Journal of Chromatography, 382 (1986) 290—296
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3284

Note

Liquid chromatographic determination of retinol and α-tocopherol in human buccal mucosal cells

N.R. BADCOCK* and D.A. O'REILLY

Department of Chemical Pathology, The Adelaide Children's Hospital, Inc., North Adelaide, South Australia 5006 (Australia)

and

C.B. PINNOCK

Department of Community Medicine, University of Adelaide, Adelaide, South Australia 5000 (Australia)

(First received March 5th, 1986; revised manuscript received June 6th, 1986)

Assessment of vitamin A status is currently focussed on circulating levels of retinol and its binding protein and establishing their relationship to liver reserves of vitamin A [1—3]. The relationship between plasma vitamin A, an insensitive measure of vitamin A status [2], and the functional availability of the vitamin at tissue level has yet to be determined. Measurement of vitamin A in human cheek epithelial (buccal mucosal) cells as a tissue source offers a convenient opportunity to study these relationships in a large number of subjects, including children, because of the non-invasive method of collection of these cells and because buccal mucosa, like other mucosal epithelial surfaces, is sensitive to vitamin A [4—6]. Vitamin E is known to influence the biological availability of vitamin A [7—9], thus a method of assessment of the costatus of these two vitamins at the tissue level is highly desirable.

We describe for the first time a high-performance liquid chromatographic (HPLC) procedure for the detection and quantification of vitamin A (as retinol) and vitamin E (as α-tocopherol) in human buccal mucosal cells. The analysis exploits the cheek cell collection method of McMurchie et al. [10], the extraction procedure of Bligh-Dyer [11], normal- and reversed-phase chromatography and the native fluorescence of vitamins A and E.

0378-4347/86/$03.50

EXPERIMENTAL

Reagents and glassware

All solvents including water were HPLC grade while all other reagents were analytical grade (Ajax Chemicals, Sydney, Australia). Chloroform was further purified by distilling from an all-glass apparatus, rejecting the first 10% and the last 25% of the distillate. All-*trans*-retinol of crystalline purity and *d*-α-tocopherol were obtained from Sigma (St. Louis, MO, U.S.A.).

The stock retinol solution, 1.0 mmol/l (286 mg/l) in absolute alcohol, was stable for at least three months at −20°C. The working retinol solution, 100 nmol/l in absolute alcohol, was prepared fresh on the day of analysis. The stock α-tocopherol solution, 1.0 mmol/l (431 mg/l) in absolute alcohol, was stable for at least three months at −20°C. The working α-tocopherol solution, 1.0 μmol/l in absolute alcohol, was prepared fresh on the day of analysis. The concentration of each working standard was confirmed spectrophotometrically by using their respective absorptivities ($A^{1\%}_{1cm}$) in absolute alcohol: retinol 1780 at 325 nm and α-tocopherol 75.8 at 292 nm.

The internal standard for retinol, 1-naphthol, was obtained from Ajax Chemicals. The stock 1-naphthol solution, 1.0 mmol/l (144 mg/l) in absolute alcohol, was stable for at least six months at −20°C. The working 1-naphthol solution, 100 nmol/l in methanol, was prepared freshly on each day of analysis. The internal standard for α-tocopherol, *d*-tocol, was a gift from Eisai Research Labs. (Tokyo, Japan). The stock tocol solution, 1.0 mmol/l (389 mg/l) in absolute alcohol, was stable for at least six months at −20°C. The working tocol solution, 25.0 nmol/l in methanol, was prepared freshly on each day of analysis.

Sample preparation

Human cheek cells were collected as follows. Subjects were first asked to rinse their mouths with distilled water. Subsequently, cells were collected by vigorously swirling 5 ml of distilled water around the mouth and spitting out the water and contents into a darkened plastic screw-cap jar containing 25 μg butylated hydroxytoluene (BHT). Collecting cycles were repeated three more times until approximately 20 ml had been collected. The resultant cheek cell suspension was filtered through a sintered-glass funnel (Pyrex Size 0, 161—250 μm).

The filtrate was successively aliquoted into a 10-ml glass quick-fit centrifuge tube, centrifuged at 2000 *g* for 10 min and decanted. The cells were resuspended twice in isotonic saline and the washed pellet, remaining following successive centrifuging and decanting, was kept either at room temperature (for up to 2 h) before extraction or stored frozen at −20°C until assay (usually within two weeks).

On the day of the analysis, the thawed cheek cell pellet was reconstituted with 2 ml distilled water by successively vortexing and sonicating for 30 s. Aliquots, 1.2 ml, 0.4 ml and 2 × 0.1 ml, were pipetted into labelled 10-ml glass quick-fit centrifuge tubes for retinol, α-tocopherol and protein determination, respectively.

Extraction procedures

Vitamin A. Known amounts of vitamin A covering the range 0—50 pmol/mg of protein were added in 10 pmol/mg of protein increments to prepared 1.2-ml cell aliquots from a cheek cell pool. To these standard tubes and to each patient sample to be assayed (tube containing a cell pellet aliquot), 3 ml of 1-naphthol working internal standard solution and 1.5 ml of chloroform were added. Tubes were stoppered, sonicated for 10 s, vortex-mixed for 60 s and allowed to stand for 5 min. Water (1.5 ml) and chloroform (1.5 ml) were added. Following gentle mixing by inversion, the tubes were centrifuged at 2000 *g* for 5 min. The infranatant was transferred to another 10-ml quick-fit centrifuge tube and evaporated to dryness at 40°C under a stream of nitrogen.

Vitamin E. Known amounts of vitamin E covering the range 0—500 pmol/mg of protein were added in 100 pmol/mg of protein increments to prepared 0.4-ml cell aliquots from a cheek cell pool. To these standard tubes and to each patient tube containing a cell pellet aliquot, 0.4 ml of a 50 mmol/l solution of pyrogallol in boiled water, 2 ml of tocol working internal standard and 1 ml of chloroform were added. Tubes were stoppered, sonicated for 10 s, vortex-mixed for 60 s and allowed to stand for 5 min. Water (1 ml) and chloroform (1 ml) were added. At this stage the procedure was followed as for cheek cell vitamin A.

Protein in cheek cells

Protein was determined in duplicate on the two 0.1-ml cell aliquots, using the method of Lowry et al. [12].

High-performance liquid chromatography

Liquid chromatographic analyses were performed using a Model 320 liquid chromatograph (Beckman Instruments) equipped with a Model F1000 fluorescence spectrophotometer (Hitachi). The analytical column for retinol was a pre-packed 250 × 4.6 mm I.D. Ultrasphere-Si column, average particle size 5 μm, and the guard column was 50 × 4.6 mm I.D., dry-packed with Spherisorb-Si, 10 μm. Tocopherol was separated on a 250 × 4.6 mm I.D. Ultrasphere ODS, average particle size 5 μm, and the guard column was 50 × 4.6 mm I.D., dry-packed with Ultrasphere ODS, 20 μm (analytical columns and packings, Beckman Instruments).

Vitamins A and E were eluted isocratically with heptane—dioxane (92:8) and methanol, respectively, at a constant flow-rate of 2 ml/min. With detector sensitivity 20, excitation 326 nm, emission 470 nm for retinol and 1, 295 nm and 330 nm, respectively, for α-tocopherol, peak heights were recorded with a 10-mV recorder at a chart speed of 0.5 cm/min.

Residues were dissolved in 300 μl of their respective mobile phases and 250-μl aliquots injected into the chromatograph. Under the above conditions, the retention times for retinol and α-tocopherol were 7.9 and 7.5 min, respectively. A calibration curve was made by plotting peak-height ratios (retinol/internal standard and α-tocopherol/internal standard) against vitamin A and E concentrations, respectively. The intercepts, which represent the endogenous levels of retinol and α-tocopherol in the cheek cell pool, were subtracted from the peak heights of the vitamin A and E standards. The ratio of corrected peak

heights of vitamin standard to internal standard was then calculated and the value of unknown specimens calculated by direct proportion.

RESULTS AND DISCUSSION

Typical chromatograms of an extract of cheek cells from an adult volunteer are illustrated in Fig. 1. For a number of reasons, it was not practical to use the same HPLC conditions for both vitamins. Unlike the excellent separation afforded α-tocopherol and tocol, retinol, at the higher sensitivity setting required for its quantitation, could not be adequately resolved on a reversed-phase column from the gross peak at the beginning of the chromatogram, even with gradient elution and a variety of solvent phase combinations. In addition,

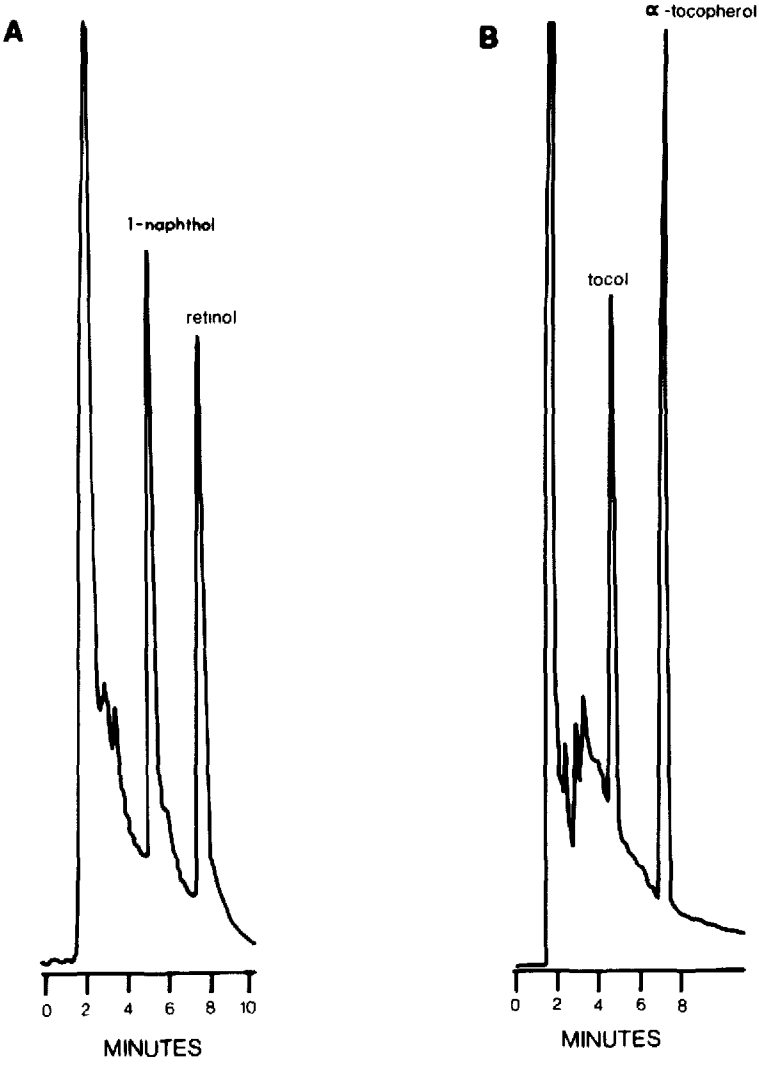

Fig. 1. Liquid chromatograms of extract of human buccal mucosal cells containing 12 pmol retinol per mg protein (A) and 240 pmol α-tocopherol per mg protein (B)

the high fluorescence intensity of α-tocopherol in methanol [13], compared with the hydrocarbon-based mobile phase (which would be necessary for its elution from a silica gel column), and the quite different fluorescent properties of each vitamin necessitated their quantitation using different chromatographic column packings and eluting solvents.

Sensitivity

Detection limit, defined as a signal twice the height of the noise level, was approximately 0.5 pmol retinol per mg protein and 5 pmol α-tocopherol per mg protein. When 1 pmol/mg of protein was added to a cheek cell aliquot with an endogenous retinol concentration of 12 pmol/mg of protein, the peak-height ratio of vitamin to internal standard was increased from 0.92 to 1.00; similarly, when 50 pmol α-tocopherol per mg protein was added to a cheek cell aliquot having an endogenous α-tocopherol concentration of 240 pmol/mg of protein, the peak-height ratio of vitamin to internal standard was increased from 1.69 to 2.04. Concentration and peak height were linearly related throughout the concentration ranges investigated, 1—50 pmol retinol per mg protein and 50—500 pmol α-tocopherol per mg protein. This adequately covers the range of vitamin A and E concentrations in cheek cell samples from normal children and adult controls.

Choice of reference standard

In selection of a reference standard, wet weight, dry weight, cell numbers and protein were considered. Of these, protein was the most reproducible [coefficient of variation (C.V.) 3%] and was correlated with dry weight by a factor 0.811 (n=18). Protein determination also provides increased sensitivity when cell yields are low, a not uncommon occurrence when collections are taken from young children.

Reproducibility

To assess the reproducibility of the method, five replicate samples from a cheek cell pool were analysed on each of five different days. For the five within-day replicates, the mean retinol concentration was 13.7 pmol/mg of protein and the C.V. was 10.6%; the mean α-tocopherol concentration was 381 pmol/mg of protein and the intra-batch C.V. was 9.8%. The corresponding between-day values were: 12.6 pmol retinol per mg protein, C.V. 12.2% and 390 pmol α-tocopherol per mg protein, C.V. 11.1%.

Analytical recovery and stability of preparations

The two vitamins were found to be stable for up to ten weeks in the washed cell pellet when prepared and stored as described above. Freezing the cheek cell collection, as received, was not attempted because of almost certain cell lysis, and storage of the whole cell collection at 4°C produced unpredictable losses of retinol and α-tocopherol, presumably from bacterial action. BHT, at the concentration employed, prevented possible losses of vitamins A and E during cell storage at −20°C. It was preferred to pyrogallol as an antioxidant at the cell collection and storage stage because of its greatly reduced handling toxicity. However, inclusion of pyrogallol during the vitamin E extraction was

essential in preventing excessive and unpredictable losses from oxidative degradation of α-tocopherol. In the absence of pyrogallol, recovery varied between 10 and 80% compared with near complete recovery (94—103%) when pyrogallol was added. Analytical recoveries of retinol were good (> 89%) without pyrogallol and were not improved by its addition, hence we elected to add none in that extraction procedure (Table I).

The high extraction recoveries of vitamins A and E from cheek cells were achieved using the Bligh—Dyer method. We failed to achieve reproducible and quantitative results with hexane procedures previously reported for the extraction of fat-soluble vitamins from plasma [14—17] and somatic afferent receptor cells [18].

TABLE I

RECOVERY OF ADDED RETINOL AND OF ADDED α-TOCOPHEROL TO BUCCAL MUCOSAL CELLS

Compound	Concentration (pmol/mg of protein)			Mean recovery (%)
	Endogenous (mean)	Added	Found (mean)	
Retinol	13.7	10.0	22.0	93
		25.0	34.4	89
		50.0	62.4	98
α-Tocopherol	381	100	452	94
		250	650	103
		500	890	101

Selectivity

The identity of retinol and α-tocopherol in cheek cells was assessed by comparing their retention times to those of the corresponding reference compounds. Sharp and isolated peaks with retention times identical to retinol and α-tocopherol were obtained when these vitamin standards were added to cheek cells, extracted and chromatographed. Moreover, the peak heights were modified as expected from the two original relative concentrations. This cochromatographic agreement was always found regardless of the chromatographic conditions (different mobile phases and C_{18} columns).

The method employed in this study for the sampling of human cheek cells yielded sufficient material (>25 μg protein per 1-ml cell collection) to allow the analysis of vitamins in cheek cells from children as young as 5 years of age. To lessen direct contamination from food, drugs, vitamin preparations, etc., and because cells are removed from buccal mucosa during eating, collection was made on fasting subjects. By filtering the resultant cheek cell suspension through a sintered-glass funnel, foreign material, such as food particles which may not have been removed by the initial rinse, was separated from the cheek cell filtrate. The cell pellet was washed twice with isotonic saline to exclude possible contamination from saliva.

The method described was developed as part of an on-going epidemiological study in which the relationship between vitamin A status and incidence of respiratory tract infections is being examined [19]. Preliminary investigations suggest that buccal cell retinol may prove a sensitive index of vitamin A status.

The reliability of the method and the relationship to other sensitive measures of vitamin A status will be reported elsewhere. The present investigation has shown liquid chromatography with fluorescence detection to be a highly sensitive and selective method for the analysis of vitamins A and E in buccal mucosal cells.

REFERENCES

1 H. Flores, F.A Campos, C.R. Araujo and B. Underwood, Am. J. Clin. Nutr., 40 (1984) 1281.
2 J.A. Olson, J. Nat. Cancer Inst., 73 (1984) 1439.
3 J.A. Olson, Am. J. Clin. Nutr., 35 (1982) 1166.
4 C.A. Squier, N.W. Johnson and R.M. Hobbs, Human Oral Mucosa; Development, Structure and Function, Blackwell Scientific, Oxford, 1st ed., 1976.
5 M.W. Hill, R.R. Harris and G.P. Carron, Cell Tissue Res., 226 (1982) 541.
6 Y.C. Wong, Exp. Mol. Pathol., 23 (1975) 132.
7 E. Sondergaard, Experientia, 28 (1972) 773.
8 M.K. Horwitt, Am. J. Clin Nutr., 29 (1976) 569
9 J.N. Roehm, Chem. Engl. News, 48 (1970) 38.
10 E.J. McMurchie, B.M. Margetts, L.J. Beilin, K.D. Croft, R. Vandongen and B.K. Armstrong, Am. J. Clin. Nutr., 39 (1984) 975.
11 E.G. Bligh and W.J. Dyer, Can. J. Biochem. Physiol., 37 (1959) 911.
12 O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J Biol. Chem., 193 (1951) 265
13 S.L. Taylor, M.P. Lamden and A L Tappel, Lipids, 11 (1976) 530.
14 G L. Catignani and J.G. Bieri, Clin. Chem., 29 (1983) 708.
15 A.P. De Leenheer, V.O.R.C. De Bevere, M.G.M. De Ruyter and A.E. Claeys, J Chromatogr., 162 (1979) 408.
16 J.G. Bieri, T J. Tolliver and G.L. Catignani, Am. J. Clin. Nutr , 32 (1979) 2143.
17 W.J. Driskell, J.W. Neese, C.C. Bryant and M.M. Bashor, J. Chromatogr., 231 (1982) 439.
18 R.A. Chole, Ann. Otol Rhinol. Laryngol., 86 (1978) 595.
19 C.B. Pinnock, R.M. Douglas and N.R. Badcock, Aust. Paediatr J., 22 (1986) 95.