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NOTES

Improved and Rapid High-Performance Liquid Chromatographic Assay for 13-*cis*-Retinoic Acid or All-*trans*-retinoic Acid

RICKEY SHELLEY, J. C. PRICE ˣ, H. WON JUN, D. E. CADWALLADER, and A. C. CAPOMACCHIA

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Abstract □ A rapid, specific, and sensitive reversed-phase high-performance liquid chromatographic (HPLC) assay for the quantitative determination of all-*trans*- retinoic acid (I) or 13-*cis*-retinoic acid (II) in rat serum without extraction or lyophilization is described. Chromatographic separation from retinol, serum components, and retinol acetate standard was achieved on octadecylsilane-coated particles with acetonitrile–1% ammonium acetate as the eluent. Serum samples (100 μl) containing as little as 10 ng of retinoid were analyzed. Serum level profiles of rats dosed with the retinoids demonstrated the utility of the assay and indicated elimination half-lives of 0.58 and 0.92 hr for I and II, respectively.

Keyphrases □ High-performance liquid chromatography—assay for 13-*cis*-retinoic acid and all-*trans*-retinoic acid □ 13-*cis*-Retinoic acid—high-performance liquid chromatographic analysis □ All-*trans*-retinoic acid—high-performance liquid chromatographic analysis

High-performance liquid chromatographic (HPLC) assay procedures for all-*trans*-retinoic acid (I) or 13-*cis*-retinoic acid (II) in serum samples differ markedly in sample preparation. One previously reported method employed lyophilization followed by extraction with methanol before analysis by reversed-phase chromatography. By this method as little as 50 ng of retinoic acid was detected in 0.5 ml of human plasma samples (1). Another report (2) showed a sensitivity limit of 25 ng/ml of plasma with reversed-phase chromatography after extraction of samples containing 1 ml of plasma with a mixture of hexane, methylene chloride, and isopropanol. In another study (3), 0.5 ml of serum was extracted with ethyl acetate, followed by evaporation and dissolution of the residue in mobile phase. Normal-phase adsorption chromatography was employed to achieve a sensitivity of 10–20 ng/ml of serum. A fourth method (4) required no extraction step and had a detection limit of 100 ng of retinoic acid when 400 μl of serum was mixed with methanol and centrifuged for 20 min and the supernatant liquid was analyzed directly by reversed-phase chromatography.

The present study describes a simple timesaving method for determining serum I or II by reversed-phase liquid chromatography with no extraction or lyophilization steps. Only 100 μl of serum is required, and many samples can be analyzed in a short time. Sensitivity compares favorably with other reported assay procedures.

EXPERIMENTAL

Reagents—All-*trans*-retinoic acid[1] (I), 13-*cis*-retinoic acid[2] (II), and all-*trans*-retinol acetate[3] (III) were used as received. All other chemicals

[1] Eastman Kodak, Rochester, N.Y.
[2] Hoffmann-La Roche, Nutley, N.J.
[3] Sigma Chemical Co., St. Louis, Mo.

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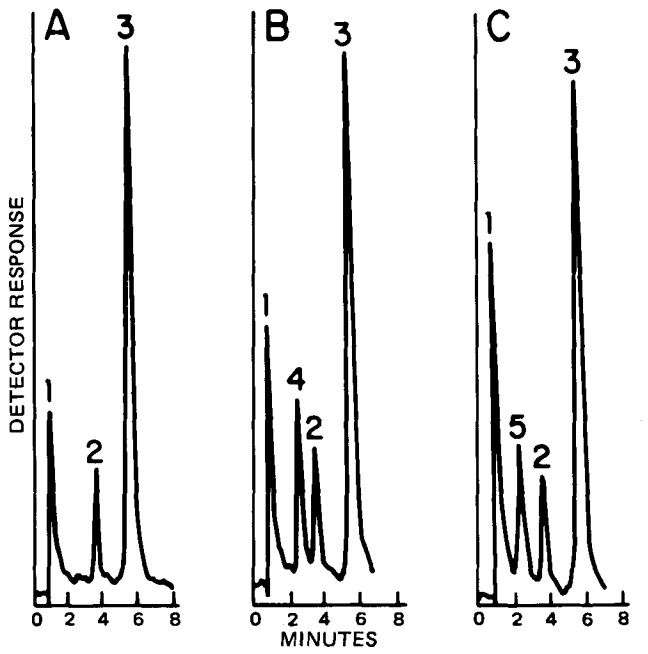

Figure 1—*Chromatograms of: A, normal rat serum with added internal standard; B, rat serum containing 15 ng of I plus internal standard; and C, rat serum containing 15 ng of II plus internal standard. Key: 1, solvent; 2, retinol (present in all serum samples); 3, internal standard; 4, I; and 5, II.*

and reagents were high grade, commercially available materials.

Chromatography—Analyses were performed on a liquid chromatograph[4] operated at ambient temperature and equipped with a UV detector (340 nm). Separations were performed on a 3.2-mm × 25-cm reversed-phase column containing 5-μm octadecylsilane-coated particles[5]. A guard column, containing 30–40 μm octadecylsilane-coated pelicular material, was employed to protect the main column. Samples were introduced into the column through a 100-μl sample loop by way of a loop filler port and sample injection valve. Chromatograms were traced on a strip-chart recorder (20 cm/hr), and peak heights were determined by an integrator–peak detector.[6] The mobile phase was acetonitrile–1% ammonium acetate solution (75:25 v/v).

Calibration—Stock solutions (0.1 mg/ml) of I and II were prepared in acetonitrile and stored at −10°. In addition, an internal standard stock solution (10 μg/ml) of III was prepared. Periodic chromatographic and spectrophotometric analyses showed that these stock solutions could be stored without alteration for 1 month at −10°. Similar stability was reported previously (5).

Blood from untreated male white rats[7] was collected from the tails (6) in serum separation tubes and centrifuged to generate a serum pool. The serum was immediately transferred to 15-ml screw-capped vials and frozen. Known amounts of I stock solutions and 50 μl of the internal standard solution were added to 100 μl of drug-free serum samples in 15-ml screw-capped test tubes.

Each sample was further diluted with acetonitrile to a final volume of 400 μl to achieve I concentrations of 0.1–7.5 μg/ml (10–750 ng/100-μl sample) and a constant III concentration of 5.0 μg/ml. Samples of II and the internal standard were obtained in the same manner. The samples were shaken vigorously on a vortex mixer, and the precipitate was separated by centrifugation (2 min at 3500 rpm). The resulting supernatant liquid was injected onto the chromatograph for analysis. In a separate calibration, water was substituted for serum in essentially the same procedure to determine whether the serum had significantly affected the assay.

Calibration curves of the peak height ratios (retinoic acid to internal standard) *versus* the retinoid concentration were prepared from the analysis of the serum and water samples at each concentration of I and II.

Serum Level Profiles—Serum concentrations of the retinoids of the male rats, weighing an average of 390 g, were determined after intravenous injection into the tail vein using the described method. All rats were fasted for 24 hr before injection. Injected retinoids were in saline solution. For I, six rats were given a bolus intravenous injection of 1.0 mg; blood samples were taken from each rat at 0.5, 1, 2, 3, 4, and 5 hr after injection. For II, eight rats received 1.25 mg iv; blood samples were taken at 0.5, 1, 1.75, 2.5, 3.25, 4, and 4.75 hr after injection. Samples were taken and analyzed as described for the calibration experiments.

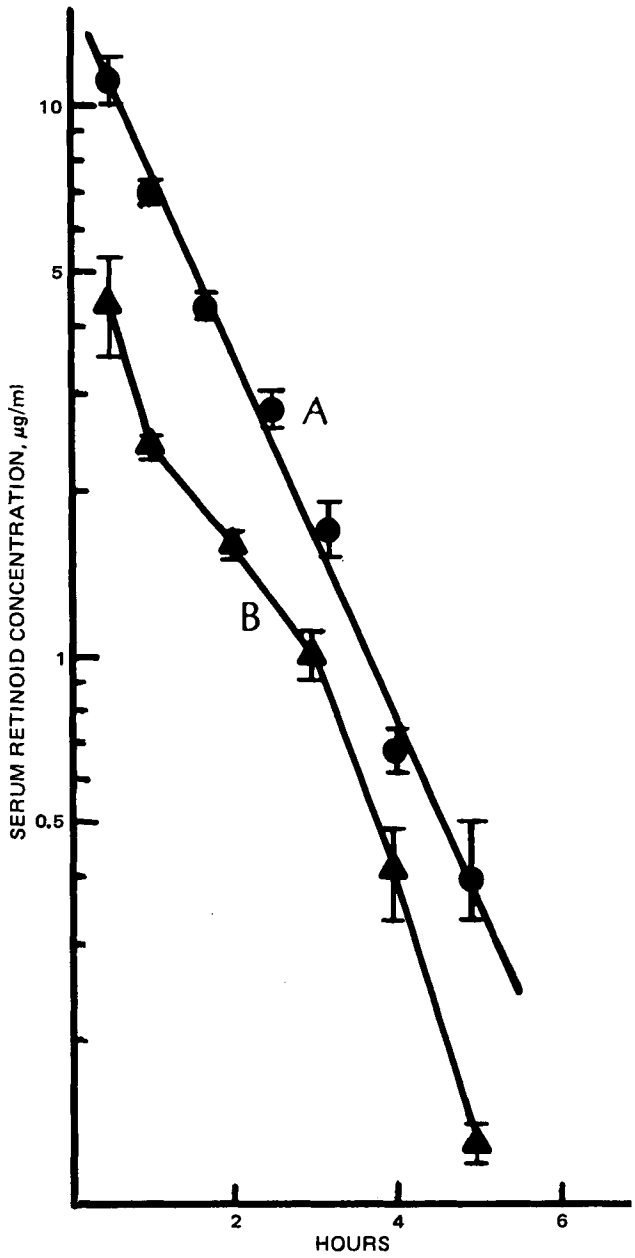

Figure 2—*Average serum concentrations of II (A) and I (B) following rapid intravenous injections of 1.25 and 1.0 mg, respectively. Each point represents data averaged from at least six rats. Bars show the standard deviation of the data.*

RESULTS AND DISCUSSION

Chromatograms of serum containing the internal standard and the internal standard plus I or II are shown in Fig. 1. All serum chromatograms showed the presence of retinol, which did not interfere with the assay. No other interfering peaks were observed.

Separation of all I from the internal standard, retinol (present in all serum samples), and the solvent front was obtained after adjusting the mobile phase of acetonitrile–1% ammonium acetate to a ratio of 75:25 (v/v) and the flow rate to 1.6 ml/min. The 1% ammonium acetate aqueous solution was needed to obtain complete separation of I from the solvent

[4] Altex model 2100 pump with model 153 UV detector, Altex Scientific, Berkeley, Calif.
[5] Spherisorb ODS, Phase Separations Ltd., Hauppauge, N.Y.
[6] Minigrator, Spectra-Physics, Santa Clara, Calif.
[7] Sprague-Dawley strain.

Table I—Retinoic Acid Assay Calibration

Number of Samples[a]	Retinoid[b] in Sample, ng	Retinoid Detected,[c] ng	Sample Concentration, ng/ml	Peak Height Ratio ± *SD* Retinoic Acid I	Retinoic Acid II
9	10	2.5	100	0.045 ± 0.015	0.055 ± 0.02
3	20	5.0	200	0.077 ± 0.002	—
9	40	10	400	0.143 ± 0.010	0.157 ± 0.02
3	60	15	600	0.217 ± 0.002	—
9	80	20	800	0.297 ± 0.016	0.308 ± 0.038
3	100	25	1000	0.375 ± 0.007	—
9	150	37.5	1500	0.601 ± 0.016	0.581 ± 0.037
3	300	75	3000	1.231 ± 0.037	—
9	450	112.5	4500	1.821 ± 0.048	1.594 ± 0.088
3	600	150	6000	2.431 ± 0.061	—
9	750	187.5	7500	3.021 ± 0.098	2.678 ± 0.103

[a] Samples of nine were determined over 3 weeks, three samples per week. [b] Contained in 0.1 ml of rat serum. [c] Detected quantity represents the calculated theoretical quantity of retinoid injected into the chromatograph.

front (Fig. 1B). Under these conditions, the retention times for I, retinol, and III were 3.0, 3.9, and 5.9 min, respectively. In addition to the spiked serum samples, a drug-free sample was run to determine if there were any absorbing substances in the region of I (Fig. 1A). Separation of II from the internal standard, retinol, and the solvent front was achieved under the same conditions as for I (Fig. 1C). The retention time for II was 2.6 min.

The results of calibration procedures for I and II in rat serum samples are shown in Table I. Linear regression analysis of chromatogram peak height ratios of sample to standard *versus* concentration showed correlation coefficients of 0.9999 for both I and II for a sample size of 10–750 ng (100–7500 ng/ml). At a sample size of 10 ng of retinoic acid, the standard deviations for data pooled from 3 different days were ±33% for I and ±36% for II and probably represent the lower working limits for the assay. At the 80-ng sample size, reproducibility was much better, with standard deviations of 7 and 13%, respectively, for the two acids. Single-day determinations usually showed lower standard deviations.

The peak height ratios of retinoids to the internal standard at each concentration for the serum and water samples were compared to determine the percent recovery of I and II from the serum samples. The recoveries of I and II were 102.9 ± 5.6% and 91.7 ± 5%, respectively.

Figure 2 shows the applicability of the assay to monitor serum levels in rats after single doses of the retinoids. Both serum level profiles showed log-linear elimination phases with correlation coefficients of ~0.99. The elimination half-lives of I and II were 0.58 and 0.92 hr, respectively. The elimination curve for I, possibly indicating saturation or storage, tends to confirm previous observations (7).

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Quantitative Analysis of Ethynodiol Diacetate and Ethinyl Estradiol/Mestranol in Oral Contraceptive Tablets by High-Performance Liquid Chromatography

G. CARIGNAN, B. A. LODGE ˣ, and W. SKAKUM

Received August 6, 1980, from the *Bureau of Drug Research, Health Protection Branch, Health and Welfare Canada, Tunney's Pasture, Ottawa, Ontario, K1A 0L2, Canada.* Accepted for publication July 7, 1981.

Abstract □ A procedure is described for the assay of ethynodiol diacetate and ethinyl estradiol/mestranol by HPLC using two UV detectors at 210 and 280 nm. The system was acetonitrile 38% (v/v) in water as mobile phase on a 250 × 3.2-mm i.d. RP-2 column, with butylated hydroxytoluene as the internal standard. There was >99% recovery from synthetic preparations and the coefficient of variation was <2.0% for formulations.

Keyphrases □ Oral contraceptives—quantitative analysis of ethynodiol, estradiol, and mestranol by high-performance liquid chromatography □ Ethynodiol—quantitative analysis by high-performance liquid chromatography, oral contraceptive tablets □ Ethinyl estradiol—quantitative analysis by high-performance liquid chromatography, oral contraceptive tablets □ Mestranol—quantitative analysis by high-performance liquid chromatography, oral contraceptive tablets □ High-performance liquid chromatography—quantitative analysis of ethynodiol, estradiol, and mestranol in oral contraceptive tablets

Ethynodiol diacetate is a synthetic steroid showing progestogenic activity. It is used in oral contraceptives in admixture with either ethinyl estradiol or mestranol. Compendial procedures (1–3) are limited to raw material or to single ingredient formulations, except the USP XX (1) which describes the analysis of the ethynodiol diace-

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