ELSEVIER

Journal of Chromatography B, 757 (2001) 365–368

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Short communication

Simultaneous quantification of retinol, retinal, and retinoic acid isomers by high-performance liquid chromatography with a simple gradiation

Michiko Miyagi, Hirokazu Yokoyama*, Haruko Shiraishi, Michinaga Matsumoto, Hiromasa Ishii

Department of Internal Medicine, School of Medicine, Keio University, 35 Shinanomachi Shinjukuku, Tokyo 160-8582, Japan

Received 21 August 2000; received in revised form 1 March 2001; accepted 5 March 2001

Abstract

A new method of high-performance liquid chromatography (HPLC) analysis to quantify isomers of retinol, retinal and retinoic acid simultaneously was established. The HPLC system consisted of a silica gel absorption column and a linear gradient with two kinds of solvents containing *n*-Hexane, 2-propanol, and glacial acetic acid in different ratios. It separated six retinoic acid isomers (13-*cis*, 9-*cis*, all-*trans*, all-*trans*-4-oxo, 9-*cis*-4-oxo, 13-*cis*-4-oxo), three retinal isomers (13-*cis*-, 9-*cis*-, and all-*trans*) and two retinol isomers (13-*cis*- and all-*trans*). Human serum samples were subjected to this HPLC analysis and at least, all-*trans* retinol, 13-*cis* retinol, and all-*trans* retinoic acid were detectable. This HPLC system is useful for evaluating retinoic acid formation from retinol via a two-step oxidation pathway. Moreover, it could be applied to monitoring the concentrations of various retinoids, including all-*trans* retinoic acid in human sera.

Keywords: Vitamins; Retinol; Retinal; Retinoic acid

1. Introduction

Retinoids are important factors for normal cell differentiation and proliferation [1]. Various retinoids have geometrical isoforms and the biological action of each isoform is known to differ. It is therefore important to assay each isomer separately to understand the characteristics of retinoids. Recently, Lanvers and co-workers established an HPLC method for simultaneously assaying various retinoids including 13-*cis*-retinoic acid, 9-*cis*-retinoic acid, all-*trans*-retinoic acid, 13-*cis*-retinol, all-*trans*-retinol, all-*trans*-4-oxo-retinoic acid and 13-*cis*-4-oxo-retinoic acid [2]. It is a useful tool to determine the concentrations of these retinoid isoforms, however, retinal isomers have not been subjected to this HPLC analysis.

Retinoic acid is formed from retinol (vitamin A) via a two-step oxidation process. Some isozymes of alcohol dehydrogenase (ADH) have been shown to oxidize retinol (an alcohol) to retinal (an aldehyde), while isozymes of aldehyde dehydrogenase (ALDH) and those of retinal dehydrogenase (RDH) oxidize retinal to retinoic acid [3]. It is therefore important to

*Corresponding author. Tel.: +81-3-3353-1211 (Ext. 62298); fax: +81-3-3353-6247.

E-mail address: yokoyama@mc.med.keio.ac.jp (H. Yokoyama).

0378-4347/01/$ – see front matter
PII: S0378-4347(01)00158-X

assay isomers of retinol, retinal and retinoic acid simultaneously to understand retinoic acid formation via two-step oxidation. Then, we applied Lanvers' method to the evaluation of the two-step oxidation process. However, the method was insufficient for the evaluation since it could not separate all-*trans* retinal and 13-*cis* retinoic acid clearly. The aim of this study was to establish a new method that could fully evaluate the two-step oxidation process by which retinoic acid is formed from retinol. In addition, we applied a new HPLC system to detect various retinoids in human sera.

2. Materials and methods

2.1. Standard retinoids

13-*cis* retinoic acid, all-*trans* retinoic acid, 13-*cis* retinal, 9-*cis* retinal, and all-*trans* retinal and 13-*cis* retinol were purchased from Sigma (St Louis, MO, USA). 9-*cis* retinoic acid (Ro 04-4079), all-*trans* retinol (Ro 01-4955), all-*trans*-4-oxo retinoic acid (Ro 12-4824), 9-*cis*-4-oxo retinoic acid (Ro 47-8078), 13-*cis*-4-oxo retinoic acid (Ro 22-6595), and ethyl-*p*-[(*E*)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)-1-propenyl] phenyl sulfone (Ro 15-1570) were kindly donated by Hoffmann-La Roche (Basel Switzerland).

2.2. HPLC conditions

All experiments were done under light shielded conditions. The Hitachi HPLC system consisted of an auto sampler (AS-4000), a pump (L-6300), an ultra violet (UV) detector (L-4000), and a chromato integrator (D-2500) with a silica gel adsorption column (Inertsil SILICA 100-5; 4.6 mm I.D.x 25 cm, GL-Science Inc., Tokyo Japan) which were used for the experiments. Various retinoids were dissolved in a solvent containing *n*-hexane (Sigma), 2-propanol (Sigma) and glacial acetic acid (Sigma) at a ratio of 1000:4.3: 0.675 (Solvent A). The new HPLC conditions are summarized in Table 1. Samples were separated by a linear gradient with Solvent A and another solvent containing *n*-hexane, 2-propanol and glacial acetic acid at a ratio of 1000: 17.5:0.675 (Solvent B). The injection volume was 50 μl on each run and the separation was done at a flow rate of 1 ml/min for 60 min at 25±1°C and samples were kept at 10°C until analysis. Each retinoid was detected by a UV detector at a wavelength of 350 nm.

Table 1
New HPLC conditions to separate various retinoids simultaneously

Time	Solvent A[a] (%)	Solvent B[b] (%)
0.0	100	0
15.0	100	0
25.0	0	100
35.0	0	100
45.0	100	0
59.0	100	0

Flow-rate 1.0 ml/min.
[a] Solvent A *n*-Hexane: Iso propanol:Acetic acid=1000:43: 0.675.
[b] Solvent B *n*-Hexane: Iso propanol:Acetic acid=1000:17.5: 0.675.

2.3. Quantification of retinoids

Retinoids of various concentrations were subjected to HPLC analysis. The relationship between each retinoid concentration and altitude of area under the curve of each peak was examined by linear least-squares regression analysis.

2.4. Detection of retinoids in human sera

Blood specimens were collected from the authors of this report. Each specimen was centrifuged at 3000 *g* for 5 min and 3.5 ml of serum fraction was collected. To the fraction, 3.5 ml of ethanol (Sigma), 1.5 ml of 2 *M* sodium hydroxide (Sigma) and 0.4 ml of 50 m*M* of ethyl-*p*-[(*E*)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)-1-propenyl] phenyl sulfone (arotenoid) as an internal standard, and 4 ml of *n*-hexane were added. After shaking for 10 min, the mixture was centrifuged at 200 *g* for 5 min. Three ml of the upper layer fraction (organic layer; Fraction A) and 3 ml of the lower layer fraction (water layer) were collected separately. To the water layer fraction, 3 ml of 2 *M* hydrochloric acid (Sigma) and 4 ml of *n*-hexane were added. After shaking for 10 min, the mixture was centrifuged at 200 *g* for 5 min and 3 ml of the top layer fraction

(organic layer; Fraction B) was collected. Organic solvents were evaporated from Fractions A and B with nitrogen gas and each residue was re-dissolved in 200 μl of *n*-hexane and subjected to HPLC analysis.

2.5. Statistical analysis

Values are expressed by mean±SD. Scattering of each parameter was assessed by coefficient of variation analysis.

3. Results and discussion

Recently, our interest has focused on retinoic acid formation from retinol via a two-step oxidation process. In the process, retinol is oxidized to retinal, and in turn, retinal is oxidized to retinoic acid. To understand the process, each isomer of retinol, retinal and retinoic acid should be simultaneously quantified. Recently, Lanvers and co-workers reported an HPLC method to separate various retinoid isomers simultaneously [2]. We applied this method to the evaluation of the two-step oxidation process. However, the conditions were insufficient for the evaluation since we could not separate all-*trans* retinal and 13-*cis* retinoic acids clearly. Thus, the HPLC conditions were improved.

To do this, the concentration of isopropanol in the mobile phase and gradient conditions were modified. However, these changes resulted in failure. Then, since increasing the specific surface area of the column is known to improve the separation capacity, the specific surface area of the column was increased from 350 m^2/g appeared in Lanvers' conditions to 450 m^2/g. This improvement enabled us to separate all-*trans* retinal and 13-*cis* retinoic acid without spoiling the other separations. Moreover, we found that the new column enabled us to separate all retinoids studied at this time by a simple gradient, which was different from the rather complicated multi step gradient in Lanvers's method.

As shown in Fig. 1, the new conditions enabled separation of 13-*cis* retinal (1), 9-*cis* retinal (2), all-*trans* retinal (3), 13-*cis* retinoic acid (4), 9-*cis* retinoic acid (5), all-*trans* retinoic acid (6), 13-*cis* retinol (7), all-*trans* retinol (8), all-*trans*-4-oxo retinoic acid (9), 9-*cis*-4-oxo retinoic acid (10), 13-*cis*-4-oxo retinoic acid (11) and Ro 15-1570 (I.S.). This HPLC assay will be useful to assess the two step oxidation process in which retinoic acid is produced from retinol.

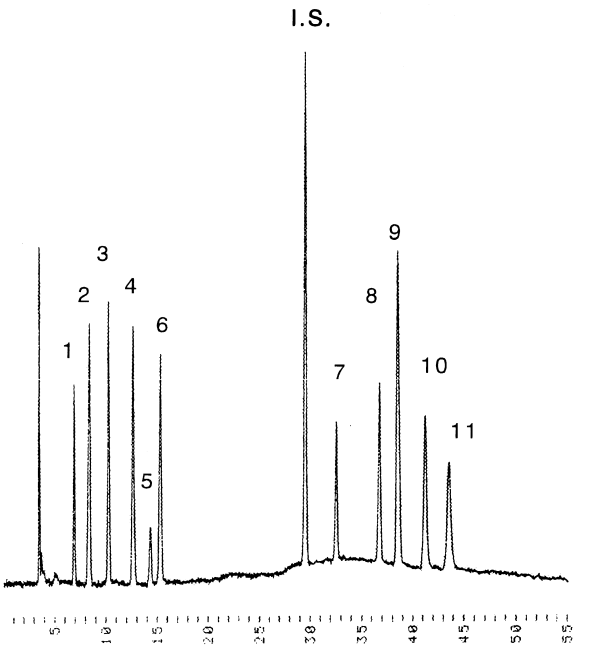

Fig. 1. Separation of various retinoids by HPLC. The newly established HPLC conditions enabled separation of 13-*cis*-retinal (1), 9-*cis* retinal (2), all-*trans* retinal (3), 13-*cis* retinoic acid (4), 9-*cis*-retinoic acid (5), all-*trans*-retinoic acid (6), 13-*cis*-retinol (7), all-*trans*-retinol (8), all-*trans*-4-oxo retinoic acid (9), 9-*cis*-4-oxo-retinoic acid (10), 13-*cis*-4-oxo-retinoic acid (11) and Ro 15-1570 (I.S.).

The retention time of each retinoid was fully reproducible on a within-day basis analysis. The coefficients of variation were from 0.11 to 1.08%. However, the variation was rather large on a day-to-day basis analysis with coefficient of variation was from 0.80 to 5.74%. Especially, those of 13-*cis* retinoic acid, 9-*cis* retinoic acid, and all-*trans* retinoic acid were large (5.74, 5.46 and 4.96%, respectively). To correct this error, authentic retinoids must be analyzed at least once per day to determining the daily retention time of each retinoid. Although we do not know the exact reason for such variation, we believe that it is at least in part caused by changes in the column conditions. Indeed, the sharp retinoic acid peaks degenerated into broad peaks after

repeated analyses and rinsing the column with ethanol was required to revive it.

The correlation between concentration and altitude of area under the peak curve was studied in each retinoid. Least-squares regression analysis demonstrated that this HPLC analysis could quantify retinoic acid from 2.5 to 600 ng/ml, retinal from 2.5 to 1000 ng/ml, or retinol from 5 to 1000 ng/ml. The quantification of retinol, retinal, and retinoic acid were fully reproducible on both within-day and day-to-day basis analyses if samples were kept at − 20°C under light shielded conditions.

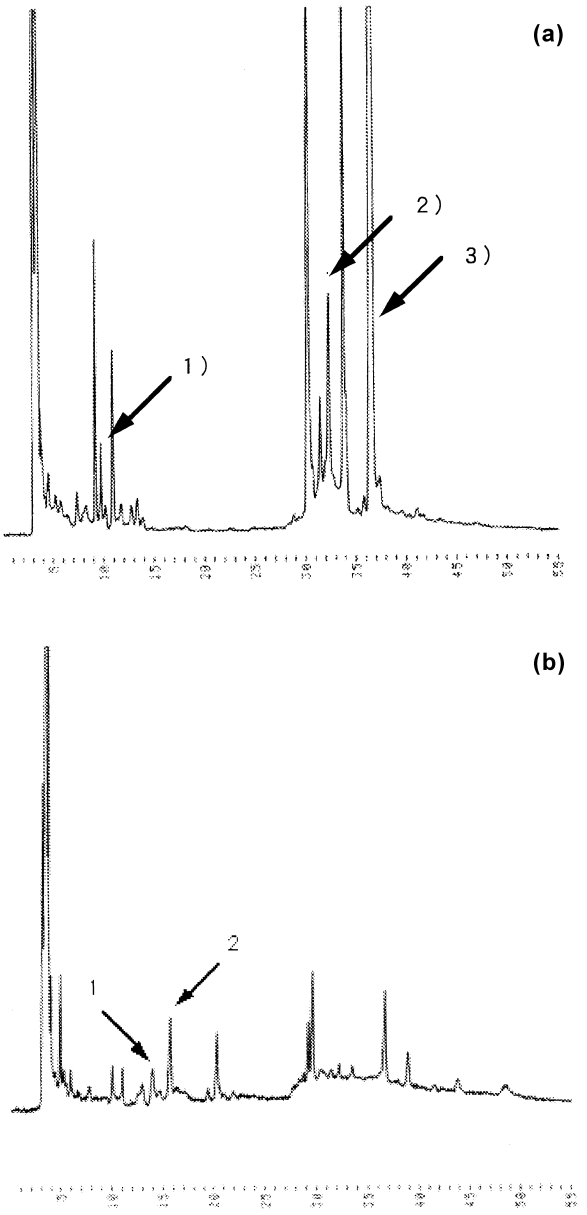

Fig. 2. (a) Detection of retinoids in sera (1). All-*trans* retinal (1), 13-*cis* retinol (2), and all-*trans* retinol (3) were detectable in Fraction A. (b) Detection of retinoids in sera (2). 13-*cis* retinoic acid (1) and all-*trans* retinoic acid (2) were detectable in Fraction B, whereas 9-*cis* retinoic acid could not be found in this condition.

Then, we examined whether the new HPLC conditions were suitable to quantify various retinoids in human sera. Retinol and retinal were extracted from human sera by *n*-hexane (Fraction A), whereas retinoic acid was, at first, collected into the water layer under alkaline conditions, then extracted by *n*-hexane after neutralization (Fraction B). As shown in Fig. 2a, at least, all-*trans* retinal, 13 *cis*-retinol, and all-*trans* retinol were detectable in Fraction A. Moreover, as shown in Fig. 2b, all-*trans* retinoic acid and 13-*cis* retinoic acid were detectable in Fraction B, whereas 9-*cis* retinoic acid could not be found in this condition. All-*trans* retinoic acid is used for treatment of a particular leukemia [4], however, it sometimes produces lethal events known as all-*trans* retinoic acid syndrome [5]. Thus, it is important to monitor all-*trans* retinoic acid levels in human sera to avoid its adverse effects. This HPLC assay will be a useful tool to prevent all-*trans* retinoic acid syndrome in clinical settings.

Acknowledgements

The authors thank Hoffmann-La Roche for kindly donating various retinoids.

References

[1] C. Hofmann, C. Thaller, T. Melia, G. Eichele, in: M.A. Livrea, G. Vidali (Eds.), Retinoids: From Basic Science To Clinical Applications, Birkhauser Verlag, Boston, 1994.
[2] C. Lanvers, G. Hempel, G. Blaschke, J. Boos, J. Chromatogr. B Biomed. Appl. 685 (1996) 233.
[3] G. Duester, Biochemistry 35 (1996) 12221.
[4] S. Castaigne, C. Chomienne, M.T. Daniel, P. Ballerini, R. Berger, P. Fenaux, L. Degos, Blood 76 (1990) 1704.
[5] S.R. Frankel, A. Eardley, G. Lauweres, M. Weiss, R.R. Warrell Jr, Ann. Intern. Med. 117 (1992) 292.