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Note

High-performance liquid chromatography of Ro 10-9359 (Tigason) and its metabolite Ro 10-1670 in human plasma

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Retinoids, analogues of vitamin A, have prophylactic and therapeutic activities in various types of experimental cancers [1—3] and dermatologic disorders [4]. All-*trans*-retinoic acid (tretinoin) is currenctly used in dermatology in topical preparations only due to its systemic toxicity [5]. Its isomer, 13-*cis*-retinoic acid is less toxic and is used orally with success in chemoprevention of lung and bladder carcinogenesis [6, 7] and in various dermatosis [8—10]. The Ro 10-9359 (Tigason), an aromatic retinoid, has a better therapeutic index than all-*trans*-retinoic acid and is experimentally used in lamellar ichtoyosis [11], epidermolytic hyperkeratosis [11], erythrokeratoderma [11] and psoriasis [12—14].

This report describes a sensitive and selective high-performance liquid chromatography (HPLC) analytical procedure for Ro 10-9359, its metabolite Ro 10-1670, and endogenous retinol in plasma, 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 light, to prevent photoisomerisation reactions.

Reagents

All solid chemicals used were of reagent grade. Solvents used for extraction and chromatography were of chromatoquality (99%).

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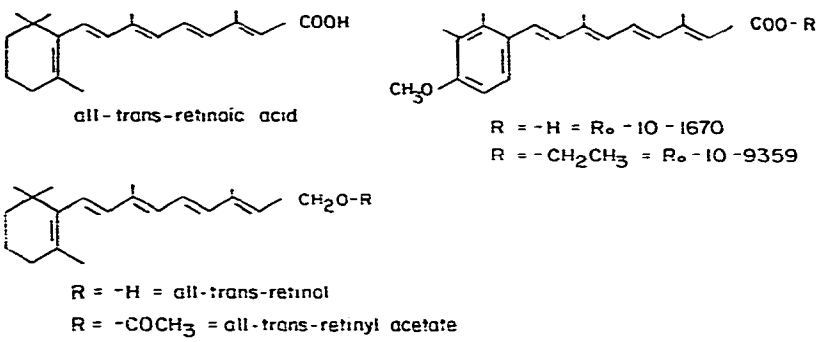

Fig. 1. Chemical structures of assayed retinoids.

Columns

The columns used were 250 mm × 4.6 mm I.D. stainless steel containing a 5-μm Spherisorb ODS reversed-phase packing.

Instruments

A dual-pump Perkin-Elmer, Model 601 high-performance liquid chromatograph was used, equipped with a Rheodyne injector permitting injection of volumes between 1 and 175 μl. The detection was carried out with a variable-wavelength UV—VIS spectrophotometer set at 350 nm, and the recorder was set at 1 mV. Peak areas were calculated with a Varian Model 485 digital integrator equipped with filtering and baseline tracking devices.

Analytical standards

All-*trans*-retinoic acid, all-*trans*-retinol and all-*trans*-retinyl acetate were purchased from Eastman Kodak (Rochester, NY, U.S.A.) and Ro 10-9359 and Ro 10-1670 were obtained from Hoffmann-La Roche (Vaudreuil, Canada). All these substances have a purity greater than 99%. Stock solutions of all retinoids were prepared by diluting 10 mg of pure substance in 100 ml of methanol. These solutions were stable for at least four months if stored at —30°C in the dark.

Mobile phase

The mobile phase was an acetonitrile—water (76:24) mixture containing 1% of ammonium acetate. The flow-rate was 1.5 ml/min, keeping the pressure between 80—95 bar which is well below the critical pressure of 205 bar.

Buffer

A 1 *M* phosphate buffer was prepared by adding 68.5 ml of an 85% phosphoric acid solution to 600 ml of water; the pH was adjusted to 6.00 with 10 *N* sodium hydroxide solution, and the volume completed to 1 l.

Procedure

In 15-ml PTFE-screw-cap tubes containing 1 ml of plasma, 1 μg of retinyl acetate as internal standard was added and the plasma was vortexed for 10 sec; then, 2 ml of buffer were added followed by 3 ml of extracting solvent

containing a mixture of diethyl ether—ethyl acetate (75:25), and the tubes were agitated for 10 min with a reciprocal movement agitator and centrifuged at 800 *g* for 5 min. The organic layer was removed and the extraction procedure repeated on the residual plasma fraction with fresh solvent. The two organic fractions were then combined, and evaporated in the dark under dry nitrogen on a hot plate set at 25°C. The dry residue was dissolved in 200 μl of extracting solvent and a 20-μl aliquot was injected for the assay.

The calibration curves were prepared by adding known amounts (Table I) of retinoids to 1 ml of plasma containing 1 μg of all-*trans*-retinyl acetate. Three spiked samples were assayed for each concentration and peak area ratios were used for the quantification of retinoids.

TABLE I

PEAK AREA RATIOS OF PLASMA Ro 10-1670, Ro 10-9359 AND ALL-*trans*-RETINOL VERSUS INTERNAL STANDARD OBTAINED FOR THE CALIBARATION CURVES

Amount of retinoids added to plasma (ng/ml)	Ratio (mean value ± S.D.)		Amount of retinol added to plasma (ng/ml)	Ratio (mean value ± S.D.) all-*trans*-retinol/ internal standard
	Ro 10-9359/ internal standard	Ro 10-1670/ internal standard		
100	0.22 ± 0.01	0.30 ± 0.02	0	0.55* ± 0.02
250	0.46 ± 0.02	0.55 ± 0.01	125	0.73 ± 0.00
400	0.61 ± 0.03	0.78 ± 0.05	250	0.81 ± 0.05
500	0.95 ± 0.04	1.06 ± 0.06	400	1.03 ± 0.06
700	1.34 ± 0.08	1.77 ± 0.04	500	1.13 ± 0.02
800	1.44 ± 0.10	1.93 ± 0.12	600	1.17 ± 0.03
1000	1.98 ± 0.13	2.52 ± 0.02	1000	1.70 ± 0.08

*This value corresponds to endogenous plasma all-*trans*-retinol.

RESULTS AND DISCUSSION

The HPLC analytical procedure reported allows the separation and quantitative determination of Ro 10-9359, its principal metabolite, Ro 10-1670 [15] and of endogenous retinol, using their UV absorbance at 350 nm and by measuring the peak area ratios relative to retinyl acetate chosen as internal standard. The method is rapid and accurate with a sensitivity limit of 25 ng/ml for each compound. Mean values ± S.D. for the elaboration of standard curves are reported in Table I for the three retinoids. The coefficients of variation are less than 7% of the mean for each concentration of the three substances studied. Routine analysis performed on plasma spiked with different concentrations of standards, were also within the variations mentioned earlier. The regression coefficients (r^2) for the standard curves were higher than 0.98 for Ro 10-9359, Ro 10-1670 and retinol. The overall recovery from plasma determined by comparison of peak areas from plasma extracts with standards injected under the same conditions, is 90 ± 5% for each substance considered and is constant for each concentration analysed.

A typical chromatogram obtained from a plasma sample containing five different retinoids is shown in Fig. 2. The retention times for Ro 10-1670, retinol, all-*trans*-retinoic acid, Ro 10-9359 and retinyl acetate are 6, 8.2, 9.5, 11 and 13.5 min, respectively. Fig. 2 illustrates the net separation between all these retinoids. The peaks are well defined with no interfering substances, these being partly eliminated by the extraction procedure or eluted within 4 min. This method has been proved to be as accurate as the one published for the dosage of 13-*cis*-retinoic acid [16]. The method was applied to determine the plasmatic concentration of Ro 10-9359 and its metabolite Ro 10-1670 in a patient receiving a 25-mg capsule of Ro 10-9359 three times a day. Following 30 days of treatment, a blood sample was withdrawn immediately before the morning dose. Fig. 3 illustrates the chromatogram obtained after analysis of this blood sample. The plasmatic concentrations calculated are 176 ng/ml, 138 ng/ml and 400 ng/ml for Ro 10-9359, Ro 10-1670 and endogenous retinol, respectively.

To ensure the reproducibility of the results, some precautions must be taken. All technical manipulations must be performed in the dark (or at least in very indirect daylight) in order to avoid photoisomerisation reactions. The evaporation of the organic phase must be carried out at a temperature not exceeding 25°C to avoid thermal degradation of the retinoids [17]. Once the extraction procedure is completed, the chromatographic analysis must be

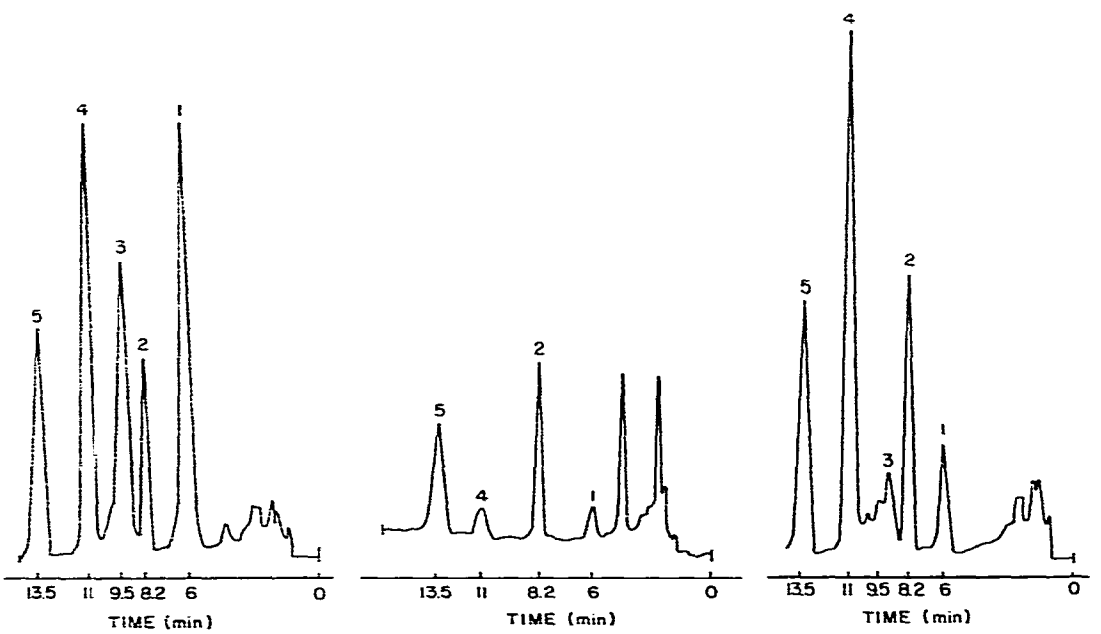

Fig. 2. Typical chromatogram of HPLC analysis of (1) Ro 10-1670, (2) retinol, (3) all-*trans*-retinoic acid, (4) Ro 10-9359 and (5) retinyl acetate (internal standard).

Fig. 3. HPLC chromatogram illustrating plasmatic concentrations from a patient of (1) Ro 10-1670, (2) retinol, (4) Ro 10-9359 and (5) retinol acetate (internal standard).

Fig. 4. Chromatogram illustrating evidence of the degradation of all-*trans*-retinoic acid (3) in plasma extract.

performed as quickly as possible. In the event that immediate HPLC analysis is impossible, the samples can be stored at −30°C in the dark for a maximum period of 24 h without significant degradation. The samples when redissolved are subject to rapid degradation, especially for Ro 10-9359, with a decrease in main peak area and occurrence of secondary peaks. This phenomenon has also been observed with all-*trans*-retinoic acid and is less striking than for Ro 10-1670. The method described in this paper differs from the assay reported by Hänni et al. [18]. Firstly, all-*trans*-retinyl acetate was chosen instead of retinoic acid as internal standard because of better stability and secondly the peak areas were evaluated instead of peak heights. We have discarded the possibility of using all-*trans*-retinoic acid due to its marked instability in our analytical conditions. The chromatogram shown in Fig. 4 illustrates the degradation of all-*trans*-retinoic acid, observed during the manipulations. The peak height is decreased and secondary peaks appear due to degradation of retinoic acid during the extraction procedure. The use of peak areas instead of peak heights insures a more precise and reproducible determination by circumventing column modification and peak broadening over a period of time. Furthermore, compared to an adsorption column, the results obtained with a reversed-phase system show a better reproducibility on a long term basis. All analytical methods should also consider endogenous retinol, even when its analysis is not the object of the exercise. The normal endogenous retinol level is between 300 and 700 ng/ml in man [19]. It has been demonstrated by Keilson et al. [20] that all-*trans*-retinoic acid affects the mobilisation of retinol. The same phenomenon could occur with other retinoids. Our method permits the separation of retinol from the retinoids studied, and thus permits its simultaneous determination. The procedure, rapid and practical, is particularly suitable for pharmacokinetic studies.

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