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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF ETRETINATE AND ALL-*trans*- AND 13-*cis*-ACITRETIN IN HUMAN PLASMA

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SUMMARY

A high-performance liquid chromatographic procedure is described for the simultaneous determination of etretinate (Tigason), all-*trans*-acitretin (Neotigason) and 13-*cis*-acitretin in human plasma. The compounds are extracted from the plasma with *n*-hexane under acidic conditions. Quantification is performed on a normal-phase column (CP-Spher Si, 5 μm), followed by UV detection at 350 nm. The limit of quantification is 3 ng/ml. The day-to-day precision was 6.7, 13.6 and 9.1% for etretinate ($\bar{x}$ = 53 ng/ml), all-*trans*-acitretin ($\bar{x}$ = 95 ng/ml) and 13-*cis*-acitretin ($\bar{x}$ = 149 ng/ml), respectively (n = 13 for each compound). The within-day precision of nine determinations was 3.2, 11.7 and 6.5%, respectively, with mean concentrations of 128, 53 and 261 ng/ml, respectively. The method was also applied to the study of the long-term pharmacokinetic behaviour of etretinate in psoriatic patients previously treated with etretinate but now on therapy with all-*trans*-acitretin.

INTRODUCTION

Etretinate (Ro 10-9359, Tigason), a synthetic second-generation retinoid, is approved for the therapy of resistant psoriasis and of various disorders of keratinization[1]. It has been demonstrated that etretinate is hydrolysed in the body to yield the corresponding acid, all-*trans*-acitretin (also called acitretin, Ro 10–1670 or Neotigason), which is then further metabolized to the 13-*cis*-isomer (Ro 13-7652, 13-*cis*-acitretin)[2]. The structures are shown in Fig. 1. Etretinate itself accumulates in subcutaneous adipose tissue[3] and is released very slowly from this compartment (half-life 80–100 days)[4,5]. As the drug is teratogenic, contraception is required for women of childbearing potential not only during treatment but even for 24 months after discontinuation of therapy with etretinate. In addition to similar clinical efficacy[6], acitretin also has the great advantage of being eliminated far more rapidly (half-life 50 h) than

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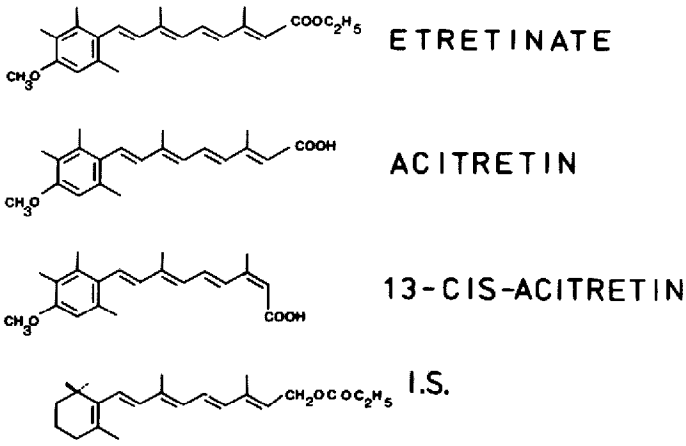


Fig. 1. Structures of etretinate, acitretin (all-*trans* form), 13-*cis*-acitretin and the internal standard (I.S.).

etretinate[7]. As a consequence, there is increased interest in all-*trans*-acitretin for the treatment of keratinizing disorders, and a large number of patients previously treated with etretinate are now placed on therapy with all-*trans*-acitretin.

Several high-performance liquid chromatographic (HPLC) procedures have already been described for the quantification of etretinate and acitretin[8–12]. A few also include the determination of 13-*cis*-acitretin[13,14], but they rely on column-switching and gradient elution. We have developed a simple, isocratic liquid chromatographic procedure for the simultaneous determination of etretinate, all-*trans*-acitretin and 13-*cis*-acitretin and applied it to blood samples from patients previously treated with etretinate for a long period but now on therapy with acitretin. In this group, the possible effect of daily administration of all-*trans*-acitretin on the late-phase elimination of etretinate was investigated.

EXPERIMENTAL

Laboratory precautions

All handling of reference compounds and biological samples was performed in a darkened room, illuminated with yellow lights. Whenever possible, amber-coloured containers were used.

Materials and reagents

Etretinate, all-*trans*-acitretin, 13-*cis*-acitretin, 4-oxoretinoic acid and 13-demethylretinoic acid all were generously supplied by Hoffmann-La Roche (Basle, Switzerland) and were stored under nitrogen at −20°C. Retinyl propionate (internal standard) was obtained from Fluka (Buchs, Switzerland), dichloromethane, isopropanol, acetic acid, ethanol and hydrochloric acid (all of analytical-reagent grade) from E. Merck (Darmstadt, F.R.G.) and *n*-hexane and tetrahydrofuran (THF) from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Water was of HPLC grade (Prosan, Ghent, Belgium).

Blood samples were collected in Monoject tubes containing ammonium and potassium oxalate (Sherwood Medical, St. Louis, MO, U.S.A.). The plasma was stored at −80°C until analysis.

Chromatographic systems

We used a Model 2150 solvent-delivery system (LKB, Bromma, Sweden), a Model 7125 injection valve (Rheodyne, Cotati, CA, U.S.A.) equipped with a 50-μl loop and a variable-wavelength detector (Pye Unicam, Cambridge, U.K.) set at 350 nm. Chromatograms were recorded on a PM 8251 recorder (Philips, Eindhoven, The Netherlands). Analyses were carried out on a 15 × 0.46 cm I.D. Spherisorb S5W 5-μm column from Chrompack (Merksem, Belgium). Other columns tested were packed with RoSil C_{18} particles (15 × 0.32 cm I.D.) (RSL, Eke, Belgium), Zorbax C_8 5-μm packing material (25 × 0.46 cm I.D.) from DuPont (Wilmington, DE, U.S.A.) or CP-Spher Si 5-μm particles (25 × 0.46 cm I.D.) from Chrompack.

Extraction

After addition of retinyl propionate (10 μl of a 9.8 μg/ml solution in methanol) as an internal standard to 0.5 ml of plasma, the proteins are denatured with 1.5 ml of ethanol and the mixture is acidified with 0.5 ml of 2 *M* HCl and vortex-mixed for 30 s. Water (5 ml) is added and the samples are vortexed again for 30 s. The extraction is then performed with 7.5 ml of *n*-hexane on a rotary mixer (Cenco Instruments, Breda, The Netherlands) for 15 min. After centrifugation (6 min at 1500 *g*), the upper organic layer is transferred into another conical tube and evaporated to dryness at room temperature under a stream of nitrogen. The residue is dissolved in 150 μl of the chromatographic solvent and a 50-μl aliquot is injected.

Calibration and calculation

For each retinoid, a calibration graph consisting of four different concentrations within the expected range of the plasma samples was generated by least-squares regression of the peak-height ratios (added drug/internal standard *versus* the concentration of the drug). The unknown concentrations of the plasma samples were then determined from the calculated peak-height ratio by interpolation from this calibration graph. Calibration samples were prepared by spiking 0.5 ml of blank plasma with appropriate amounts (5, 10, 15 and 20 μl) of methanolic solutions of etretinate (2.1 μg/ml), all-*trans*-acitretin (2.4 μg/ml) and 13-*cis*-acitretin (7.3 μg/ml). Subsequently, the calibration samples were treated in an identical way to the unknowns.

Precision

Within-day precision was estimated from the analysis of nine 0.5-ml aliquots from the same plasma sample, and day-to-day reproducibility was established from the variation of the results of analyses of aliquots from a plasma pool over more than 3 months.

RESULTS AND DISCUSSION

We have already reported the quantification of etretinate and its main metabolite (acitretin) on an RSIL 5-μm column with *n*-hexane–acetonitrile–acetic acid

(99.5:0.2:0.3) (all mixture proportions by volume) as eluent[12]. However, under these conditions the 13-*cis*-isomer of acitretin coeluted with acitretin itself. Later it became clear that 13-*cis*-acitretin was not only the main metabolite of all-*trans*-acitretin itself, but that it was also present in plasma samples from patients under long-term therapy with etretinate.

On a Spherisorb S5W 5-μm column eluted with *n*-hexane–THF–acetic acid–acetonitrile (89.5:10:0.3:0.2) we were able to separate all-*trans*- from 13-*cis*-acitretin and from 4-oxoretinoic acid (internal standard)[15]. However, under these conditions, etretinate eluted too close to the solvent front. Lowering the percentage of THF or even replacing the THF with either diisopropyl ether (5%) or dichloromethane (5%) did not yield better separations. On the other hand, on a RoSil reversed-phase C_{18} column (5 μm, 15 × 0.32 cm I.D.) eluted with acetic acid–water–methanol or acetonitrile (0.2:15:85), *cis*- and all-*trans*-acitretin were only partially separated whereas the internal standard (4-oxoretinoic acid) showed almost no retention. Another possible internal standard (13-demethylretinoic acid) coeluted with acitretin itself. Also, on a Zorbax C_8 5-μm column (25 × 0.46 cm I.D.) eluted with a non-aqueous mobile phase (methanol–acetonitrile–acetic acid, 10:90:0.2) the isomers of acitretin were not separated. Finally, we eluted a 25 × 0.46 cm I.D. CP-Spher Si 5-μm column with dichloromethane–*n*-hexane–acetic acid mixtures. By lowering the concentration of, and finally omitting, the *n*-hexane, the separation between etretinate and all-*trans*- and 13-*cis*-acitretin was improved.

However, the choice of a suitable internal standard was still a problem. 4-Oxoretinoic acid did not dissolve easily in the eluent, 13-demethylretinoic acid coeluted with acitretin and retinyl acetate was not completely separated from etretinate. Finally, retinyl propionate proved to be a suitable internal standard for our analysis. Fig. 2A illustrates the net separation of the different retinoids under the latter analytical conditions. The sample was from a psoriatic patient previously treated with etretinate, but now on therapy with all-*trans*-acitretin. The trace of a blank plasma sample showed no interfering peaks on the chromatogram at the elution positions of the compounds of interest (Fig. 2B).

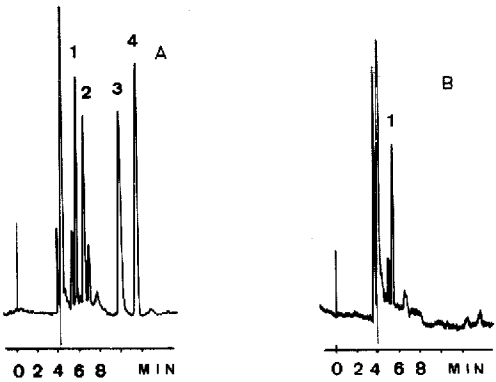


Fig. 2. (A). Representative chromatogram of a plasma sample from a patient previously on etretinate, but now on all-*trans*-acitretin treatment. Peaks: 1 = internal standard; 2 = etretinate; 3 = 13-*cis*-acitretin; 4 = all-*trans*-acitretin. The levels were 17, 24 and 26 ng/ml for etretinate, 13-*cis*-acitretin and all-*trans*-acitretin, respectively. (B) Trace of a blank plasma sample.

TABLE I

WITHIN-DAY AND DAY-TO-DAY REPRODUCIBILITY DATA

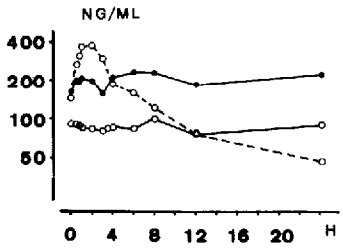
Compound	*Mean level (ng/ml)*	*n*	*Relative standard deviation (%)*
Etretinate:			
Within-day	128	9	3.2
Day-to-day	53	13	6.7
All-*trans*-acitretin			
Within-day	53	9	11.7
Day-to-day	95	13	13.6
13-*cis*-acitretin			
Within-day	261	9	6.5
Day-to-day	149	13	9.1

Assay performance

The precision was determined for etretinate and all-*trans*- and 13-*cis*-acitretin and the results are given in Table I. For all compounds the method was linear up to at least 400 ng/ml, corresponding to the plasma concentrations found during therapy (correlation coefficients >0.99). Further, the intercepts of the calibration graphs did not differ significantly from zero. The detection limit for the three retinoids was estimated to be 3 ng/ml. The extraction recovery exceeded 80% for all compounds.

Applications

The HPLC procedure was applied to the determination of the three retinoids in samples from psoriatic patients. The time–concentration courses for etretinate and all-*trans*- and 13-*cis*-acitretin in plasma from a patient who had received 25 mg of all-*trans*-acitretin daily for 160 days are presented in Fig. 3. The etretinate found in the plasma results from the slow release of this drug accumulated in the adipose tissue during previous treatment of this patient with etretinate. It can be seen that even after 160 days after changing the therapy from etretinate to all-*trans*-acitretin, etretinate is still present at a level of 70–80 ng/ml.



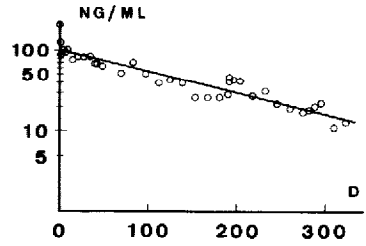


Fig. 3. Observed concentrations of etretinate (○——○), 13-*cis*-acitretin (●——●) and all-*trans*-acitretin (○- - -○) following an oral dose of 25 mg of acitretin 160 days after switching over from a daily dose of 25 mg of etretinate to a daily dose of 25 mg of all-*trans*-acitretin.

Fig. 4. Concentration–time profile of etretinate after the last dose of etretinate (day zero). After day zero, the patient received a daily alternate oral dose of 25 and 50 mg of all-*trans*-acitretin.

We also applied the method to a long-term pharmacokinetic study of etretinate in the same group of patients previously treated with etretinate and now on therapy with all-*trans*-acitretin. Blood samples were taken every 2 weeks over more than 300 days starting from day zero (change in therapy). Fig. 4 shows the time course of etretinate in such a patient. The calculated half-life for etretinate is 120 days.

Up to now, more than 500 samples have been analysed on the same HPLC column, clearly demonstrating that the method can be used for routine determinations. Additionally, the long-term pharmacokinetic study of etretinate has revealed once again the need for serial determinations of this teratogenic retinoid, especially in those patients who may consider a future pregnancy.

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