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Improved quantitation of 13-*cis*- and all-*trans*-acitretin in human plasma by normal-phase high-performance liquid chromatography

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ABSTRACT

A reliable analytical method has been developed for measurement of 13-*cis*- and all-*trans*-acitretin (Neotigason) in human plasma by normal-phase high-performance liquid chromatography, with ultraviolet detection. Human plasma was obtained after centrifugation of whole blood samples and deproteinized by ethanolic denaturation. After liquid–liquid extraction with water–*n*-hexane, an aliquot was chromatographed on a silica column using isocratic elution with *n*-hexane–methylsalicylate–acetic acid (200:18:0.6, v/v). The wavelength was set at 360 nm, and for plasma samples a limit of quantification of 3–4 ng/ml was obtained. All manipulations were carried out under dim light conditions to prevent photoisomerization.

INTRODUCTION

Acitretin (Ro 10-1670, Neotigason) is a synthetic second-generation retinoid that proved to be a very efficient drug when applied to cases of resistant psoriasis and in various disorders of keratinization [1]. The pharmacokinetic behaviour of acitretin in the plasma of psoriatic patients treated with this drug, has been studied extensively [2–4]. We have previously described a high-performance liquid chromatographic (HPLC) system allowing sensitive measurements and pharmacokinetic studies of acitretin [5–7].

Recently, our laboratory entered a multi-centre inter-laboratory quality control study on the pharmacokinetics of all-*trans*-acitretin in the plasma of psoriatic patients. Reliable analytical methods are important for the monitoring of all patients under treatment with drugs, especially if those drugs have severe side-effects. In the case of acitretin, which is a teratogenic molecule, it is important to stress the detectability of low-nanogram levels [8].

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Already during the first quality control round, we had difficulties with the chromatography performed according to the original publication [3]. In order to meet the very rigorous criteria that were set for this study, implicating also the aspect of automation, we decided to adapt our system. We report here an optimized method for HPLC analysis of acitretin in human plasma, which proved to be very suitable for routine analysis.

EXPERIMENTAL

Materials

All solvents were of analytical-reagent grade. Absolute ethanol was supplied by Riedel-de Haën (Seelze, Germany), water and *n*-hexane by Prosan (Eke, Belgium), methylsalicylate by Fluka (Buchs, Switzerland) and acetic acid by Lab Chemistry (Geer, Belgium). Both isomers of acitretin, *i.e.* the 13-*cis* (Ro 13-1672) and the all-*trans* form (Ro 10-1670), as well as the internal standard (13-demethylretinoic acid), were generous gifts from F. Hoffmann-La Roche (Basel, Switzerland) and were stored under nitrogen at −20°C. The analytical column was purchased from Chrompack (Merksem, Belgium).

Sample preparation

Blood samples were obtained from the Department of Dermatology, where they were kept in black plastic bags at 4°C immediately after prelevation. After being centrifuged at 1500 *g* for 10 min, potassium and ammonium oxalate plasma was stored at −20°C until processing. As previously described [7], calibration samples were prepared by spiking 0.5 ml of blank plasma with appropriate amounts of methanolic solutions of standards. Subsequently, the calibration samples were treated similarly to the unknowns.

To 0.5 ml of plasma spiked with 10 μl of the internal standard solution, 1.5 ml of absolute ethanol were added and proteins were denatured during 30 s of full-speed vortex-mixing. Then 0.5 ml of 2 *M* aqueous HCl and 5.0 ml of HPLC water were added, followed by another 30-s period of vortex-mixing. After 7.5 ml of *n*-hexane were added to the aqueous mixture, the two-phase extraction was performed during 15 min on a rotating mixer. The low pH of the aqueous layer was essential to obtain the carboxylic acid end-group of the retinoids in the undissociated, hydrophobic form. The HPLC water had to be added in order to obtain the desired degree of polarity of the ethanolic aqueous phase for an optimal extraction of the compounds of interest. The structures are shown in Fig. 1.

Instrumentation

HPLC was performed using the same chromatographic system as described previously [7], except for the components necessary to automate the procedure. For this purpose, the following features were included: a WISP autosampler (Waters), a 3396A integrator and a 9114B disc drive (Hewlett Packard). Thus

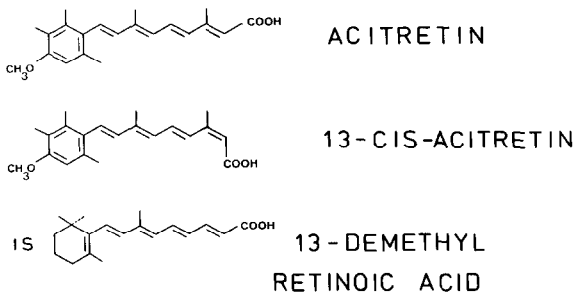

Fig. 1. Structures of acitretin (all-*trans*-form), 13-*cis*-acitretin, and the internal standard (IS).

injections could be performed automatically, and chromatographic profiles, peak retention times and integrated peak heights were stored and could easily be processed statistically.

Chromatography

Originally, we used a Chromspher silica column (15 cm × 0.46 cm I.D., 5 μm particle size) [7]. The mobile phase contained 2-propanol as a modifier and consisted of dichloromethane–2-propanol–acetic acid (250:0.55:0.4, v/v). Capacity factors for the three peaks of interest *i.e.* 13-*cis*-, all-*trans*-acitretin and the internal standard, were satisfactory. The isomers were clearly separated, and initially a good baseline was observed. From the start, a major interfering peak could be observed in the chromatograms, very probably the endogenous retinol peak, as it coeluted with retinol standard. No coelution with any of the peaks of interest was observed. However, problems started when a second major interfering peak showed up. Together with the first one, it consistently bracketed the region of the chromatogram containing the peaks of interest. Also, it became more and more difficult to produce a proper baseline, owing to some small interfering peaks that were coeluting with the compounds of interest (Fig. 2). Those problems provoked

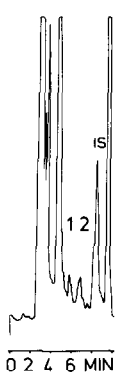

Fig. 2. Typical "problem" chromatogram, demonstrating the bracketing phenomenon. The shapes of all three peaks are unacceptable because of poor baseline stability. Peaks: 1 = 13-*cis*-acitretin; 2 = all-*trans*-acitretin; IS = internal standard.

us to find a proper solution, in order to obtain a reliable method for routine analysis and quality-control purposes.

First, we checked both the analytical column by using other packing materials, and the purity of our reagents by extracting them without plasma sample and the eluent by direct injection. Then we started to modify the ratios of the components of the mobile phase, in order to vary the retention of the interfering peaks according to the amount of modifier. This approach was not successful, as the compounds of interest started to migrate together with the interfering peaks and the selectivity was not improved. Because of these observations, and the fact that the highly compressible and reactive dichloromethane caused problems, such as trapping of air bubbles in the pump-heads (even after degassing by vacuum combined with ultrasonication), we changed to a totally different mobile phase composition with a more convenient main component, namely *n*-hexane.

We began with a mixture of *n*-hexane, methylsalicylate, ethyl acetate and propionic acid. However, the decision was made to omit ethyl acetate, because it always contains a minor percentage of water, and accumulation of water on a normal-phase column should be avoided at all times. We also returned to our initial acid (acetic acid) instead of propionic acid. As for the methylsalicylate, this oily substance was essential to obtain a baseline separation between the *cis* and the *trans* isomers.

Varying the relative amounts of the methylsalicylate and the acetic acid fractions, we obtained acceptable capacity factors (2.9 for the internal standard, 3.6 for 13-*cis*- and 4.0 for all-*trans*-acitretin) with a mobile phase composition of *n*-hexane–methylsalicylate–acetic acid (200:18:0.6, v/v).

Precision

Day-to-day reproducibility was established from the variation of the results of the analysis of aliquots from three pools of quality control levels over more than four months. Within-day precision could be estimated from the analysis of four aliquots of each of those levels.

RESULTS

Sample preparation and assay performance

As the extraction procedure did not differ from the previously described method, we refer to that publication for any details [7]. To validate the new method, the precision for all-*trans*- and 13-*cis*-acitretin was determined. The results are given in Table I.

Method development and peak identification

Under the modified chromatographic conditions, the order of elution of the peaks changed. A typical chromatogram is shown in Fig. 3. Compared with the initial system (Fig. 2), the internal standard is now the first-eluting peak instead of the third one. The 13-*cis* and all-*trans* isomers still elute in the same order.

TABLE I

WITHIN-DAY AND DAY-TO-DAY REPRODUCIBILITY DATA

Compound	Mean level (ng/ml)	*n*	Coefficient of variation (%)
All-*trans*-acitretin			
Within-day	5.0	4	4.7
	52.7	4	1.9
	541.5	4	1.4
Day-to-day	5.0	19	22.0
	50.4	23	8.1
	538.4	15	8.2
13-*cis*-acitretin			
Within-day	4.5	4	3.4
	39.2	4	1.7
	415.5	4	2.9
Day-to-day	4.0	20	21.9
	39.3	23	5.4
	418.7	15	7.8

This was verified by an experiment based on the principle of coelution. A plasma sample from a psoriatic patient was injected on the first chromatographic system. Then both peaks were collected separately, evaporated under nitrogen, redissolved and subsequently reinjected on the second HPLC system. The retention times of both isomers were identical with those of pure standards in both systems.

Additional confirmation of peak identity was obtained by a second experiment. This consisted of a stepwise (10 nm) variation of the wavelength in a range

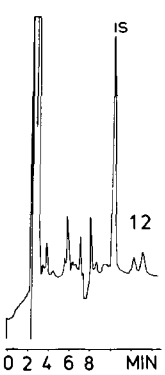

Fig. 3. Lowest calibration point obtained under the new conditions. Concentrations added were 3.73 and 4.19 ng/ml for 13-*cis*- and all-*trans*-acitretin, respectively. Peaks as in Fig. 2.

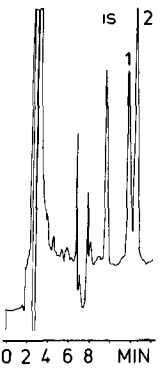

Fig. 4. Representative chromatogram of a plasma sample from a patient on a 30-mg daily dosing regimen. The levels were 34.8 and 53.5 ng/ml for 13-*cis*- and all-*trans*-acitretin, respectively. Peaks as in Fig. 2.

from 320 to 420 nm. The same plasma sample was reinjected eleven times and the absorption was recorded each time. For both isomers a typical spectrum was obtained, comparable with that of a standard, showing one maximum at *ca.* 360 nm.

Calibration curves for both 13-*cis*- and all-*trans*-acitretin are linear from 0 up to at least 500 ng/ml. Least-squares regression analysis performed on the relationship between peak-height ratio (y) and concentration of the standards (x) gave a high correlation ($r > 0.995$). The reproducibility of our equation and the coefficient of variation was improved by pooling the calibration graphs.

Some minor differences between the two systems remain to be mentioned. We now use a slightly higher flow-rate, *i.e.* 0.85 instead of 0.7 ml/min. Wavelength settings changed from 350 to 360 nm at a detector sensitivity of 0.01 a.u.f.s. Fig. 4 shows a typical chromatogram of a plasma extract from a psoriatic patient.

To cope with the extensive workload and to avoid a too labour-intensive schedule, we decided to partially automate the system. It was semi-automated by replacing the manual injector by a WISP autosampler, and the recorder by an integrator with disc drive. Extracts were evaporated to dryness with nitrogen, redissolved in mobile phase, and kept in a cooled (10°C) carousel in amber-coloured containers prior to injection. With the integrator, data acquisition and statistical calculations could be performed in a very smooth and flexible way.

DISCUSSION

From our experience we can once again confirm that plasma is a complex matrix to analyse. It contains many UV-absorbing and lipophilic molecules, which may influence the accurate monitoring of UV-absorbing drugs in samples from patients. Even the choice of a selective wavelength like 360 nm cannot prevent such interferences. With the change of detection wavelength from 350 to

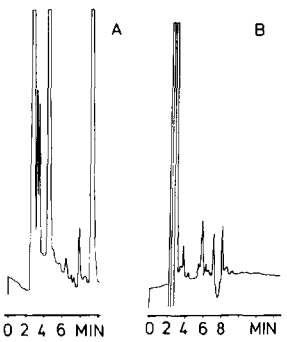

Fig. 5. Chromatograms of blank plasma samples, injected on (A) the first and (B) the second chromatographic system.

360 nm, we managed only to decrease the height of some peaks, such as the late-eluting and thus potentially interfering retinol peak. The sensitivity for the peaks of interest, however, was not affected significantly by this change.

Using the optimized chromatographic conditions, we believe we now have a reliable system for the analysis of acitretin in psoriatic patients on a routine basis. As demonstrated in Figs. 3 and 4, the peak shapes of the compounds have again become symmetric. Comparison of a blank plasma extract run in both the original and the new system (Fig. 5A and B) clearly reveals the improvement achieved with the second method. Furthermore, the important decrease in the signal-to-noise ratio allows the use of calibration graphs with a limit of quantification of 3–4 ng/ml.

The gain in baseline stability has allowed us to start to examine the extraction of biological fluids other than plasma, and skin samples. For these purposes we now use 0.005 a.u.f.s., *i.e.* the maximum detector sensitivity, and a lower calibration point (1–2 ng/ml) for our calibration.

In conclusion, the reliability of the improved and semi-automated chromatographic system should help to improve the quality of routine analysis of acitretin levels in plasma. For this kind of drug, where low-nanogram levels are of the utmost importance with regard to its teratogenic side-effects, future developments should be focused on reaching even greater sensitivity and accuracy.

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