Journal of Chromatography, 341 (1985) 187—192
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2550

Note

Simple high-performance liquid chromatographic method for the separation of retinoids including N-(4-hydroxyphenyl)-all-*trans*-retinamide

THERESA A. HULTIN*, RAJENDRA G. MEHTA and RICHARD C. MOON

Laboratory of Pathophysiology, IIT Research Institute, Chicago, IL 60616 (U.S.A.)

(First received September 12th, 1984; revised manuscript received January 14th, 1985)

Many retinoids, both natural and synthetic, have a demonstrated prophylactic and in some cases therapeutic effect in a variety of chemically induced epithelial cancers in experimental animals [1, 2]. Earlier studies from our laboratory have provided evidence that the inhibition of carcinogenesis by retinoids is target organ specific, for example, 13-*cis*-retinoic acid is effective in suppressing urinary bladder carcinogenesis but ineffective against mammary cancers. Similarly, retinyl acetate can effectively inhibit mammary carcinogenesis but has little effect against two-stage skin tumorigenesis [2]. Among the retinoids tested against mammary and urinary bladder carcinogenesis, N-(4-hydroxyphenyl)retinamide (4-HPR) appears to be the most promising in terms of its effectiveness relative to toxicity [3]. The usefulness of a retinoid for protection against carcinogenesis depends not only on the lack of toxicity but also upon the tissue distribution, blood level and metabolism of the compound. Essential information on these pharmacokinetic parameters with respect to the breast and urinary bladder is lacking. Although Swanson et al. [4] have examined the pharmacokinetics of 4-HPR in male rats, such studies in the female rat have not been performed. Since 4-HPR is an effective inhibitor of breast cancer, it is of critical importance to study the distribution and metabolism of 4-HPR in female rats and mice.

Several methods exist for the high-performance liquid chromatographic (HPLC) separation of retinoids, however the majority of these procedures involve complex mobile phases and flow-rate changes [5—7]. Swanson and co-workers [4, 8] reported two separate HPLC systems to analyze the processing of 4-HPR in tissues. However, the first method failed to separate retinol from the 4-HPR metabolites [4] whereas in the second procedure, separation of polar metabolites of 4-HPR could not be achieved [8]. In this

0378-4347/85/$03.30

study, we describe a simple, sensitive HPLC method for the separation and quantitation of 4-HPR and its potential metabolites, as well as the separation of these compounds from retinol, retinyl acetate, and retinyl palmitate.

MATERIALS AND METHODS

All manipulations were carried out under yellow light or with the use of amber glassware or aluminum foil. All the solvents used for extraction and chromatography were chromatoquality grade and were purchased from E.K. Industries (Addison, IL, U.S.A.).

HPLC instrument and conditions

A Spectra Physics Model 8700 high-pressure liquid chromatograph equipped with a Model 8440 variable-wavelength detector set at 350 nm was used. This wavelength was intermediate between the maximum absorption wavelengths of several of the retinoids of interest. Peak areas were calculated with a Model 4100 computing integrator.

Separations were performed on a 250 mm × 4.6 mm I.D., 10-μm, bonded octodecylsilane, reversed-phase column (Partisil 10 ODS-2; Whatman, Clifton, NJ, U.S.A.). A 70 mm × 2.1 mm I.D. guard column containing Co:Pell ODS was positioned between the injector and the analytical HPLC column.

The column was eluted with a 30-min linear gradient of methanol—water (70:30) (pH ≈ 6) to 100% methanol (pH ≈ 7) at a flow-rate of 1.2 ml/min. Chromatography was continued at the final conditions for 40 min.

Analytical standards

All-*trans*-4-HPR, 13-*cis*-4-HPR, and all-*trans*-4-ethoxyphenylretinamide (4-EPR) were obtained from Southern Research Institute (Birmingham, AL, U.S.A.) through the National Cancer Institute; all-*trans*-retinoic acid, all-*trans*-retinol, and all-*trans*-retinyl palmitate from Sigma (St. Louis, MO, U.S.A.); all-*trans*-retinyl acetate and 13-*cis*-retinoic acid from BASF (Ludwigshafen, F.R.G.); and all-*trans*-4-methoxyphenylretinamide (4-MPR) from McNeil Pharmaceuticals (Springhouse, PA, U.S.A.). All-*trans*-5,6-epoxyretinoic acid was generously provided by Drs. Andre LaCroix and Pangala Bhat (Clinical Research Institute of Montreal, Montreal, Canada). All standards were stored under argon in amber vials at − 20 °C.

A solution of all standards was made by dissolving 2 mg of each dry compound and 10 μl retinyl palmitate in 200 ml of methanol. Of the standard solution 1 ml was concentrated to 0.5 ml by evaporation under nitrogen and 10 μl (200 ng of each standard) were injected onto the HPLC column.

Treatment of animals

Female Sprague—Dawley rats, 55—60 days of age, were injected intraperitoneally with 5 mg/kg 4-HPR per day for five days. Control rats received dimethyl sulfoxide (DMSO) vehicle alone. Rats were sacrificed 6—8 h after the last 4-HPR injection at the end of day 5. Liver, mammary gland, urinary bladder, and serum were collected, frozen under liquid nitrogen and stored at − 80 °C.

Extraction of retinoids

Tissues were lyophilized overnight, ground to a fine powder or cut into small pieces, and added to 100 x 16 mm glass-stoppered tubes. Retinyl acetate (80 μg) was added as an internal standard to control for end volume differences and potential spillage. The tissues were extracted twice with several volumes (6—10) of chloroform—methanol (2:1) by rotating on a Labquake shaker (Labindustries, Berkeley, CA, U.S.A.) for 1 h each. The samples were centrifuged at 800 *g* for 10 min, the liquid was transferred into amber vials and evaported immediately under a gentle stream of nitrogen. The residue was redissolved in 0.5 ml methanol (concentrated to 0.1 ml for mammary gland and urinary bladder) and a 10-μl aliquot was analyzed.

In order to calculate the concentration of 4-HPR in the samples, known amounts of 4-HPR were injected into isolated tissues from untreated rats prior to lyophilization. Retinyl acetate was added to dry tissue as the internal standard. Peak area ratios of 4-HPR relative to retinyl acetate were calculated, and a linear peak area versus 4-HPR concentration curve was constructed. This curve was linear from approximately 200 ng to 20 μg 4-HPR. However, below this level, peak height ratios were used in the construction of the standard curve. This curve was linear down to 10 ng 4-HPR. Amounts of 4-HPR in biological samples were quantitated by reference to the standard curves.

RESULTS AND DISCUSSION

Although several systems exist for the separation of retinoids, they involve complex assay conditions. In addition, suitable systems for the separation of

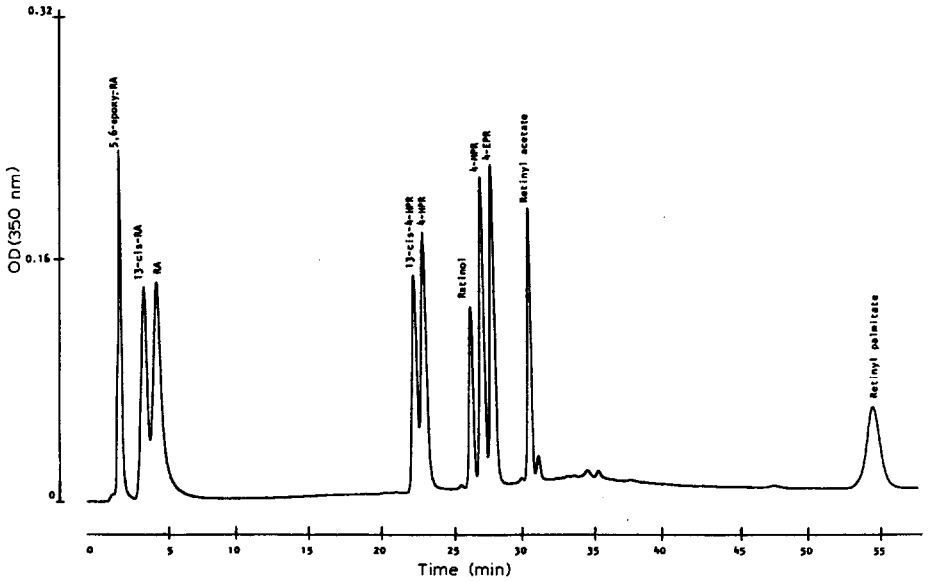

Fig. 1. HPLC separation of a mixture of retinoid standards. Retinoids were eluted from a Partisil 10 ODS-2 reversed-phase column with a 30-min linear gradient of methanol—water (70:30) to 100% methanol at a flow-rate of 1.2 ml/min. Chromatography was continued at the final conditions for 40 min. Each peak represents 200 ng of retinoid standard. RA = retinoic acid.

4-HPR and its metabolites are unavailable. This study was undertaken to develop a simple, sensitive method for the separation of 4-HPR, its possible metabolites, and the endogenous retinoids.

The separation of a mixture of ten retinoid standards is shown in Fig. 1. The retinoids were all clearly separable and eluted with retention times shown in Table I. The detection limit for retinoids using this procedure was approximately 5—10 ng. The signal-to-noise ratio at this level for 4-HPR was approximately 2—2.5, and the intra- and inter-assay variability was less than 5%.

TABLE I

HPLC ELUTION OF RETINOID STANDARDS

Retinoid	Retention time (min)
5,6-Epoxyretinoic acid	2.3
13-*cis*-Retinoic acid	4.0
All-*trans*-retinoic acid	4.9
13-*cis*-4-HPR	22.7
All-*trans*-4-HPR	23.4
Retinol	26.6
4-MPR	27.4
4-EPR	28.1
Retinyl acetate	30.7
Retinyl palmitate	54.6

Previous investigators have concentrated their efforts on separating only 4-HPR and/or on separating 4-HPR esters which are more hydrophobic than 4-HPR. The conditions used in those studies did not distinguish potential polar metabolites of 4-HPR such as retinoic acid [3, 4, 8]. Using the conditions described here retinoic acid and 4-HPR separated by about 18 min. This allows one to evaluate the polar metabolites of 4-HPR, if any, in the tissue. Moreover, all the phenyl retinamides tested separated very well under the conditions described here (Fig. 1).

Retention times for most of the retinoids were constant. However, all-*trans*-retinoic acid and 13-*cis*-retinoic acid showed variability (up to 1.5 min) in their elution profiles. Nevertheless, they were always separable from each other and clearly distinguishable from neighboring peaks. If necessary, a better separation of the two compounds with longer, more constant retention times could be accomplished either by increasing the amount of water or by including ammonium acetate, or another appropriate buffer, in the mobile phase [6].

Three small peaks were visible in the profile of standards (Fig. 1). One peak eluted immediately after retinyl acetate and appeared to be a minor contaminant in the retinyl acetate standard. In addition, two small peaks eluted approximately 4 min after retinyl acetate and corresponded with the end of the gradient and the beginning of 100% methanol mobile phase. These two peaks appeared to be due to a change in the percentage of organic modifier and were visible in every HPLC assay carried out using this gradient procedure.

The separation of retinoids extracted from the liver of 4-HPR-treated female rats is shown in Fig. 2. Liver was used as the example to demonstrate the separation in the presence of large amounts of endogenous retinoids found in

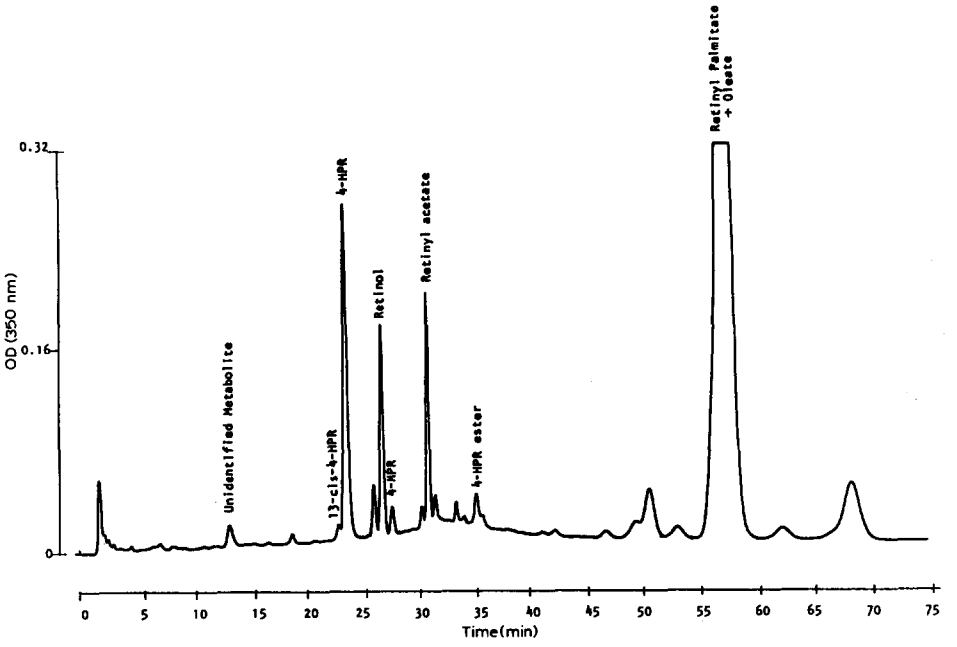

Fig. 2. Liver extract from 4-HPR-treated female rats. Rats were injected intraperitoneally with 5 mg/kg 4-HPR per day for five days and sacrificed on day 5, 6—8 h after the last injection. Liver was lyophilized, extracted with chloroform—methanol (2:1), and analyzed by HPLC as described in Fig. 1.

this tissue. Again, all retinoids were separable and exhibited a similar elution profile and retention times as shown in Fig. 1 and Table I. Compounds which coeluted with retinol and retinyl palmitate were extracted from the liver. In addition, several other peaks which appeared to be retinyl esters eluted with 100% methanol. Unfortunately, 100% methanol does not separate retinyl palmitate from retinyl oleate, but Bhat and LaCroix [9] have shown that separation on a 5-μm ODS column could be achieved with methanol—water (98:2). A 4-HPR peak was readily detectable as were peaks corresponding to 4-MPR, 13-*cis*-4-HPR, and a 4-HPR ester, all probable metabolites of 4-HPR. A more polar compound which peaked at 13.6 min, and which was not present in vehicle-treated animals was considered to be an unidentified metabolite of 4-HPR. All other peaks, including retinyl acetate which was added as the internal standard, were present in vehicle-treated animals.

In summary, we have developed a simple, sensitive method which separated 4-HPR from its metabolites, from retinol and retinyl palmitate, and from the internal standard, retinyl acetate. Currently, this procedure is being utilized successfully in our laboratory to study pharmacokinetics of 4-HPR in several tissues including normal and cancerous breast and urinary bladder samples [10].

REFERENCES

1 M.B. Sporn, N.M. Dunlop, D.L. Newton and J.M. Smith, Fed. Proc., Fed. Amer. Soc. Exp. Biol., 35 (1976) 1332.
2 R.C. Moon, D.L. McCormick and R.G. Mehta, Cancer Res., 43 (1983) 2469s.

3 R.C. Moon, H.J. Thompson, P.J. Becci, C.J. Grubbs, R.J. Gander, D.L. Newton, J.M. Smith, S.L. Phillips, W.R. Henderson, L.T. Mullen, C.C. Brown and M.B. Sporn, Cancer Res., 39 (1979) 1339.
4 B.N. Swanson, D.W. Zaharevitz and M.B. Sporn, Drug Metab. Dispos., 8 (1980) 168.
5 P.V. Bhat and A. LaCroix, Biochim. Biophys. Acta, 752 (1983) 451.
6 C.A. Frolik, T.E. Tavela and M.B. Sporn, J. Lipid Res., 19 (1978) 32.
7 T. Annesley, D. Giacherio and K. Wilkerson, J. Chromatogr., 305 (1984) 199.
8 B.N. Swanson, D.L. Newton, P.P. Roller and M.B. Sporn, J. Pharmacol. Exp. Ther., 219 (1981) 632.
9 P.V. Bhat and A. LaCroix, J. Chromatogr., 272 (1983) 269.
10 T.A. Hultin, R.G. Mehta and R.C. Moon, unpublished results.