.34
	65.42		74.14		83.73
	57.41		77.73		81.34
	59.70		64.30		92.27
	59.24		68.05		95 17
	52.71		63.44		100.97
Mean	58.82		71.25		89.14
S.D.	3 74		6 37		7.50
C V (%)	6.36		8.94		8.41
2.0	53.79	4.0	71.04	4.0	79.81
	62.26		75.07		77.99
	60.21		74.27		81 02
	62.80		73 53		77.99
	53.66		72.03		70.74
	53.90		70.68		72.19
Mean	57 77		72.77		76.62
S.D	4.06		1.63		3.82
C.V (%)	7.04		2.25		4.98
4.0	57.23	8.0	81.11	8.0	79.01
	60.77		87.52		75 39
	58.93		83.94		78.71
	54.89		91 41		69.36
	55.01		88.51		70.10
	61.47		90.05		74.51
Mean	58.05		87.09		74.51
S.D.	2.57		3.54		3 76
C.V. (%)	4.43		4.07		5.04

[a]Amount of pure standard added (fortified) to a pooled sample of human buccal mucosa cells prior to extraction.

mental injection to the peak height of pure standards and then multiplying by 100. For buccal mucosa cell concentrations up to 5 mg/ml of protein, the recovery percentages for 4 ng of β-carotene, 8 ng of retinol and 8 ng of retinol palmitate exogenously added to the cell deviates no more than a standard deviation of 6% from the mean. However, for buccal mucosa cell concentrations

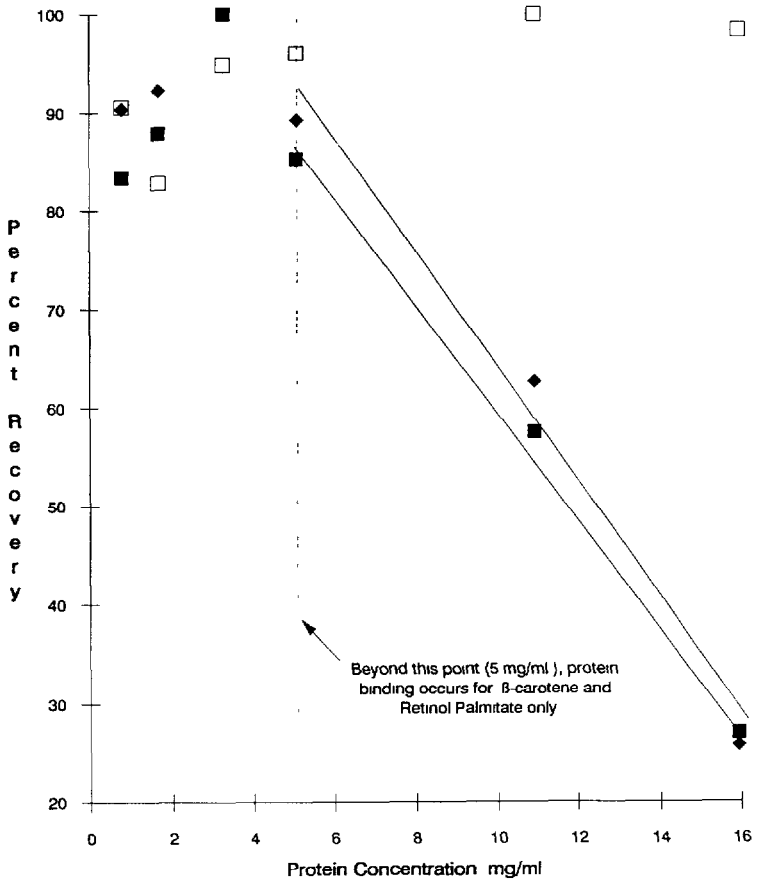

Fig. 2 Binding of β-carotene (■), retinol (□) and retinol palmitate (◆) to buccal mucosa cells (protein) Standards (4 ng β-carotene, 8 ng retinol and 8 ng retinol palmitate) were added to 500-μl aliquots containing buccal mucosa cell protein concentrations ranging from 0.8 to 15 9 mg/ml Percent recovery was calculated by normalizing the peak height from each experimental injection to the peak height of pure standards and then multiplying by 100.

above 5 mg/ml of protein, both compounds show a significant decrease in recovery percentage. A possible explanation is protein binding of β-carotene and retinol palmitate in the buccal mucosa cell matrix. When the matrix is spun down to remove the extraction layer (hexane), the protein and carotenoids or retinoids may go with it. Recovery of retinol was not affected by the protein concentration.

Measurements of endogenous levels

Eight individuals within the local community volunteered to donate buccal mucosa cells for analysis. Two cell collections were made from each person in

TABLE III

ENDOGENOUS LEVELS OF β-CAROTENE, RETINOL AND RETINOL PALMITATE

Patient No.	Age (years)	Sex	Buccal mucosa cells extracted ($\times 10^8$)	Concentration (ng/mg of protein)		
				β-Carotene	Retinol	Retinol palmitate
1	35	F	1.88	0.05	N.D.[a]	N.D.
2	31	F	0.88	0.12	N D.	N.D.
3	24	M	3.79	0.04	N.D.	N.D.
4	23	M	2.82	0.04	N.D.	N.D.
5	21	M	1.87	0.04	N.D.	N.D.
6[b]	23	M	1.31	0.24	N.D.	N.D.
7	41	M	3.46	0.08	N.D.	N D.
8	21	F	3.27	0.19	N.D	N.D
Mean	27.38		2.41	0.10	–	–
S.D	7.44		1.07	0.08	–	–
C.V. (%)	27.19		44.46	77.28	–	–

[a]Not detected.
[b]Patient is a vegetarian

order to harvest enough cells for accurate, multiple quantitation. These individuals were not screened in any way, except that their diets could not be supplemented with β-carotene within the three months preceding the collection. The objective was to sample a normal, disease-free population. The findings are presented in Table III. Patient 6 is a vegetarian and his β-carotene level was significantly higher. This agrees with previous findings that individuals who adhere to a vegetarian diet have higher endogenous levels of β-carotene [17]. Although the sample size is small ($n=8$), the data do show that there is a wide variation of endogenous levels of β-carotene within our population. No endogenous retinol or retinol palmitate were detected and several explanations are possible for this observation. Retinol and retinol palmitate were present, but at very low levels below our limits of detection, were not stored in the cells or were metabolized quickly in the dynamic growth cycle of buccal mucosa cells.

Pharmacokinetic study

To study the absorption and storage of β-carotene in buccal mucosa cells, a disease-free patient group ($n=4$) was dosed with 60 mg of β-carotene per day for two weeks. Harvested cells during the dosing period were analyzed and the resulting levels normalized to protein content. Fig. 3 shows each individual's buccal mucosa cell β-carotene concentration during the trial period. This study was not meant to be a definitive pharmacokinetic study of the uptake and storage of β-carotene in buccal mucosa cells. Rather, it was a survey to probe cellular levels of β-carotene in dosed individuals. A wide variation was noted

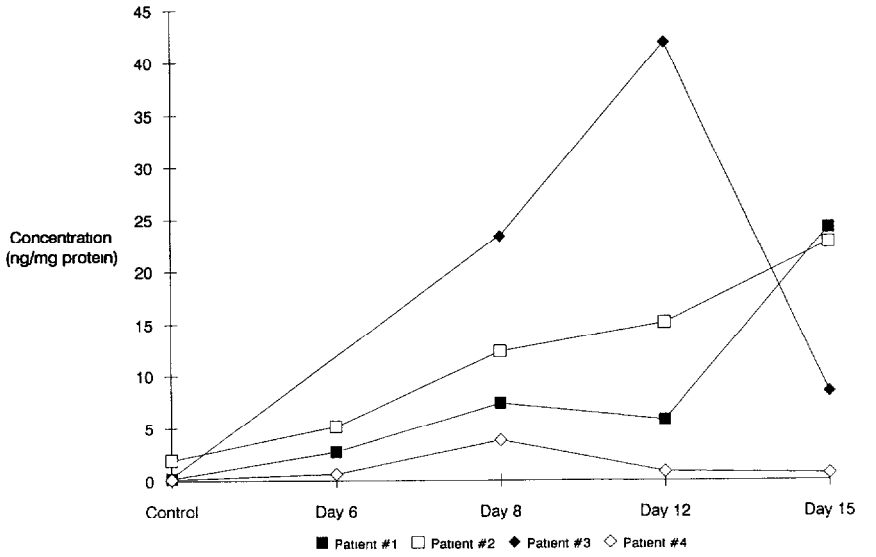

Fig. 3. Endogenous β-carotene levels from a pharmacokinetic study of four patients dosed with 60 mg β-carotene per day for two weeks. Patient 4 was confirmed to be not in compliance with dosage at the end of the study. Levels of β-carotene, retinol and retinol palmitate were determined using the external standard method based on peak-height values obtained from an extracted standard curve. All β-carotene and retinoid levels were normalized to protein concentration.

between different individuals. In our study, three of the patients showed marked uptake of β-carotene. The fourth showed little uptake. Part of the explanation for this reduced rate is non-compliance by subject 4 at the end of the study. In addition to the variation between individuals indicated from this survey, one should anticipate variable β-carotene concentrations in a single individual over the course of a year, primarily due to changing diets.

In summary, we have developed a simple and sensitive reversed-phase HPLC method for determining β-carotene, retinol and retinol palmitate in human buccal mucosa cells. In 1981, a review article by Peto et al. [10] called for large population studies to determine if β-carotene has a beneficial effect on reducing cancer incidence rates in humans. The analytical method that we have developed can be used for large chemoprevention trials by allowing the non-invasive quantitation of picogram quantities of β-carotene in buccal mucosa cells. The sensitivity is low enough for the quantitation of endogenous levels of β-carotene in a single sample harvesting of cells from a patient, but not retinol or retinol palmitate. The observation that retinol and retinol palmitate are below the low nanogram (high picogram) detection limit of this assay is interesting because it provides the first evidence that human buccal mucosa cells are not a good cellular depot for these two retinoids. However, buccal

mucosa cells still offer an ideal opportunity for large population chemoprevention or nutrition studies because they are a known tissue depot for β-carotene which can be sampled in a non-invasive manner.

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