

High-Performance Liquid Chromatography Determination of Acitretin in Plasma and Its Application to a Pharmacokinetic Study in Human Subjects

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INTRODUCTION

Etretinate (Tigason, Tegison), an aromatic retinoid, has now been used for over a decade in the treatment of dermatologic diseases such as psoriasis and congenital disorders of keratinization. Acitretin, previously etretin (Neotigason, Soriatane), is the main and active metabolite of etretinate, with clinical effects comparable to those of etretinate (Fig. 1) (1). In contrast to etretinate, acitretin is not sequestered into fatty tissue and, as a consequence, is eliminated more rapidly from the body (2). Etretinate is extremely slowly eliminated, with a terminal plasma half-life of up to 120 days. This is possibly due to the lipophilicity of the drug causing accumulation in fatty tissues from where it is slowly released. Acitretin, the corresponding carboxylic acid, is less lipophilic and has been found to be eliminated much faster ($t_{1/2} = 2-4$ days) (2). The initial and major blood metabolite of acitretin (all-*trans*-acitretin) is the 13-*cis* isomer of acitretin (13-*cis*-acitretin) (Fig. 1) (3). Several methods for quantification of etretinate and acitretin in biological fluids by high-performance liquid chromatography (HPLC) with detection limits of 2–5 ng/ml have been described previously (4–6). These methods usually use all-*trans*-retinoic acid or 13-*cis*-retinoic acid as internal standard. However, retinoic acids are endogenous and can be detected in the range of 1 to 2 ng/ml in blank plasma samples (7–9). This may introduce errors measuring low acitretin concentrations when using low, adequately adjusted concentrations of internal standard. This paper describes a simple and sensitive isocratic HPLC method for the simultaneous and specific analysis of all-*trans*-acitretin and 13-*cis*-acitretin in plasma. The method

was validated and subsequently applied to a pharmacokinetic study in subjects receiving multiple oral doses of acitretin.

MATERIALS AND METHODS

Human Subjects. Nine healthy subjects (all male; mean age 30.3 years; range, 26–33 years; mean weight, 71.4 kg; range 61–81 kg) took part in the study. A medical history was elicited from each subject and all underwent a complete physical and laboratory examination before, twice during, and once after the study. All subjects totally free of any preexisting dermatologic disease had to have normal blood values and had not to have been treated previously with any natural or synthetic retinoids. After the briefing, they were asked to sign a consent form. The trial was approved by the local Ethics Committee.

Dosing and Sample Collection. Capsules containing 25 mg acitretin were supplied by Roche Dermatologics, Nutley, NJ. The dose was 50 mg/day and the drug was taken with a standard breakfast once daily. Blood (10 ml) was collected by venipuncture into oxalated Vacutainers at appropriate points of time for 6 weeks. Group I ($n = 4$) was treated for 3 weeks. Blood was drawn on days 0, 7, and 14 before and 3 hr after dosing and on day 21 before and 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 24, 48, and 96 hr after dosing. Additional blood samples were taken 7 and 14 days after the last dose. Group II ($n = 5$) was treated for 4 weeks. Blood was drawn on days 7 and 14 before dosing and on days 21 and 28 before and 3 hr after dosing. Additional blood samples were drawn 7 and 14 days after the last dose.

Photoprotection. Because of photodegradation and photoisomerization of the retinoids, all manipulations with the samples were performed under minimal light exposure (e.g., yellow light, total light protection).

Reagents and Standard Solutions. All solvents were of analytical grade (Carlo Erba RS HPLC, France). HPLC-grade water was prepared from deionized water by purification with the Milli-Q Reagent Water System (Millipore Corp., France). All *trans*-acitretin (Ro 10-1670), 13-*cis*-acitretin (Ro 13-7652), and internal standard (arotinoid ethyl sulfone; Ro 15-1570) (Fig. 1) were supplied by Hoffmann-La Roche Ltd. (Basel, Switzerland). Stock methanolic solutions (100 μ g/ml) of all-*trans*-acitretin, 13-*cis*-acitretin, and the internal standard were prepared every 2 months, stored at 4°C in amber glass volumetric flasks, and screened from light. Working solutions of these compounds were freshly prepared every week by successive dilutions of the stock solutions in methanol.

Extraction Procedure. The plasma extraction procedure has been reported previously (8). Briefly, after the addition of a suitable volume of internal standard and pH 7 buffer, extraction from 0.3 to 1 ml plasma was performed by diethyl ether/ethyl acetate (1/1, v/v). The organic solvent layer was separated and evaporated under a stream of nitrogen. The residue was then dissolved in 30–50 μ l methanol and transferred into an injection vial for HPLC analysis. This provided an extraction recovery better than 80%.

Chromatography. Analyses were performed with a Kontron liquid chromatograph equipped with an autosampler (Model 460) and a variable-wavelength UV detector

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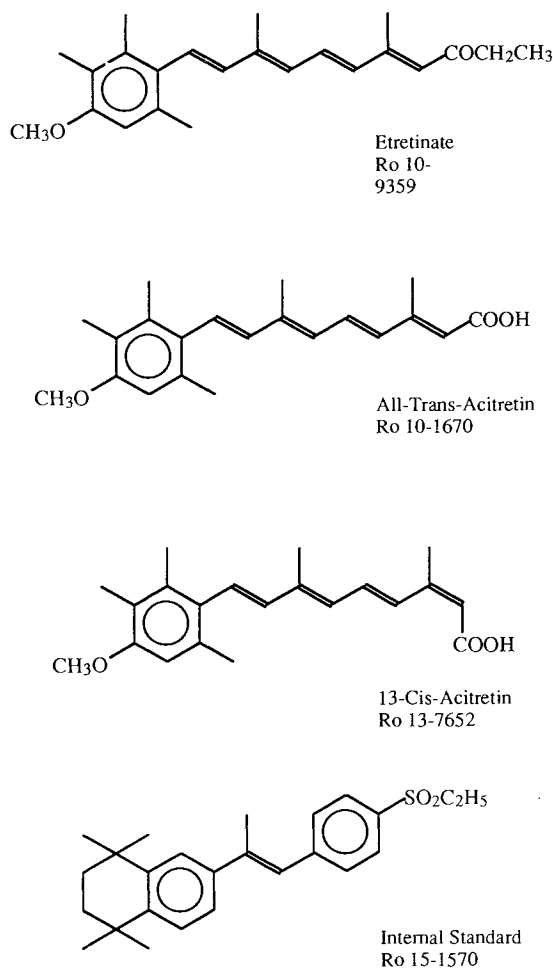


Fig. 1. Structural formulae of etretinate, the stereoisomere of acitretein, and the internal standard arotinoid ethyl sulfone.

Uvikon (Model 430). A 250×4.2 -mm (inner diameter) reversed-phase column with Nucleosil C18, $5 \mu\text{m}$, was used at ambient temperature. The following mobile phase was used: organic phase, methanol/acetonitrile (7/3, v/v); and aqueous phase, purified water with 98.5/1.5 (v/v) acetic acid. The components of the mobile phase were filtered before degassing through a $0.2\text{-}\mu\text{m}$ membrane (organic phase) and a $0.45\text{-}\mu\text{m}$ membrane (aqueous phase). The elution solvent was obtained by mixing the organic and aqueous phases (85/15, v/v). The flow rate was 1.2 ml/min and the detection wavelength was 350 nm. Under these conditions, the retention

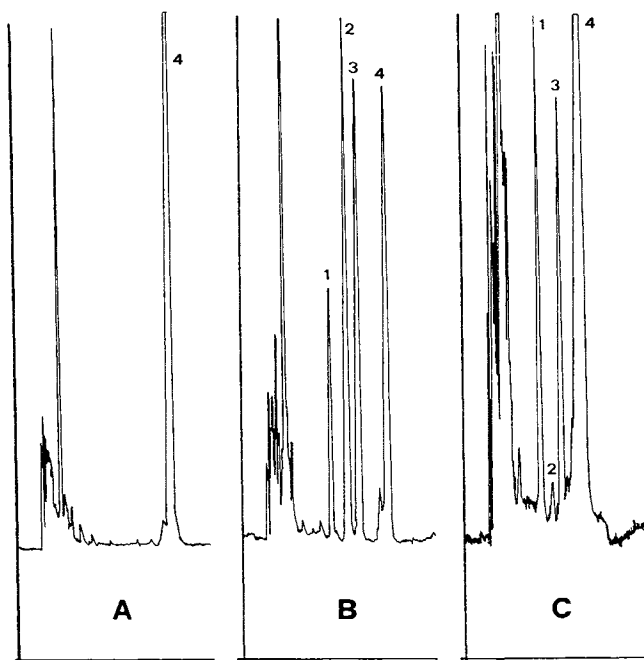


Fig. 2. Typical chromatograms obtained under the described analytical conditions for plasma samples obtained (A) before the first administration and (B) 3 hr after and (C) 96 hr after administration of the last dose of the 28-day treatment. In chromatogram C the all-*trans*-acitretein concentration is 1 ng/ml. The chromatograms show a satisfactory selectivity for all-*trans*-acitretein (peak 2; retention time, 6.9 min), 13-*cis*-acitretein (peak 1; retention time, 6.0 min), internal standard (peak 3; retention time, 7.8 min), and endogenous plasma components (peak 4).

times were 6.0, 6.9, and 7.8 min for 13-*cis*-acitretein, all-*trans*-acitretein, and internal standard, respectively (Fig. 2). Peak-height ratios were computed by means of Kontron data system D450 and calibration curves for plasma were obtained from least-squares linear regression established daily from at least four calibration points.

Data Analysis. Pharmacokinetic parameters were calculated for group I. For all-*trans*-acitretein we used APIS software (10) on an IBM PS2 8555 microcomputer for data processing with a two-compartment model. For 13-*cis*-acitretein we used a noncompartmental calculation. The data from group II were included for the determination of the disappearance of drug from plasma after cessation of the treatment.

Table I. Intraassay Precision and Accuracy for (A) All-*trans*-Acitretein and (B) 13-*cis*-Acitretein in Plasma^a

ng added (X_0)	n	A				B			
		X_m	S_x	CV %	Bias %	X_m	S_x	CV %	Bias %
1	6	1.01	0.12	12	0.05	1.13	0.20	18	13.4
5	6	4.46	0.43	9	10.8	4.76	0.28	6	-4.7
10	6	9.99	0.42	4	0.07	9.75	0.65	7	-2.5
50	6	47.05	2.02	4	-5.9	48.28	2.60	5	-3.4
200	6	216.79	10.14	5	8.4	216.77	9.34	4	8.3

^a X_0 , drug amount added; X_m , mean value; S_x , standard deviation ($n - 1$); CV %, coefficient of variation $S_x \times 100/X_0$; bias %, $(x_m - X_0) \times 100/X_0$.

Table II. Interassay Precision and Accuracy for (A) All-*trans*-Acitretin and (B) 13-*cis*-Acitretin in Plasma^a

ng added (X_0)	n	A				B			
		X_m	S_x	CV %	Bias %	X_m	S_x	CV %	Bias %
5	7	5.09	0.53	10	1.86	5.10	0.28	14	1.97
50	7	48.47	1.28	3	-3.07	48.58	1.91	4	-2.83
200	6	191.18	9.67	5	-4.40	199.83	11.94	6	-0.33

^a X_0 , drug amount added; X_m , mean value; S_x , standard deviation ($n - 1$); CV %, coefficient of variation $S_x \times 100/X_0$; bias %, $(X_m - X_0) \times 100/X_0$.

Table III. Linearity Test for (A) All-*trans*-Acitretin and (B) 13-*cis*-Acitretin in Plasma

Concentration added (ng/ml), $y = ax + b$	A			B		
	a	b	r	a	b	r
1, 2, 5, 10, 20	16.34	-0.37	0.9997	23.13	1.18	0.9998
2, 5, 10, 25, 50, 100, 200	1.46	-0.49	0.9997	1.42	0.51	0.9999

RESULTS

Apart from dry lips and dry mouth, no clinical signs of retinoid related adverse effects were noted. Laboratory parameters of all subjects were within the normal range during and after treatment.

Analytical Results

Chromatograms. Figure 2 shows typical chromatograms obtained under the described analytical conditions for plasma samples collected (A) before the first administration, (B) 3 hr after, and (C) 96 hr after administration of the last dose of the 28-day treatment. In chromatogram C, the acitretin concentration is 1 ng/ml. The chromatograms show an excellent selectivity for all-*trans*-acitretin (peak 2; retention time, 6.9 min), 13-*cis*-acitretin (peak 1; retention time, 6.0 min), and internal standard (peak 3; retention time, 7.8 min). Endogenous plasma components (peak 4) are well separated from the peaks of interest.

Precision and Accuracy. The precision and accuracy (11) were estimated on spiked samples containing 1–200 ng of each compound, i.e., over a concentration range of 1–600 ng/ml for plasma samples of 0.3–1 ml. Intraassay variation coefficients were lower than 20% for both compounds at the limit of quantification and lower than 10% over 5 ng/ml (Table I). The bias was lower than 14% for both compounds at the limit of quantification and lower than 10% over 5 ng/ml (Table I). Interday reproducibility results obtained between 5 and 200 ng per sample (0.3–1 ml) were satisfactory (Table II).

Limit of Quantification. The limit of quantification was defined as the lowest concentration that can routinely be determined with acceptable precision and accuracy (11). The limit of quantification was estimated to be 1 ng/ml for both all-*trans*-acitretin and 13-*cis*-acitretin (Table I).

Linearity. In the range 1–20 and 2–200 ng per sample (0.3–1 ml), linearity results were good, with correlation coefficients better than 0.999. Intercepts of the calibration curves were not significantly different from zero (Table III).

Pharmacokinetic Results

Plasma concentration–time profiles for acitretin and its main metabolite 13-*cis*-acitretin were monitored in group I, between 0 and 336 hr after cessation of the treatment. A typical plasma concentration–time profile is shown in Fig. 3. The plasma concentration of 13-*cis*-acitretin is lower than that of all-*trans*-acitretin in the first hours but passes beyond the all-*trans*-acitretin concentration between 8 and 24 hr after the last dose. Experimental all-*trans*-acitretin data were fitted with a two-compartment model (10). The pharmacokinetic parameters identified for each volunteer are shown in Table IV. The terminal elimination half-lives of 13-*cis*-acitretin calculated for the four subjects are (A) 73 hr, (B) 69 hr, (C) 54 hr, and (D) 85 hr, respectively. Residual plasma concentrations of both compounds (groups I and II) 7 and 14 days after cessation of the treatment are shown in Fig. 4.

DISCUSSION

While acitretin is more rapidly eliminated than etretinate, the potential teratogenicity of retinoids (12) requires

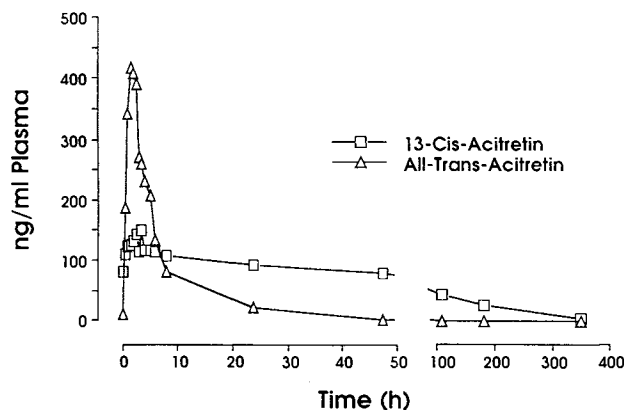


Fig. 3. Plasma concentration–time profiles for all-*trans*-acitretin and its main metabolite 13-*cis*-acitretin in subject B between 0 and 336 hr after cessation of the treatment.

Table IV. Pharmacokinetic Parameters of All-*trans*-Acitretin in Four Healthy Subjects Following Multiple (50-mg) Oral Doses

Pharmacokinetic parameter	Subject A (33 yr/71 kg)	Subject B (32 yr/67 kg)	Subject C (34 yr/73 kg)	Subject D (33 yr/81 kg)
T_{max} (hr)	1.5	1	2.5	2
C_{max}/C_{min} (ng/ml)	423/27	578/35	358/12	452/13
$T_{1/2}$ (hr)	39	37	38	87
Apparent volume of distribution (L)	51	30	71	50
Apparent clearance (L/hr)	24	14	21	20

improved analytical assays. The sensitivity and selectivity were enhanced over previously proposed and comparable assays for all-*trans*-acitretin and its main metabolite 13-*cis*-acitretin (4-6) using a simple isocratic HPLC system. A further improvement is the use of arotinoid ethyl sulfone (synthetic retinoid) as internal standard. Further the use of a newly designed mobile phase enhanced the separation of

all-*trans*-acitretin, 13-*cis*-acitretin, and the internal standard of approximately 1 min. Pharmacokinetic data obtained are consistent with previously reported data (2). A recent report (13) on the detection of etretinate after oral dosing with acitretin in a few patients has again focused the interest on the simultaneous and specific analysis of acitretin, its main metabolite, and the etretinate. With our method, etretinate was also well separated (retention time, 14.4 min) and detectable at a level of 2 ng/ml. Even though our method has not been validated for quantitative determination of etretinate, we tried to evaluate qualitatively the presence of this compound. In plasma samples taken during and after the treatment at 3 and 24 hr after administration of the drug, no peak with a retention time corresponding to that of etretinate was observed.

In conclusion, our HPLC method has provided a suitable tool for pharmacokinetic and drug monitoring studies of all-*trans*-acitretin and 13-*cis*-acitretin that can be performed by any laboratory with a darkroom and a basic isocratic HPLC system. With minor modifications the method is applicable to the analysis of other retinoids such as 13-*cis*-retinoic acid (Roaccutane, Accutane) and its metabolites.

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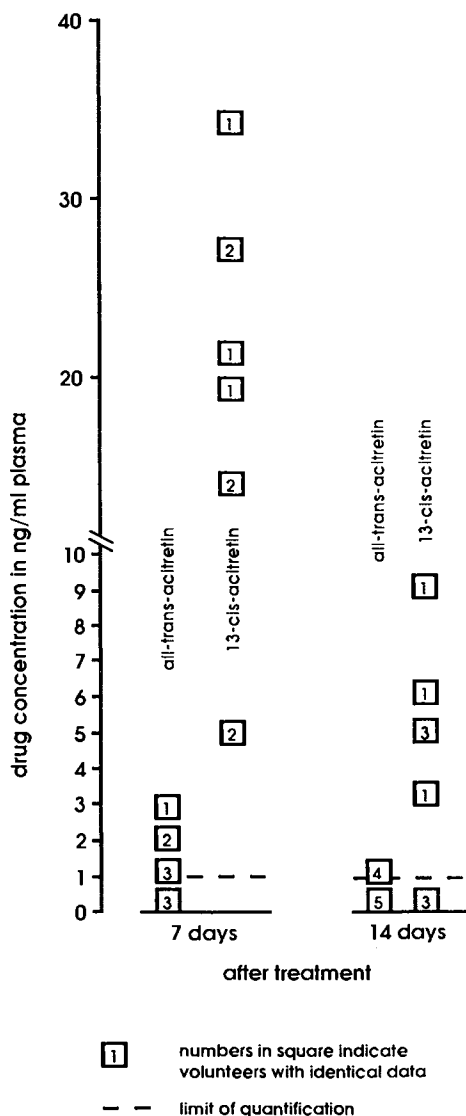


Fig. 4. Residual plasma concentrations of both groups (I + II) 7 and 14 days after cessation of the treatment.

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