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Note

Simultaneous determination of the aromatic retinoids etretin and etretinate and their main metabolites by reversed-phase liquid chromatography

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The aromatic retinoid etretinate (I, Fig. 1) is used in the treatment of severe psoriasis and dyskeratoses [1]. Etretinate is rapidly metabolized by hydrolysis to the biologically active metabolite etretin (II, Fig. 1), which appears in blood plasma at about the same time as the parent compound [2]. The biological half-life of etretinate following continuous treatment with the drug has been shown to be in the range of 80–120 days [2,3] which is a great disadvantage because of the teratogenicity of the drug. It has been shown that the metabolite, etretin, is also a potent drug in the treatment of psoriasis and that the biological half-life of etretin is considerably shorter than that of etretinate [4]. In vivo etretin is further transformed into a 13-*cis* isomeric metabolite (III, Fig. 1) [4]. Several methods for the determination of etretinate, including normal- and reversed-phase high-performance liquid chromatography (HPLC) [5–8], have been published.

We report here a simple, selective and sensitive reversed-phase HPLC method that simultaneously determines etretinate, etretin and the *cis* isomeric metabolite of etretin using isotretinoin (IV, Fig. 1) as an internal standard.

EXPERIMENTAL

Reagents

Compounds I–IV and the *trans* isomer of isotretinoin, tretinoin (V), were provided as pure crystalline compounds by F. Hoffmann-La Roche (Basle, Switz-

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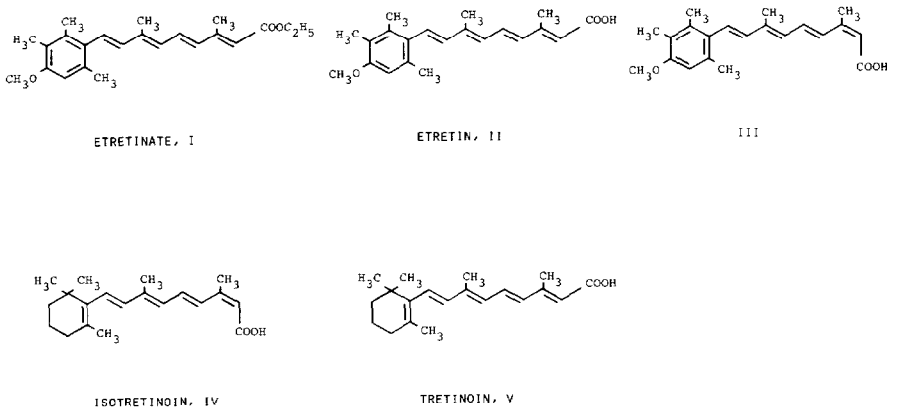

Fig. 1. Structures of the retinoids.

erland). Standard solutions were prepared by dissolving 10 mg of the retinoid in a few millilitres of dimethylsulphoxide and subsequent dilution with ethanol to the desired concentration. Diethyl ether, methanol (Merck, Darmstadt, F.R.G.) and acetonitrile (Rathburn Chemicals, Walkerburn, U.K.) of HPLC grade were used without further purification. All handling of the retinoids was carried out in a darkened room and solutions were kept in the dark at 4 °C under a nitrogen atmosphere.

Chromatography

A Waters Assoc. (Milford, MA, U.S.A.) Model 600 liquid chromatographic pump and a U6K injection system were used. The absorbance was measured at 365 nm using a Waters 440 dual-channel UV detector. Separations were achieved using a Knauer 250 mm × 4.6 mm I.D. steel column slurry-packed with Nucleosil 5 C_{18} (Macherey, Nagel & Co., Düren, F.R.G.). The isocratic solvent was acetonitrile–0.1 *M* ammonium acetate (80:20, v/v) recycled through the chromatographic system. The flow-rate was 2.5 ml/min and the pressure drop through the column was 140 bar.

Procedure

Blood samples, drawn into 10-ml amber-glass tubes containing 20 mg of potassium oxalate and 25 mg of sodium fluoride, as recommended by Vane et al. [9], were immediately centrifuged at 1200 *g*. Plasma was separated and stored at −70 °C. Frozen plasma samples were thawed at room temperature in the dark and 1.0 ml was transferred into a round-bottomed centrifuge tube equipped with glass stoppers. The tube was immediately placed in a cardboard container. Then, 0.1 ml of 28 m*M* ascorbic acid, 0.1 ml of 14 m*M* disodium ethylenediaminetetraacetate, 0.1 ml of 1% of ammonium acetate (pH 6), 200 μl of the internal standard (isotretinoin, 2 μg/ml) (when plasma concentrations of the aromatic retinoids are expected to be in the range 0–500 ng/ml), and 5 ml of diethyl ether were added. The cardboard containers were placed in a horizontal shaker and

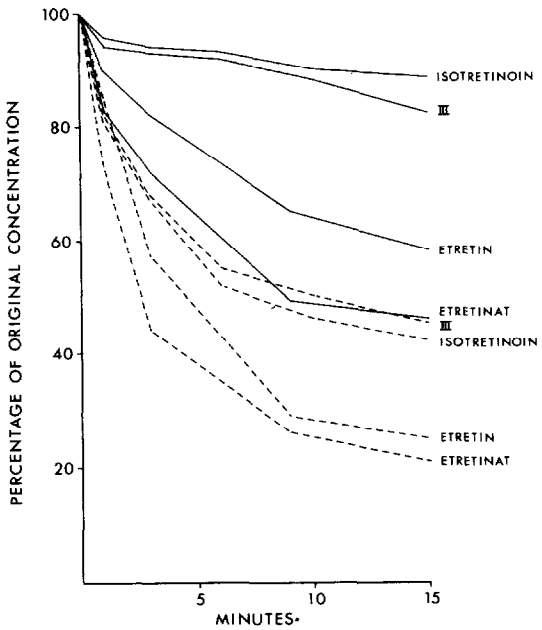

Fig. 2. Degradation of retinoids in solution: the samples were left at room temperature in the laboratory in normal daylight. Solid lines, solutions in amber-glass tubes; broken lines, solutions in transparent glass tubes.

extracted for 20 min. After centrifugation at 1200 *g*, 4 ml of the organic phase were transferred into an amber-glass tube and evaporated to dryness under nitrogen at room temperature. A 100-μl volume of methanol was added to the residue and kept at 4°C until analysis. A 25-μl volume of this solution was chromatographed. The whole procedure was carried out in the dark or in a darkened room under light from Philinea tubes (Philips Lampe, Copenhagen, Denmark).

Biological samples

Blood was drawn from two psoriatic patients receiving 40 mg of etretin or 40 mg of etretinate. Blood was collected at different time intervals during the first 24 h of treatment. The plasma samples were stored at −70°C until analysis.

RESULTS AND DISCUSSION

The most difficult part of the determination of retinoids is to keep the samples protected from light. Fig. 2 shows the degradation of different retinoids exposed to normal laboratory light when solutions were kept in transparent and in amber-glass tubes. All the retinoids were partly transformed into their *cis–trans* isomers and it is obviously not adequate to keep the solutions in amber glassware only. In contrast, solutions kept in a darkened room illuminated with light from Philinea tubes were stable for more than 1 h in transparent normal glass tubes. When plasma samples were treated as described under *Procedure*, only 1–2% of the

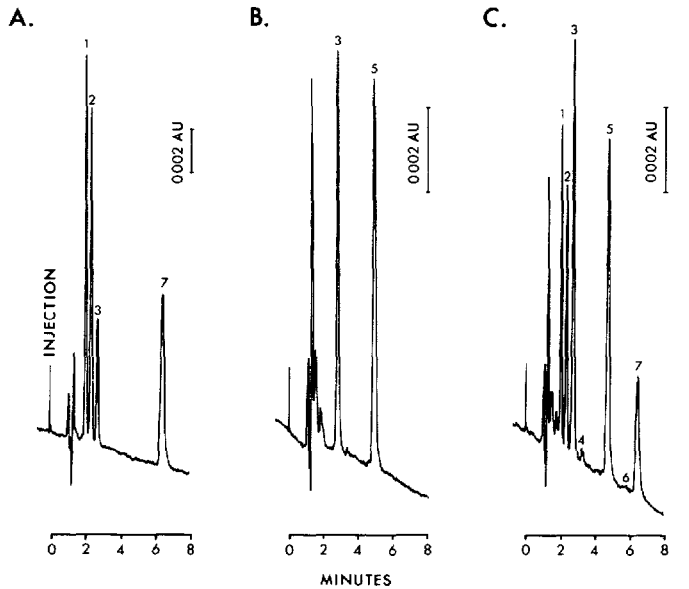

Fig. 3. HPLC of the retinoids. 2 = Etretin; 3 = isotretinoin; 7 = etretinate; 1, 4 and 6 = *cis-trans* isomers of the retinoids, respectively; 5 = an endogenous compound, probably retinol, from plasma. (A) 25 ng of pure 1, 2, 3 and 7 injected; (B) and (C) blank and spiked plasma samples carried through the extraction procedure. Internal standard: 200 ng of isotretinoin. The spiked plasma samples contained 50 ng/ml of III, etretin and etretinate.

retinoids were transformed into the *cis-trans* isomeric compounds.

Fig. 3A shows the chromatograms of pure retinoids and Fig. 3B shows a chromatogram of a plasma blank carried through the extraction procedure. Peak 3 is the internal standard (200 ng added to 1 ml of plasma) and peak 5 is an endogenous compound, probably retinol, always present in the plasma samples. Fig. 3C shows the chromatogram of an extracted plasma sample spiked per millilitre of plasma with 50 ng of each of etretin, etretinate and III and 200 ng of isotretinoin. Peaks 4 and 6 are the *trans* isomer of isotretinoin and the *cis* isomer of etretinate, respectively. The short retention times enable all the compounds to be determined within 7 min. The slight change in the baseline of the chromatograms is due to the recirculation of the eluent, but compared with the high cost of solvents this is preferable. About 500 plasma samples can be analysed within three weeks, using the same 1 l of eluent.

Diethyl ether, as recommended by Palmskog [5] and Besner et al. [6], was found to be a suitable solvent for the extraction of the retinoids. Table I shows the recoveries of the retinoids from plasma samples treated as described under *Procedure* together with the minimum detectable concentrations, retention times, precision and the equations of the calibration graphs. Day-to-day variations in the slopes of the calibration graphs were below 5%. The method is sensitive and allows the analysis of small amounts of plasma samples. Fig. 4 shows the results of concentration measurements on plasma samples from the two patients mentioned under *Biological samples*. Patient No. 1 received 40 mg of etretin and patient

TABLE I

RECOVERY, DETECTION LIMITS, RETENTION TIMES, PRECISION AND SLOPES OF CALIBRATION GRAPHS FOR THE DETERMINATION OF AROMATIC RETINOIDS

Retinoid	Recovery* (mean ± S.D., $n=6$) (%)	MDC** (ng/ml)	Retention time (min)	C.V.*** (%)		Calibration graph§: $y=\alpha x+\beta$		
				25 ng/ml	100 ng/ml	α	β	r
III	101.7 ± 2.2	1	2.1	4.2	5.1	0.666	0.018	0.9964
Etretin	97.0 ± 3.5	1	2.3	4.0	4.2	0.592	0.004	0.9989
Etretinate	85.6 ± 3.8	2	6.8	6.2	7.2	0.292	−0.008	0.9941
Isotretinoin	102.6 ± 2.0		2.7					

*Corrected for loss of solvent during the extraction procedure.
**Minimum detectable concentration.
***Within-analysis coefficient of variation ($n=6$).
§Calibration graphs constructed on four independent days. α = slope; β = intercept; r = regression coefficient.

No. 2 40 mg of etretinate orally. The metabolite of etretin, III, was present in trace amounts (< 2 ng/ml) in the patient receiving etretin and was not detected in plasma samples from the patient treated with etretinate. In this patient, the metabolite of etretinate, etretin, appeared very early after administration of etretinate.

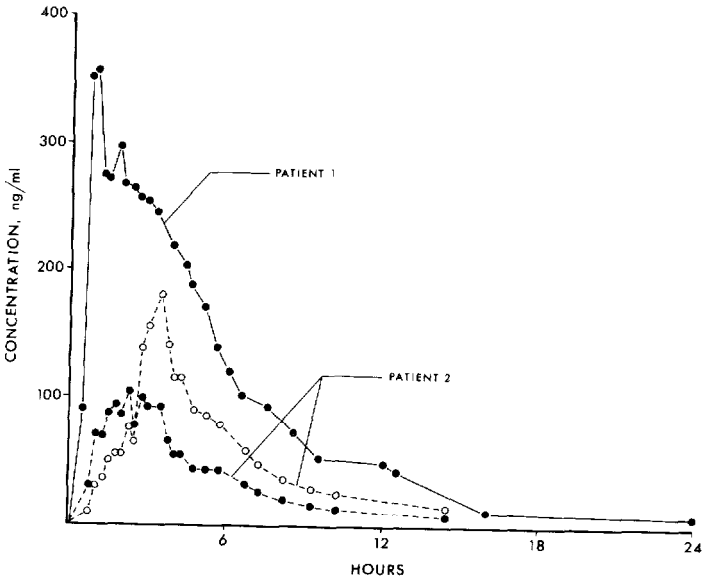

Fig. 4. Plasma concentration–time profiles from two patients receiving either 40 mg of etretin (patient 1) or 40 mg of etretinate (patient 2) orally ○, Etretinate; ●, etretin.

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