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Note

High-performance liquid chromatography of 13-*cis*-retinoic acid and of endogenous retinol in human plasma

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Retinoids (vitamin A and analogues) are essential for normal growth and differentiation of epithelial tissues [1], and possess prophylactic and therapeutic activity in a variety of chemically induced epithelial cancers in experimental animal tumor systems [2—4]. To circumvent the toxicity of

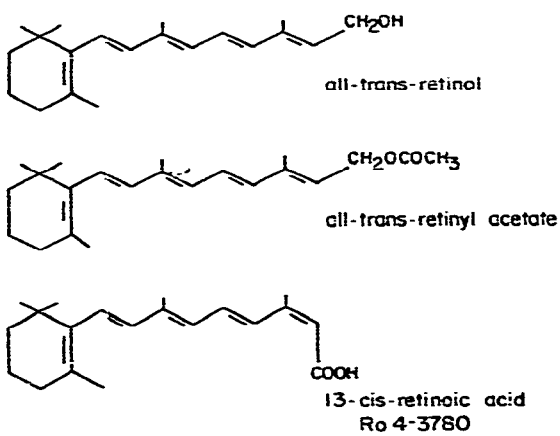

Fig. 1. Chemical structures of assayed retinoids.

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pharmacologic doses of natural retinoids, less toxic derivatives have been synthesized [5]. The synthetic structural analogue of all-*trans*-retinoic acid (RA), 13-*cis*-retinoic acid (13-*cis*-RA), has been shown to be less toxic than the parent compound [6], to be effective in the prevention of lung and bladder carcinogenesis in rodents [7, 8], and has been used successfully in the treatment of various dermatoses in man [9—11]. In view of the potential chemopreventive effect of 13-*cis*-RA in human epithelial cancer [12], we have undertaken a clinical Phase I pharmacologic and toxicologic study of 13-*cis*-RA, and have modified the method of Frolik et al. [13] to assay the drug. This report describes a sensitive and specific high-performance liquid chromatographic (HPLC) assay for the simultaneous determination of 13-*cis*-RA and of endogenous plasma retinol (R-OL), using all-*trans*-retinyl acetate as the internal standard (Fig. 1).

MATERIALS AND METHODS

All manipulations were carried out in the dark allowing only slight indirect bulb light. Fluorescent light was totally avoided to prevent photoisomerisation reactions.

Reagents

All solid chemicals used were of reagent grade; solvents used for extraction and chromatography were of chromatoquality (99%) grade and were purchased from Canlab Ltd., Montreal, Canada.

Columns

The columns were 250 mm × 3.2 mm I.D. stainless steel containing 10 μm Altex LiChrosorb C_{18} reversed-phase packing.

Instruments

A Perkin-Elmer Model 601 dual-pump high-performance liquid chromatograph was used, equipped with a Rheodyne injector permitting injection of volumes between 1 and 150 μl.

The detector was a Perkin-Elmer Model LC-55 set at 350 nm, and the recorder a Perkin-Elmer Model 56 set at 1 or 2 mV. Peak areas were calculated with a Varian integrator Model 485 equipped with filtering and baseline tracking devices.

Mobile phase

Throughout the procedures, the mobile phase was constituted of a mixture of acetonitrile and water containing 1% of ammonium acetate (80:20). The flow-rate was 1.5 ml/min, keeping the pressure at 70—105 bars, well below the critical pressure of 205 bars.

Analytical standards

All-*trans*-R-OL, all-*trans*-RA, and all-*trans*-retinyl acetate were purchased from Eastman Kodak, Rochester, NY, U.S.A.; 13-*cis*-RA (RO-4-3780) was

obtained from Hoffmann-LaRoche, Nutley, NJ, U.S.A. All these substances have a purity greater than 99%.

Preparation of standards

Stock solutions of all retinoids were made by diluting 10 mg of pure substance in 100 ml of methanol; these solutions were stable for over four months. Working standard solutions of 1 mg% were made by dilution with methanol and discarded after single use.

Buffer

An acetate buffer of 0.2 *M* was made by adding 12 ml of 0.2 *M* to 700 ml of water; the pH was adjusted to 3.00 with 0.2 *M* sodium hydroxide, and the volume completed to 1 liter.

Extraction procedure

To 15-ml Teflon screw-cap tubes containing 1 ml of plasma, 0.5 μg of retinyl acetate as internal standard was added to 1 ml of methanol. The plasma was vortexed for 15 sec to ensure good protein precipitation; 1 ml of buffer was then added to 2 ml of extracting solvent containing hexane—methylene chloride—isopropanol (80:19:1, v/v), and the tubes were vortexed for 1 min and centrifuged. The organic layer was removed and the extraction procedure repeated on the residual plasma fraction. The two organic fractions were then combined and evaporated under dry nitrogen at 45°C in the dark. The residue was dissolved in 100 μl of acetonitrile and a 10-μl aliquot was used for the assay.

Calibration curve

To aliquots of 1 ml of plasma, previously tested for the absence of interfering substances, were added 0, 50, 100, 150, 200, 300, 500, and 700 ng of R-OL and of 13-*cis*-RA; to each tube 500 ng of all-*trans*-retinyl acetate were also added. Analysis at each of these concentrations was repeated five times.

RESULTS

The individual values obtained for the construction of standard curves are shown in Table I. Standard deviations at each of the concentrations studied were less than 10% of the mean for both 13-*cis*-RA and R-OL. Routine analyses performed on different days of plasma spiked with standards were also within the S.D. indicated in Table I. The coefficients of regression were 0.99 for both 13-*cis*-RA and R-OL calibration curves. To allow for a more quantitative determination of the tested compounds and to circumvent biases induced by possible variations of peak shapes over time, surface areas instead of peak heights were evaluated. Comparisons of peak areas of spiked plasma extracts of R-OL, 13-*cis*-RA, and all-*trans*-retinyl acetate with standards injected under the same conditions indicated a recovery from plasma of 85 ± 10% for each substance.

Typical chromatograms obtained with blank plasma, and with plasma from a patient who received a single oral dose of 60 mg (35 mg/m^2) of 13-*cis*-RA are shown in Fig. 2. The peaks are well defined with no extraneous substance

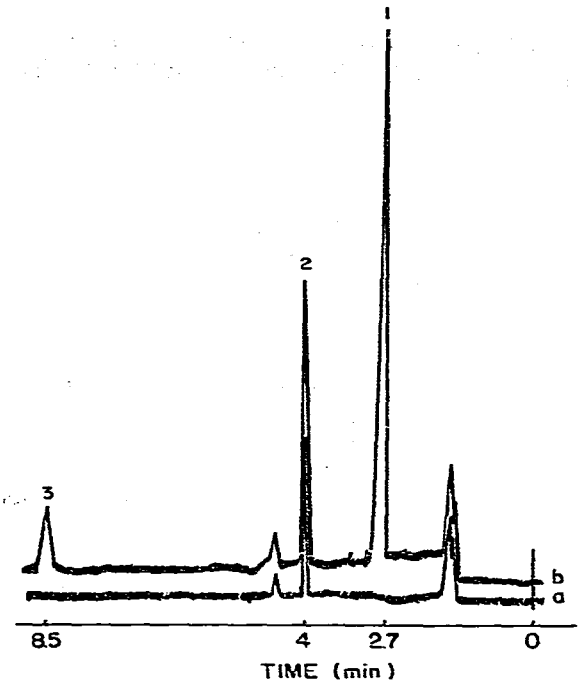

Fig. 2. Chromatograms of the HPLC analysis of (a) control plasma containing endogenous all-*trans*-retinol (peak 2); and (b) plasma of a patient treated with a single oral dose of 60 mg of 13-*cis*-retinoic acid (peak 1); peak 3 represents the internal standard (all-*trans*-retinyl acetate).

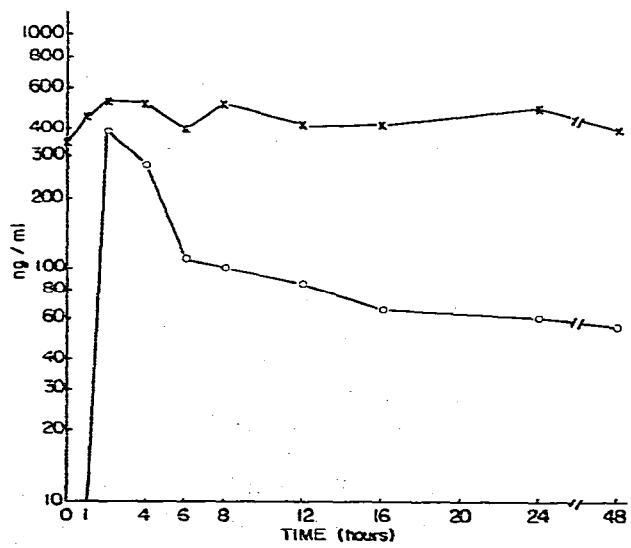

Fig. 3. Plasma levels of endogenous retinol (x) and of 13-*cis*-retinoic acid (o) in a patient receiving a single dose of 60 mg of 13-*cis*-retinoic acid.

TABLE I

SURFACE RATIOS OF PLASMA 13-*CIS*-RETINOIC ACID AND OF ALL-*TRANS*-RETINOL OVER INTERNAL STANDARD OBTAINED FOR THE CALIBRATION CURVES

Amount of retinoids added to plasma (ng/ml)	Surface ratios			
	All-*trans*-retinol / Internal standard		13-*cis*-retinoic acid / Internal standard	
	Values	Mean ± S.D.	Values	Mean ± S.D.
0	1.26, 1.21, 1.17 1.12, 1.25*	1.20* ± 0.06		
50	1.52, 1.38, 1.64 1.72, 1.61	1.57 ± 0.13	0.35, 0.38, 0.32 0.31, 0.30	0.33 ± 0.03
100	1.95, 1.82, 2.20 2.30, 2.28	2.11 ± 0.21	0.56, 0.57, 0.54 0.67, 0.56	0.58 ± 0.05
150	2.35, 2.30, 2.48	2.44 ± 0.15	0.76, 1.04, 0.82 0.69, 0.80	0.83 ± 0.13
200	2.80, 2.70, 2.64 3.02, 2.88	2.81 ± 0.15	1.16, 1.20, 1.12	1.14 ± 0.05
300	3.24, 3.34, 3.42 2.98, 3.18	3.23 ± 0.17	1.72, 1.75, 1.73 1.60, 1.53	1.67 ± 0.10
400	3.72, 3.89, 4.02 3.81, 3.59	3.81 ± 0.17	2.33, 2.34, 2.25 2.17, 2.21	2.26 ± 0.07
500	4.83, 4.79, 4.62 4.55, 4.52	4.66 ± 0.14	2.72, 2.72, 2.50 2.83, 3.03	2.76 ± 0.19
700	6.08, 5.98, 5.65 5.96, 5.87	5.91 ± 0.16	4.10, 4.10, 3.29 4.08, 3.84	3.88 ± 0.35

*In the standard curves these values, corresponding to endogenous plasma all-*trans*-retinol, were subtracted from all other ratios.

interfering with the assay. Fig. 3 illustrates the pharmacokinetics of 13-*cis*-RA in the same patient. The drug is absorbed rapidly and follows a bicompartmental model with a distribution and an elimination phase. Endogenous plasma R-OL concentrations are also shown.

DISCUSSION

All-*trans*-retinyl acetate, a closely related structural analogue of R-OL and 13-*cis*-RA, was chosen as the internal standard because its elution time is long and occurs in a region of the chromatogram free from interference. Although the maximum UV absorbance of retinyl acetate is at 326 nm, sensitivity at 350 nm is sufficient to obtain good surface ratios with the retinoids studied.

The method outlined is rapid and accurate with a limit of sensitivity of about 25 ng/ml for both R-OL and 13-*cis*-RA. All technical procedures, however, must be performed in the dark as degradation occurs when plasma extracts are submitted to light. Also, one must ensure that the patients avoid the intake of exogenous vitamin A preparations, particularly those containing retinyl acetate.

The HPLC assay reported in this paper differs from the method originally described by Frolik et al. [13]. Firstly, the extraction procedure is directly performed on plasma without prior lyophilization. The use of a solvent—solvent partition system gives a clear separation of the retinoids assayed from interfering substances which are rapidly eluted in the chromatogram, and the analytical separation procedure is completed within 10 min (Fig. 2). Secondly, to avoid variations in peak shapes and elution times, the column pressure was kept below 105 bars. This enabled us to inject about 100 samples before changing the inlet frit to ensure quality and reproducibility of results. All-*trans*-RA has a retention time of 3.5 min (not shown in Fig. 2) just between those of R-OL and of 13-*cis*-RA. Due to the possibility of photoisomerisation between 13-*cis*-RA and all-*trans*-RA, an endogenous metabolite of R-OL present in the plasma [14], the separation of both isomers is of importance. Further, Keilson et al. [15] have noted a decrease in endogenous plasma R-OL in rats deficient in vitamin A and given all-*trans*-RA. In our clinical study [16] we have observed a similar decrease in plasma R-OL in some patients chronically treated with 13-*cis*-RA. Our analytical method provides the means to study the kinetic parameters of 13-*cis*-RA, to assay R-OL and all-*trans*-RA, and to evaluate the possible interactions between these retinoids. The procedure, rapid and practical, is particularly suitable for multiple sample analysis.

ACKNOWLEDGEMENT

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