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Note

Determination of the aromatic retinoids (etretin and isoetretin) in biological fluids by high-performance liquid chromatography

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Etretin (all-*trans*-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoic acid; Ro 10-1670) is the hydrolysis product of the ethyl ester etretinate (Ro 10-9359, Tigason®) and thus the main metabolite of etretinate (Fig. 1) [1–3]. The antipsoriatic activity of etretin was recently demonstrated [4,5]. Another active metabolite [6] of etretinate is the *cis* isomer of etretin at position 13 (Fig 1), isoetretin (Ro 13-7652). This metabolite was detected after administration of etretinate [6] or etretin [7].

The thermal instability of retinoids [8,9] excludes gas–liquid chromatographic procedures for their determination. Several methods for the quantification of etretinate and etretin in biological fluids by high-performance liquid chromatography (HPLC) have been described [10–15], but only a few allowed the determination of isoetretin [10,13].

The HLPC method described by Bugge et al. [10] allowed a simultaneous quantification of etretin and isoetretin, but its sensitivity (10 ng/ml) was insufficient for kinetic studies after administration of a single oral dose of 30 mg of etretin. Moreover, the use of a ternary mixture as mobile phase, and the analysis by gradient elution, required a sophisticated apparatus and relatively long analysis times; such a method was not very reliable for routine determination in clinical practice. The method previously described by Paravicini and Busslinger [13] did not allow a specific determination of etretin and isoetretin, owing to incomplete resolution between the chromatographic peaks of these two compounds.

This paper describes a simple and rapid HPLC method for the simultaneous and specific analysis of etretin and isoetretin in biological fluids (blood, plasma and urine). This method was applied to a pharmacokinetic study in psoriatic

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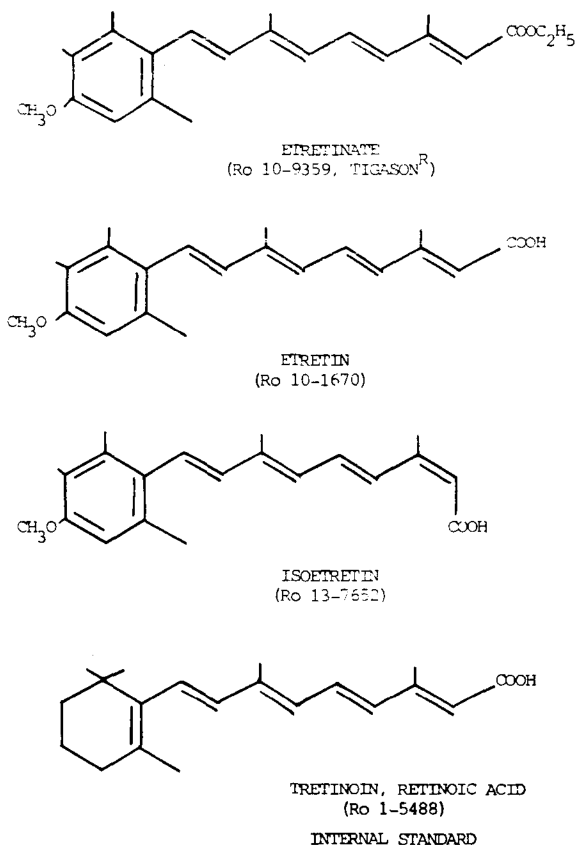

Fig. 1. Chemical structures of etretinate, its main metabolites and the internal standard used.

patients receiving a single oral dose of 30 mg of etretin, followed by the repeated administration of 30 mg of etretin once a day for 42 days.

EXPERIMENTAL

Chromatography

HPLC analyses were performed with an HP 1090 apparatus (Hewlett-Packard, Les Ulis, France) equipped with an autosampler (maximum injection volume 25 μl) and a variable-wavelength UV detector Uvikon 735 LC (Kontron Instruments, Montigny-le-Bretonneux, France). Nucleosil C_{18} columns (25 cm $\times$ 4 mm I.D.) with 5-μm particles (Chromoptic, Montpellier, France) were used at ambient temperature. The mobile phase was methanol–1% aqueous acetic acid (85:15, v/v) at a flow-rate of 1.5 ml/min. The detection wavelength was 350 nm. Under such conditions, the retention times of compounds etretin, isoetretin and the internal standard (tretinoin) were 8.9, 7.5 and 11.2 min, respectively.

Standard solutions and reagents

Stock solutions of etretin, isoetretin and tretinoin (internal standard), obtained from Hoffmann-La Roche (Basle, Switzerland), were prepared every two months by carefully weighing ca. 10 mg into a 100-ml volumetric flask and dissolving in methanol by ultrasonication. Working solutions of these compounds at two concentrations, 1 ng/μl and 0.1 ng/μl were freshly prepared every week by successive dilutions of the stock solutions in methanol. These solutions were stored at 4°C in yellow amber glass volumetric flasks. Yellow lighting was used in the laboratory to minimize photodecomposition.

Diethyl ether and ethyl acetate (Pestipur grade) were supplied by S.D.S. (Peypin, France), and methanol (RPE-ACS grade) was from Carlo Erba (Milan, Italy). Phosphate buffer (pH 7) was obtained from Merck (Darmstadt, F.R.G.). The components of the mobile phase were filtered before degassing through an FG 0.2-μm membrane for organic solutions and an HA 0.45-μm membrane (Millipore, Bedford, MA, U.S.A.) for aqueous solutions.

Extraction procedure

In a 10-ml yellow amber tube, a suitable volume (25–100 μl) of the internal standard solution was evaporated to near dryness under a stream of pure nitrogen. After addition of 0.5–3 ml of biological fluids (depending on the expected concentrations) and 0.1 ml of phosphate buffer (pH 7), the tube was extracted for 5 min with 2 ml of a diethyl ether–ethyl acetate (50:50, v/v) mixture by vortexing. After centrifugation at 3000 *g* for 10 min at 4°C, the organic layer was evaporated to dryness. The residue was dissolved in 30–100 μl of methanol and transferred to an injection vial for HPLC determination.

Data relating to plasma concentrations of etretin and isoetretin were obtained from least-squares linear regression curves, established daily from four or five calibration points. Peak-height ratios were computed by means of an HP 3390 system.

RESULTS AND DISCUSSION

Chromatograms

Fig. 2 shows typical chromatograms obtained under the described analytical conditions for plasma samples obtained (A) before administration, (B) 6 h after a single oral administration of 30 mg of etretin and (C) 5 min before the fourteenth daily administration of 30 mg of etretin. A satisfactory specificity was achieved for endogenous plasma components (peak IV).

Precision and accuracy

The precision (given by the relative standard deviation) and the accuracy (defined by the difference between obtained and expected concentrations) were checked for plasma concentrations ranging from 2 to 400 ng/ml. The results were acceptable within these therapeutic concentration ranges (Table I).

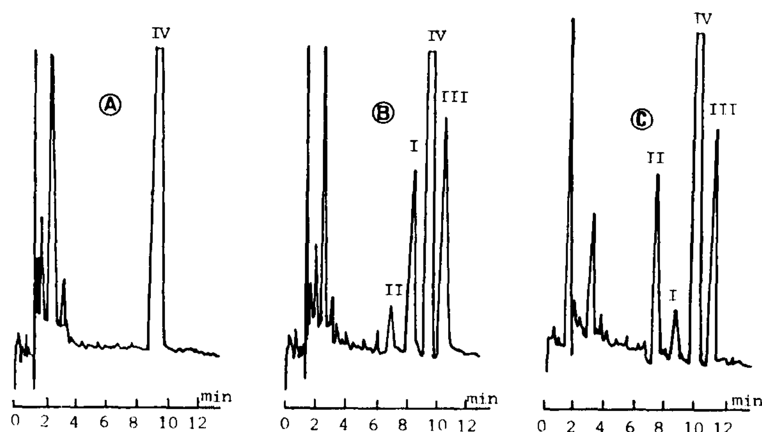

Fig. 2. Chromatograms obtained after extraction of: (A) a plasma sample from a patient before administration (control plasma); (B) a plasma sample from the same patient 6 h after a single oral administration of 30 mg of etretin (40 ng/ml etretin, 7 ng/ml isoetretin); (C) a plasma sample from this patient 5 min before the fourteenth daily administration of 30 mg of etretin (7 ng/ml etretin and 42 ng/ml isoetretin). Peaks: I = etretin; II = isoetretin; III = tretinoin; IV = endogenous plasma components.

Linearity

The linearity of the method was checked for concentrations of compounds etretin and isoetretin in biological fluids (plasma, whole blood and urine) in the range from ca. 2 to ca. 1200 ng/ml. Owing to this very large concentration range, and to avoid a great discrepancy between the respective concentrations of the compounds to be measured and the internal standard, successive linearity tests were carried out using smaller concentration ranges (i.e. 2–14 ng/ml, 5–30 ng/ml, 20–80 ng/ml) (Table II). The correlation coefficients obtained ranged from 0.9931 to 0.9999, and the intercepts of the calibration curves did not differ significantly from zero.

Limit of quantification

The limit of quantification was estimated to be ca. 2 ng/ml plasma for etretin and isoetretin (Fig. 3). Near this detection limit, the intra-assay reproducibility and accuracy were less than 10% (Table I).

Stability

Stability under different light conditions The stability of the working solutions of etretin and isoetretin was tested under natural light, normal artificial light, yellow light, and complete darkness, for periods of time up to 5 h, at 20°C. Etretin and isoetretin were stable under yellow light and in darkness, and very unstable under other light conditions.

Stability in human plasma. Control plasmas separately spiked with etretin and isoetretin at two concentrations, 50 and 100 ng/ml plasma, were stored successively for 2, 6, 10 and 24 h at ambient temperature, and for 7, 21 and 90 days at −20°C. For each storage duration and temperature, these spiked plasmas were

TABLE I

INTRA-ASSAY REPRODUCIBILITY AND ACCURACY FOR BIOLOGICAL FLUIDS DETERMINATION OF ETRETIN AND ISOETRETIN

$n=8$ in all cases.

Concentration expected (ng/ml)	Concentration obtained (ng/ml)	C.M.I.* (%)	Difference between expected and obtained concentration (%)
Etretin in plasma			
2	1.9	9.5	−5.0
5	4.8	5.4	−4.0
10	10.1	6.1	+1.0
20	20.5	4.8	+2.5
50	48.7	1.8	−2.6
100	99.4	4.5	−0.6
200	192.1	1.6	−3.9
400	400.7	4.0	+0.2
Isoetretin in plasma			
3	2.8	10.3	−6.7
6	5.9	7.0	−1.6
9	9.3	4.9	+3.3
18	18.7	4.3	+3.9
60	59.5	1.3	−0.8
120	120.7	0.9	+0.5
210	210.2	0.7	+0.1
420	414.2	1.4	−1.4
Etretin in blood			
2	1.8	10.0	−10.0
5	5.0	2.9	0
15	15.1	7.3	+0.6
30	29.7	4.9	−1.0
50	48.7	1.0	−2.0
100	98.4	2.1	−1.6
200	205.1	2.5	+2.5
Isoetretin in blood			
2	1.8	9.7	−10.0
5	4.9	3.7	−2.0
15	15.8	10.0	+5.3
30	30.5	3.2	+1.7
50	48.6	5.3	−1.4
100	96.8	2.4	−3.2
200	205.1	0.9	+2.5
Etretin in urine			
2	1.9	10.0	−5.0
5	5.1	10.0	+2.0
15	13.9	7.3	−7.3
30	28.3	3.9	−5.6
50	48.1	5.7	−3.8
100	93.6	2.7	−6.4
200	196.1	1.9	−1.9
Isoetretin in urine			
2	1.8	9.5	−10.0
5	4.9	8.9	−2.0
15	15.6	9.9	+4.0
30	28.7	2.9	−4.3
50	49.1	6.9	−1.8
100	95.9	3.1	−4.1
200	196.1	1.7	−1.9

*C.M.I. = confidence interval of mean (significance level = 0.05).

TABLE II

LINEARITY TESTS FOR DETERMINATION OF ETRETIN AND ISOETRETIN IN BIOLOGICAL FLUIDS

Concentration added (ng/ml)	Equation of the non-weighted linear regression curves	Correlation coefficient
Etretin in plasma		
2,6,10,14	$0.0321X+0.0032$	0.9948
5,10,15,30	$0.0522X+0.0064$	0.9995
20,30,40,80	$0.0245X-0.0064$	0.9969
50,100,150,300	$0.0156X-0.0139$	0.9994
200,400,800,1200	$0.0039X-0.0200$	0.9997
Isoetretin in plasma		
2,6,10,14	$0.0416X+0.0024$	0.9939
6,9,18,36	$0.0698X+0.0051$	0.9969
18,27,36,72	$0.0322X+0.0351$	0.9931
60,120,180,360	$0.0195X-0.0038$	0.9999
210,420,630,1260	$0.0044X+0.0085$	0.9999
Etretin in blood		
2.5,5,7.5,10,15	$0.0196X-0.1376$	0.9958
10,20,40,70	$0.0248X+0.0208$	0.9959
50,100,150,250	$0.0116X+0.0094$	0.9969
75,150,300,600	$0.0075X+0.0660$	0.9991
Isoetretin in blood		
2.5,5,7.5,10,15	$0.0539X-0.1599$	0.9985
10,20,40,70	$0.0283X-0.0064$	0.9960
50,100,150,250	$0.0136X-0.0527$	0.9985
75,150,300,600	$0.0087X-0.0196$	0.9983
Etretin in urine		
2.5,5,7.5,10,15	$0.0168X+0.0422$	0.9889
10,20,40,70	$0.0316X+0.0482$	0.9878
50,100,150,250	$0.0217X+0.0459$	0.9993
75,150,300,600	$0.0085X+0.0728$	0.9983
Isoetretin in urine		
2.5,5,7.5,10,15	$0.0176X+0.0299$	0.9985
10,20,40,70	$0.0330X+0.0157$	0.9989
50,100,150,250	$0.0225X-0.0211$	0.9992
75,150,300,600	$0.0088X-0.0703$	0.9990

analysed in duplicate using a calibration curve established daily. The results obtained are presented in Tables III and IV for storage at ambient temperature and for storage at $-20°C$, respectively. No significant differences ($p=0.05$) were observed for these two compounds under the storage conditions checked.

Applications

This HPLC method was applied to the plasma determination of etretin and its isomer isoetretin in a psoriatic patient who received a daily oral administration

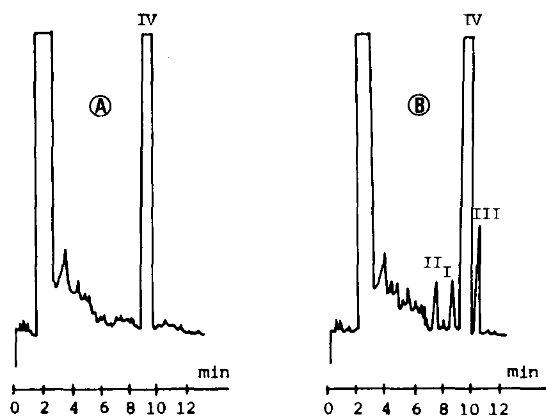

Fig. 3. Chromatograms obtained after extraction of: (A) a control plasma; (B) a control plasma spiked with 2 ng/ml etretin and 2 ng/ml isoetretin (with 10 ng of tretinoin as internal standard). Peaks: I = etretin; II = isoetretin; III = tretinoin; IV = endogenous plasma components.

TABLE III

STABILITY IN HUMAN PLASMA OF ETRETIN AND ISOETRETIN AT AMBIENT TEMPERATURE

Added concentration (ng/ml)	Experimentally found concentration (ng/ml) after storage for:				
	0	2 h	6 h	10 h	24 h
Etretin					
50	46	50	44	48	46
100	94	102	96	101	97
Isoetretin					
50	47	46	47	49	49
100	99	98	97	101	102

TABLE IV

STABILITY IN HUMAN PLASMA OF ETRETIN AND ISOETRETIN AT −20°C

Added concentration (ng/ml)	Experimentally found concentration (ng/ml) after storage for:			
	0	7 days	21 days	90 days
Etretin				
50	47	48	44	48
100	92	96	95	94
Isoetretin				
50	46	45	46	49
100	96	92	96	99

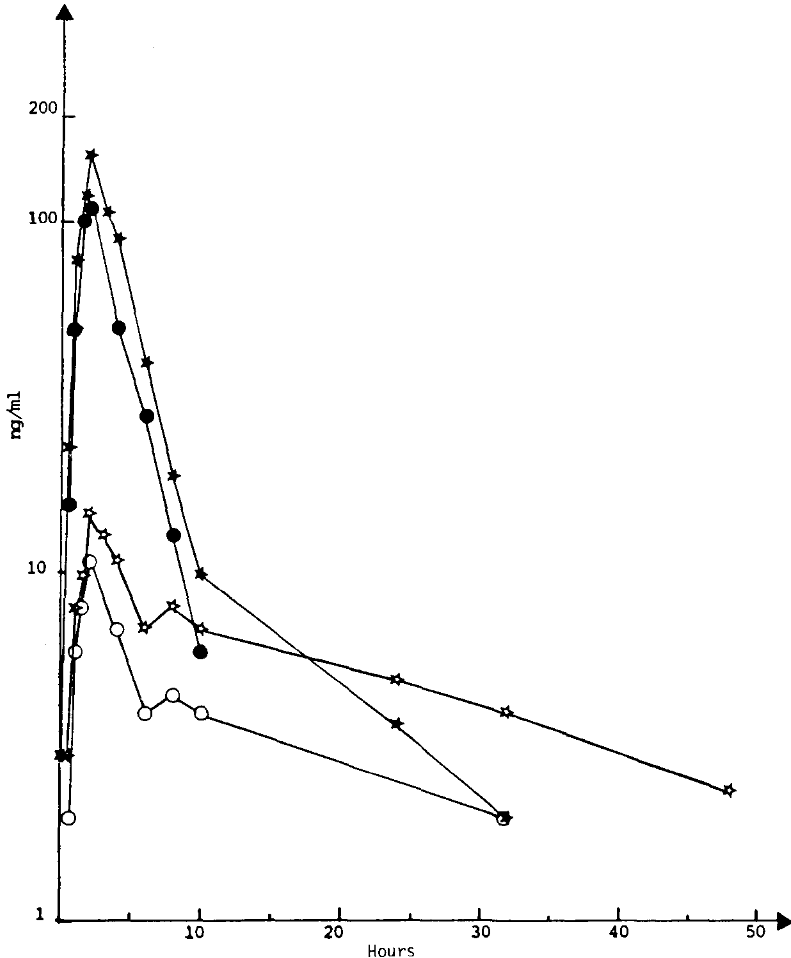

Fig. 4. Time courses of plasma and whole blood levels of etretin and isoetretin following a single oral administration of 30 mg of etretin to a patient. Data points: ★ = etretin in plasma; ☆ = isoetretin in plasma; ● = etretin in whole blood; ○ = isoetretin in whole blood.

of 30 mg of etretin (with 200 ml of water) for 42 days. Blood samples were taken before treatment, after the first administration (test dose) at 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 24 and 48 h, during multiple dose on days 14, 21, 28, 35 and 42 before each daily administration, and after the last administration (washout) at 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 24, 48, 72, 96, 120, 144, 168 and 192 h. The time courses of the concentrations of etretin and isoetretin in plasma and whole blood after a single oral administration of 30 mg of etretin are presented in Fig. 4. Note that urinary concentrations are below the detection limit of 2 ng/ml for etretin and isoetretin. Fig. 5 shows the time courses of etretin and isoetretin plasma concentrations during the multiple dose administration and after completion of the treatment. An extensive pharmacokinetic treatment of the results will be the subject of a further publication.

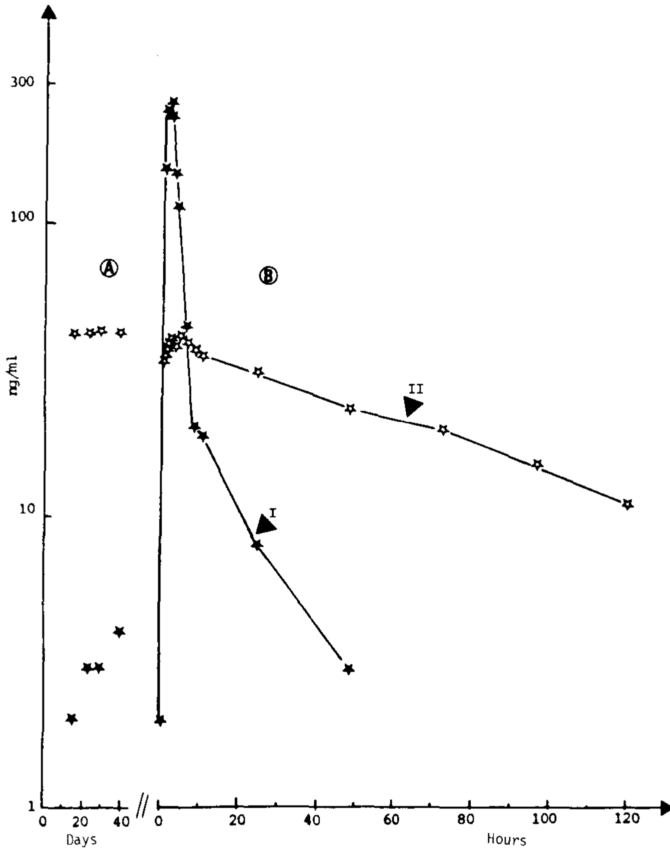

Fig. 5. Etretin (I) and isoetretin (II) plasma concentrations measured after a multiple oral administration of etretin (30 mg per day) to a patient: (A) concentrations during the 42 days treatment; (B) time course of plasma levels after administration of the last 30 mg of etretin.

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