Journal of Chromatography, 553 (1991) 179–186
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2234

Determination of several retinoids, carotenoids and E vitamers by high-performance liquid chromatography

Application to plasma and tissues of rats fed a diet rich in either β-carotene or canthaxanthin

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ABSTRACT

A method, using two different systems, is described for the high-performance liquid chromatographic analysis of retinol, retinal, retinoic acid, retinyl acetate, retinyl palmitate, α-, β- and γ-carotene, β-apo-6′-, β-apo-8′-, β-apo-10′- and β-apo-12′-carotenal, ethyl β-apo-8′-carotenoate, α-tocopherol and α-tocopheryl acetate. The first system consists of a laboratory-packed Hypersil-ODS 3-μm column and a mobile phase of acetonitrile–methylene chloride–methanol–water (70:10:15:5, v/v). The second system consists of a laboratory-packed Nucleosil C_{18} 3-μm column and a mobile phase of acetonitrile–0.1 *M* ammonium acetate (80:20, v/v). The detection limits in standard solutions were 10 ng/ml for retinoids and carotenoids and 60 ng/ml for the E vitamers. Analysis of the tissues and plasma of rats, after 2 weeks on a diet supplemented with either β-carotene or canthaxanthin (both 2 mg/g), led to the conclusion that the rats were able both to transport and store β-carotene and canthaxanthin and to convert β-carotene to retinol. Incubation of cytosol preparations from the mucosa of the small intestine of rat with 1 μg of β-carotene resulted in the formation of 10–20 ng of retinal within 1 h.

INTRODUCTION

Vitamin A and β-carotene may be important agents in the prevention of cancer [1–3]. After absorption by the small intestine, most β-carotene is believed to be converted to vitamin A, while a small part may be transported as β-carotene [4]. It is not clear whether β-carotene is protective after conversion to vitamin A, or by itself, for instance as an antioxidant [5].

The primary interest is in the absorption of β-carotene in the intestine and its

0021-9673/91/$03.50

possible cleavage following absorption. Two cleavage theories exist: the first assumes a central cleavage by 15,15′-dioxygenase, resulting in two molecules of retinal, whereas the second assumes a random cleavage, resulting in retinal, various β-apo-carotenals and other products.

To validate these two theories it should suffice to measure the amount of retinal formed from β-carotene. However, the experience is that, under experimental conditions *in vitro*, the amount of retinal formed is too small to confirm the first theory [6]. To confirm the second theory products other than retinal (and not originating from retinal) should be demonstrated.

It is therefore important to measure the possible products resulting from random β-carotene cleavage. Several high-performance liquid chromatographic (HPLC) procedures have already been described for the determination of retinol, retinoic acid, retinyl esters and/or carotenoids [7–9]. Furr [10] described a method for the analysis of retinal, among other retinoids, using a gradient programme. Hansen and Maret [11] measured retinoids, carotenoids and β-apo-carotenals using three different HPLC systems.

In this paper a method is described for the HPLC analysis (using two different systems) of retinol, retinal, retinoic acid, retinyl acetate, retinyl palmitate, α-, β- and γ-carotene, β-apo-6′-, β-apo-8′-, β-apo-10′- and β-apo-12′-carotenal, ethyl β-apo-8′-carotenoate, α-tocopherol and α-tocopheryl acetate. In addition, two applications of the method are described. The first is the analysis of tissues obtained from female rats fed a diet enriched with either β-carotene or canthaxanthin. The second is the analysis of samples of an *in vitro* cleavage assay with intestinal cytosol preparations, described as the 15,15′-dioxygenase assay by Goodman *et al.* [12].

EXPERIMENTAL

Chemicals

β-Apo-6′-caretenal was a gift from BASF (Arnhem, The Netherlands). Retinyl stearate, γ-carotene, β-apo-10′-carotenal and β-apo-12′-carotenal were gifts from Hoffmann-La Roche (Mijdrecht, The Netherlands). β-Carotene was obtained from Merck (Darmstadt, Germany). β-Apo-8′-carotenal, ethyl β-apo-8′-carotenoate and canthaxanthin were obtained from Fluka (Buchs, Switzerland). Other reference compounds were purchased from Sigma (St. Louis, MO, U.S.A.). Acetonitrile (HPLC grade) from Westburg (Leusden, The Netherlands) was used. All other chemicals were of analytical-reagent grade.

Chromatography

HPLC analyses were performed using a system incorporating a Gynkotek 300 C constant-flow pump (Kipp Analytica, Delft, The Netherlands), an ISS-100 automatic injector with cool tray (Perkin-Elmer, Gouda, The Netherlands) and two programmable 783 absorbance detectors (Applied Biosystems, Rotterdam, The Netherlands). Two stainless-steel Hyperchrome HPLC columns (125 mm × 4.6 mm I.D.) were packed in the laboratory with Hypersil ODS 3 μm (Shandon Southern Products, Astmoor, U.K.) or Nucleosil 120-3 C_{18} (Machery-Nagel, Düren, Germany) by the balanced-density slurry technique on a column-packing installation designed at the TNO Toxicology and Nutrition Institute, using a Haskel DSTV-150 pump

(Ammann Technik, Stuttgart, Germany). The slurry and packing solvents were isopropanol and methanol, respectively. Elution profiles were displayed on a Kipp BD 41 recorder (Kipp Analytica).

The mobile phase used for the Hypersil column consisted of acetonitrile–methylene chloride–methanol–water (70:10:15:5, v/v). A flow programme of 0.5–2.0 ml/min was used (0–13 min at 0.5 ml/min; 14–24 min at 1.0 ml/min; 25–46 min at 1.5 ml/min; 47–57 min at 2.0 ml/min; 58–60 min at 0.5 ml/min). The Nucleosil column was eluted at a flow-rate of 1.0 ml/min with acetonitrile–0.1 *M* ammonium acetate (80:20, v/v).

Detection after separation on the Hypersil column was carried out using one detector set at 350 nm and a second detector switching after 16 min from 445 to 292 nm and returning 9 min later to 445 nm for the last part of the HPLC run.

Procedures

Standard solutions were prepared in methanol. The preparation of standard solutions and the extraction of plasma and tissue samples and of incubation mixtures from the dioxygenase assay were carried out under subdued light. The actual concentrations of the standards were determined by measuring the absorbance of diluted stock solutions using a Ultrospec K spectrophotometer (LKB, Cambridge, U.K.) and calculating the concentrations based on published spectral data [13–15].

Livers were dismembrated in liquid nitrogen using a Mikro-dismembrator II (Braun, Melsungen, Germany). All other tissues were homogenised with an Ultra-Turrax homogeniser (Wilten Woltil, De Bilt, The Netherlands). Samples were homogenised in brown-coloured test-tubes with 3 ml of doubly distilled water containing EDTA (10 m*M*), ascorbic acid (1 mg/ml) and acetic acid (1%, v/v). Plasma samples of 100 μl were mixed with 100 μl of 0.9% (w/v) NaCl. To precipitate proteins, 2 ml (0.2 ml for plasma samples) of methanol [containing 1 mg of butylated hydroxytoluene (BHT) per ml added as an antioxidant] were added and the mixture was vortexed for 30 s. After 10 min, 4 ml (0.4 ml for plasma) of chloroform (containing 1 mg/ml BHT) were added and the sealed tubes were vortexed for 4 min. After centrifugation the chloroform layer was separated and evaporated under nitrogen. The residue was dissolved in methanol, transferred into brown HPLC injection vials and placed in the HPLC tray of the injector, which was cooled down to about +4°C to increase stability, especially of the aldehyde forms of the compounds of interest.

For the incubation mixtures of the dioxygenase assay the same procedure was followed, except that ethanol was used to precipitate the proteins and hexane was used for extraction. The residues were dissolved in water-free eluent.

RESULTS AND DISCUSSION

Characteristics of the high-performance liquid chromatographic methods

With the Hypersil column the HPLC method described here yielded a complete resolution of retinol, retinyl acetate, retinyl palmitate, α-, β- and γ-carotene, β-apo-6′-, β-apo-8′-, β-apo-10′- and β-apo-12′-carotenal, ethyl β-apo-8′-carotenoate, α-tocopherol and α-tocopheryl acetate. The method using the Nucleosil column gave a complete resolution of retinoic acid, retinal, retinol and retinyl acetate. It was not possible to separate all the compounds in a single run using the Hypersil column;

retinoic acid eluted in the void volume and no baseline separation could be obtained for retinol, retinal and retinyl acetate. Separation could be improved by increasing the polarity of the eluent. However, this extends the elution time of β-carotene by several hours. As the detector was used in a very sensitive setting, gradient elution was not useful because of a rising baseline. Flow programming shortened the run to 45 min.

Using the Hypersil column two spectrophotometric detectors were needed as the time between the elution of the retinyl acetate and β-apo-12′-carotenal and the time between the elution of the β-carotene and retinyl palmitate were too short for reliable wavelength switching. This is why the carotenoids and tocopherols were measured

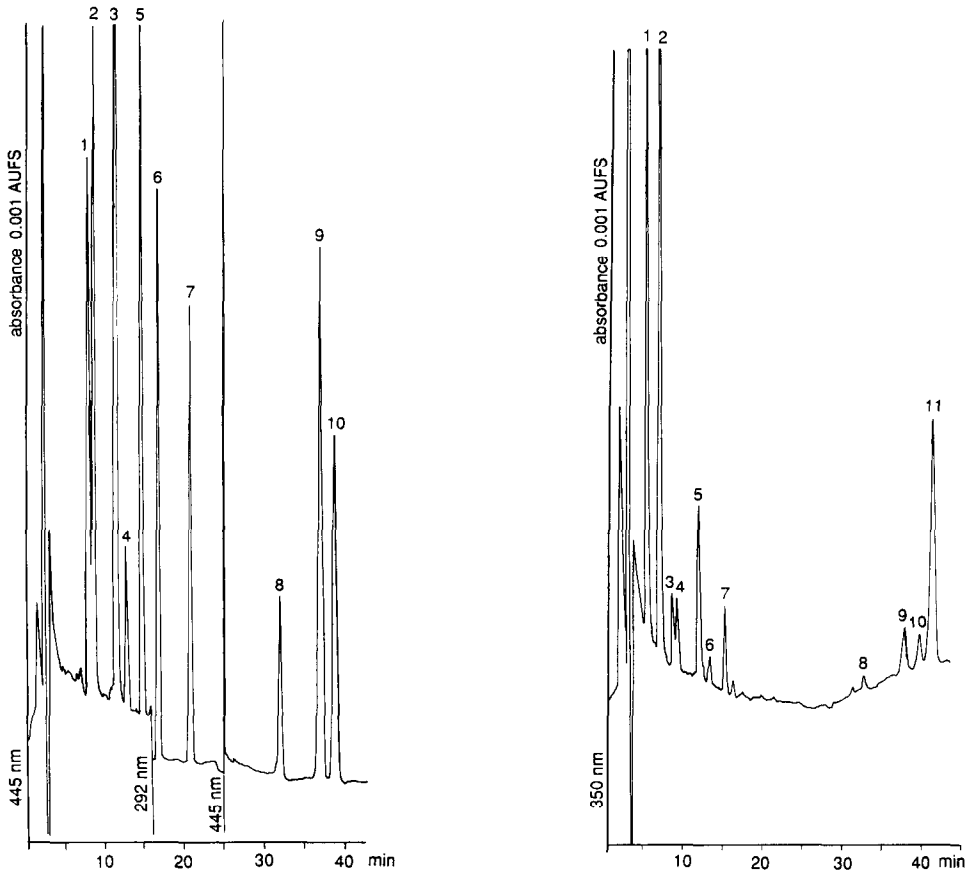

Fig 1. HPLC elution profile of a mixture of reference compounds after injection onto the Hypersil column and detection at a wavelength switching from 445 to 292 nm and back to 445 nm. Peaks: 1 = β-apo-12′-carotenal; 2 = β-apo-10′-carotenal; 3 = β-apo-8′-carotenal; 4 = β-apo-6′-carotenal; 5 = ethyl β-apo-8′-carotenoate; 6 = α-tocopherol; 7 = α-tocopheryl acetate; 8 = γ-carotene; 9 = α-carotene; 10 = β-carotene.

Fig. 2. HPLC elution profile of a mixture of reference compounds after injection onto the Hypersil column and detection at a wavelength of 350 nm. Peaks: 1 = retinol; 2 = retinyl acetate; 3 = β-apo-12′-carotenal; 4 = β-apo-10′-carotenal; 5 = β-apo-8′-carotenal; 6 = β-apo-6′-carotenal; 7 = ethyl β-apo-8′-carotenoate; 8 = γ-carotene; 9 = α-carotene; 10 = β-carotene; 11 = retinyl palmitate.

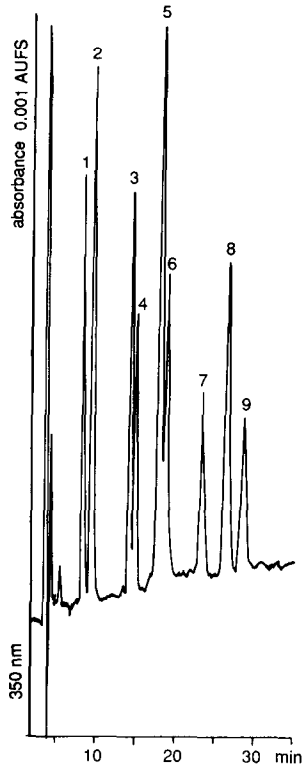

Fig. 3. HPLC elution profile of a mixture of reference compounds after injection onto the Nucleosil column and detection at 350 nm. Peaks: 1 = 13-*cis*-retinoic acid, 2 = all-*trans*-retinoic acid; 3 = 13-*cis*-retinol; 4 = all-*trans*-retinol; 5 = 9- and 13-*cis*-retinal; 6 = all-*trans*-retinal; 7 = etretinate; 8 = 13-*cis*-retinyl acetate; 9 = all-*trans*-retinyl acetate.

with one detector switching from 445 to 292 nm and back to 445 nm (see Fig. 1 for an elution profile), whereas retinol, retinyl acetate and retinyl palmitate were determined using a second detector set at 350 nm (Fig. 2). Fig. 3 shows the results of the separation of a standard retinoid mixture on the Nucleosil column.

The use of BHT as an antioxidant in the extraction procedure resulted in a BHT peak on the Nucleosil column with a retention time of 6 min, which did not disturb the measurements. However, on the Hypersil column BHT eluted after 11 min, and hence interfered with the peaks of β-apo-10′-carotenal and β-apo-8′-carotenal. In a first trial without BHT no negative effects were found, therefore in further studies BHT was not used if the presence of β-apo-carotenals was expected in a sample.

To calculate recoveries, liver samples to which known amounts (comparable to endogenous levels) of β-carotene, retinoic acid, retinol and retinal had been added were analysed in duplicate together with untreated samples. Recoveries, (mean ± S.D.) were 102.5 ± 0.7% for β-carotene, 102.5 ± 10.6% for retinoic acid, 95.5 ± 4.9% for retinol and 95.5 ± 6.4% for retinal.

Assuming that the signal-to-noise ratio should be at least 3, and using an injection volume of 50 μl, the detection limits of the method correspond to a 10 ng/ml

standard solution for retinoids and carotenoids and 60 ng/ml for α-tocopherol and α-tocopheryl acetate.

Using the method described it is possible to measure more compounds than, to the authors' knowledge, are described in the literature, using not more than two systems.

Application to tissue samples

Two groups, each of five female Wistar rats (post-weaning) were fed regular laboratory feed (containing 2 mg/g retinol) supplemented with either 2 mg/g β-carotene or 2 mg/g canthaxanthin (no pro-vitamin A activity). β-Carotene and canthaxanthin were added as beadlets (a gift from Hoffmann-La Roche, Basle, Switzerland). The rats had free access to food and water and consumed around 10 g of food per day.

After 2 weeks the rats were sacrificed. The animals were anaesthesized with diethyl ether, blood was collected by cardiac puncture and whole body perfusion with 0.9% (w/v) NaCl was carried out before the tissues were collected. The tissues were immediately frozen in liquid nitrogen and stored at −80°C. Plasma was separated from the blood and also stored at −80°C.

Retinyl stearate and canthaxanthin were measured using the Hypersil column. Retinyl stearate was detected at 350 nm and eluted about 10 min after retinyl palmitate. Canthaxanthin was detected at 445 nm and eluted at the same time as β-apo-10′-carotenal. As canthaxanthin and β-apo-10′-carotenal were not expected to be present together in the samples, this was no real problem. In fact, by using more water in the eluent, canthaxanthin can be separated from the apocarotenals.

Figs. 4 and 5 show the chromatographic traces of a lung sample analysed on the Hypersil column. In this example the detection was carried out with one detector set at 445 nm and the second switching from 350 to 292 nm, then returning to 350 nm. The Nucleosil column was used to determine retinoic acid.

Table I shows the results of the plasma and tissue analyses. Variable amounts of β-carotene and canthaxanthin were stored in subcutaneous fat in which 1–105 μg/g β-carotene and 2–113 μg/g canthaxanthin were found.

Apocarotenals could not be confirmed in any sample. In liver and lung samples some indications for apocarotenals were found, but these did not exceed the detection limits.

From Table I it can be concluded that the rats were able to convert β-carotene to retinol, as the rats fed β-carotene showed higher levels of retinol in liver, lung and mamma than rats fed canthaxanthin. Furthermore, β-carotene was found in tissues, so the rats were also able to transport unconverted β-carotene to the liver, lung and mamma. Canthaxanthin was also transported to these organs.

Application to 15,15′-dioxygenase assay samples

The proximal 60 cm of the small intestine was removed from male or female Wistar rats and flushed with ice-cold 0.9% NaCl. The mucosa was scraped off in 0.1 *M* potassium phosphate buffer and cytosol was isolated by differential centrifugation. Incubation was carried out as described by Goodman *et al.* [12] with 200 μl of cytosol (4 mg of protein), 0.125 g/l α-tocopherol and 1 μg of β-carotene in 0.1 *M* potassium phosphate buffer, pH 7.7, containing 15 m*M* nicotinamide, 2 m*M* $MgCl_2$, 5 m*M*

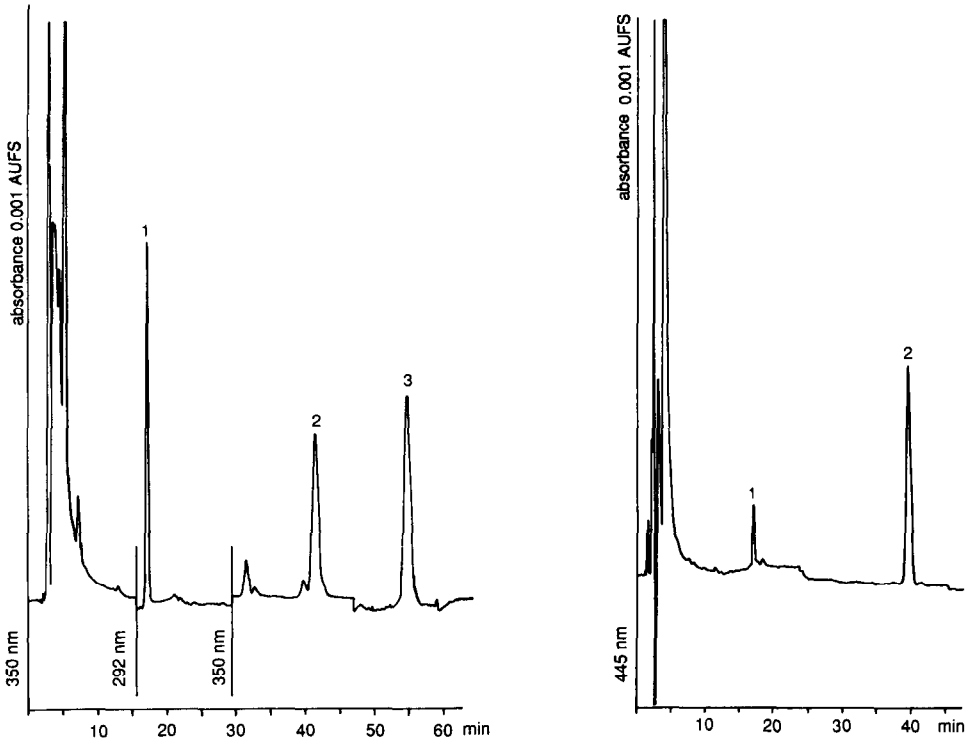

Fig. 4. HPLC elution profile of a lung sample after injection onto the Hypersil column and detection at a wavelength switching from 350 to 292 nm and back to 350 nm. Peaks: 1 = α-tocopherol; 2 = retinyl palmitate; 3 = retinyl stearate.

Fig. 5. HPLC elution profile of a lung sample after injection onto the Hypersil column and detection at a wavelength of 445 nm. Peaks: 1 = α-tocopherol; 2 = β-carotene.

TABLE I

RETINOID AND CAROTENOID CONCENTRATIONS IN PLASMA AND TISSUES OF RATS

Rats had been fed a diet containing either 0.2% β-carotene (group 1) or 0.2% canthaxanthin (group 2) for 2 weeks.

Compound	Plasma (μmol/l)		Liver (μg/g)		Lung (μg/g)		Mamma (μg/g)	
	1	2	1	2	1	2	1	2
Retinol	0.62	0.63	7.8	1.3	3.61	0.67	0.38	0.09
Retinyl palmitate	–	–	395	48	18.9	5.6	0.63	–
Retinyl stearate	–	–	61	8.5	8.15	2.75	–	–
Retinoic acid	–	–	0.09	–	0.03	–	0.05	0.01
β-Carotene	0.33	–	51	0.35	2.10	–	0.09	
α-Carotene	–	–	1.19	0.08	–	–	–	–
Canthaxanthin	–	0.73	–	294	–	6.44	–	2.38
α-Tocopherol	24.4	37.8	61.6	53.0	28.7	37.3	36.7	22.6

glutathione, 1.7 m*M* sodium dodecyl sulphate and 0.2 g/l L-α-phosphatidylcholine. After 1 h incubation with 1 μg of β-carotene, 10–20 ng of retinal were formed. To optimise the measurement of retinal, the wavelength can be switched to 380 nm. No other products could be demonstrated, although only about 80% of the added β-carotene was left. More experiments are in progress to optimise the assay and to find out whether other products are formed and whether the β-carotene is partially lost during the procedure.

From the results obtained, no definite conclusion can be drawn as to the correct β-carotene cleavage theory. To study the β-carotene cleavage further, experiments with intestinal cell lines are currently being undertaken and *in vivo* experiments with rats are being planned.

REFERENCES

1 R. C. Moon, *J. Nutr.*, 119 (1989) 127.
2 R. Peto, R. Doll, J. D. Buckley and M. B. Sporn, *Nature (London)*, 290 (1981) 201.
3 D. L. Hill and C. J. Grubbs, *Anticancer Res.*, 2 (1982) 111.
4 D. S. Goodman, R. Blomstrand, B. Werner, H. S. Huang and T. Shiratori, *J. Clin. Invest.*, 45 (1966) 1615.
5 N. J. Temple and T. K. Basu, *Nutr. Res.*, 8 (1988) 685.
6 J. Ganguly and P. S. Sastry, *World Rev. Nutr. Diet.*, 45 (1985) 198.
7 W. A. MacCrehan and E. Schönberger, *Clin. Chem.*, 33 (1987) 1585.
8 R. K. Aaran and T. Nikkari, *J. Pharm. Biomed. Anal.*, 6 (1988) 853.
9 D. I. Thurnham, E. Smith and P. S. Flora, *Clin. Chem.*, 34 (1988) 377.
10 H. C. Furr, *J. Chromatogr.*, 309 (1984) 299.
11 S. Hansen and W. Maret, *Biochemistry*, 27 (1988) 200.
12 D. S. Goodman, H. S. Huang, M. Kanai and T. Shiratori, *J. Biol. Chem.*, 242 (1967) 3543.
13 R. M. C. Dawson, D. C. Elliott, W. H. Elliott and K. M. Jones, *Data for Biochemical Research*, Clarendon Press, Oxford, 3rd ed., 1986, p. 240.
14 J. Fragner, *Vitamine Chemie und Biochemie*, Part I, Gustav Fisher Verlag, Jena, 1964, Ch. 11, p. 185.
15 O. Isler and P. Schudel, in K. Lang (Editor), *Wissenschaftliche Veröffentlichungen der Deutschen Gesellschaft für Ernährung. 9. Carotine und Carotinoide*, Dietrich Steinkopff Verlag, Darmstadt, 1963, Ch. 3, p. 54.