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Quantification of acitretin in human plasma by microbore liquid chromatography–negative chemical ionization mass spectrometry

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ABSTRACT

A highly sensitive liquid chromatographic–mass spectrometric procedure has been developed to quantitate plasma concentrations of acitretin, a dermatologic agent used to treat severe psoriasis. The assay utilizes the combination of normal-phase microbore high-performance liquid chromatography, negative chemical ionization mass spectrometry, selective ion monitoring and stable isotope dilution. The method has been used to measure acitretin and its metabolite, 13-*cis*-acitretin, over a range of 1–20 ng/ml in human plasma. The inter-assay precision was 5.3% for acitretin and 3.9% for 13-*cis*-acitretin, while the intra-assay precisions for acitretin and 13-*cis*-acitretin were 10.8 and 12.7%, respectively. Reproducibility of the assay for acitretin and 13-*cis*-acitretin, which was determined by the relative standard deviation of multiple analyses of the same quality assurance sample, was 5.9 and 8.1%, respectively.

INTRODUCTION

The aromatic retinoid, etretinate (Fig. 1), has been shown to be useful in the treatment of psoriasis. Etretinate, a highly lipophilic compound [1], has a terminal elimination half-life in man of 100–120 days [2,3]. The long half-life has been attributed to etretinate being stored in adipose tissue and then slowly released. Etretinate, like all other retinoids, is teratogenic. Thus, women of childbearing age have to use contraceptives during and two years after treatment with etretinate. As a consequence, etretinate has limited use in women of childbearing age.

The main metabolite of etretinate is its enzymatic hydrolysis product, acitretin (Fig. 1) [4]. Several clinical studies have determined that acitretin is as efficacious as its prodrug, etretinate, in the treatment of psoriasis [5–8]. From preliminary results, it has been determined that the half-life of acitretin (50–57 h) is much shorter than that of etretinate [9,10]. Because acitretin is also teratogenic, it is important to accurately measure the terminal elimination half-life of both acitretin and its main metabolite, 13-*cis*-acitretin (Fig. 1) with a sufficiently sensitive and reproducible analytical method. Several analytical methods for determining plasma concentrations of both compounds have been published. These methods include high-performance liquid chromatography (HPLC) methods with UV de-

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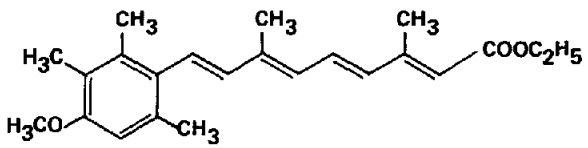
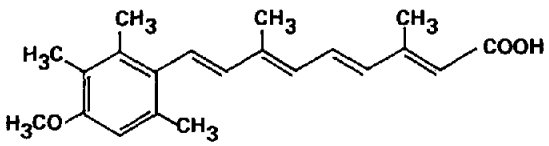
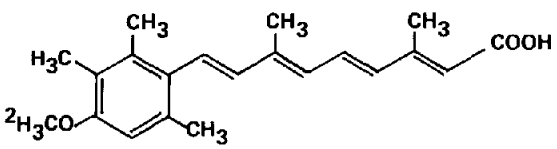


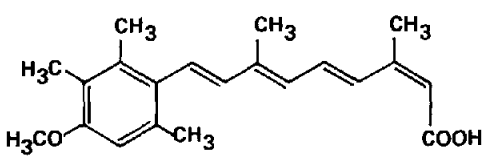
ETRETINATE



ACITRETIN



TRIDEUTERATED ACITRETIN



13-CIS ISOMER OF ACITRETIN

Fig. 1. Structures of compounds of interest.

tection [11,12] that either lack sensitivity (limits of detection between 10 and 20 ng/ml) or do not report on assay characteristics (precision, reproducibility, etc.) at their limit of quantitation [13,14]. Al-Mallah and co-workers [15,16] published a sensitive reversed-phase HPLC–UV method that measures acitretin in human plasma. However, this technique requires a large volume (between 0.5 and 3 ml) of plasma.

In this report we describe a method that combines the resolving power of normal-phase HPLC and the sensitivity of negative chemical ionization mass spectrometry (NCI-MS) to quantitate low concentrations of acitretin and its metabolite, 13-*cis*-acitretin, in human plasma.

EXPERIMENTAL

Materials and reagents

HPLC-grade methanol, hexane, benzene, water and toluene were obtained from Burdick and Jackson (Muskegon, MI, USA). Ethanol (200 proof) was purchased from Quantum Chemical Corporation (Newark, NJ, USA). Ultrex hydrochloric acid and acetic acid were obtained from J. T. Baker (Phillipsburg, NJ, USA). α-Bromo-2,3,4,5,6-pentafluorotoluene (PFBBr) was purchased from Aldrich (Milwaukee, WI, USA). Potassium acetate was purchased from Fisher Scientific (Fairlawn, NJ, USA) and 18-crown-6-ether was purchased from Eastman Kodak (Rochester, NY, USA). Acitretin and 13-*cis*-acitretin were provided by Quality Control, Hoffmann-La Roche (Nutley, NJ, USA). Trideuterated acitretin was obtained from Dr. A. Liebman, Department of Isotope Synthesis, Hoffmann-La Roche. All procedures were performed under yellow lights, including the LC–MS analysis. All glassware was amberized.

Solutions and standards

Stock solutions of acitretin and 13-*cis*-acitretin were prepared in 10-ml volumetric flasks by dissolving approximately 1 mg in 10 ml of methanol. The stock solutions were diluted 1:10 with ethanol in 10-ml volumetric flasks. Appropriate amounts of the diluted stock solutions of acitretin or 13-*cis*-acitretin were diluted to give calibration solutions in the range 0.02–0.4 ng/μl. A stock solution of trideuterated acitretin (reference standard) was prepared in a 10-ml volumetric flask by dissolving approximately 0.5 mg in 10 ml of methanol. An appropriate amount of this stock solution was diluted with ethanol to give a final concentration of 0.2 ng/μl. A 0.5% PFBBr solution was prepared by adding 50 μl of PFBBr to 10 ml of benzene containing 150 mg of 18-crown-6-ether.

Preparation of samples

The calibration curve of 1, 2, 5, 10 and 20 ng/ml solutions was obtained by adding known amounts of acitretin (25 μl), 13-*cis*-acitretin (25 μl) and the trideuterated reference standard (25 μl) to 500 μl of plasma. The patient samples were prepared by adding 50 μl of ethanol and 25 μl of the trideuterated reference standard to 500 μl of the sample. The quality assurance samples, which contained 12.50 ng/ml acitretin and 13-*cis*-acitretin, were prepared by adding 50 μl of ethanol and 25 μl of the reference standard to 500 μl of the quality assurance sample.

Extraction and analytical HPLC isolation

Calibration, quality assurance and experimental samples were acidified with 500 μl of 0.2 *M* methanolic HCl and then extracted three times with 500 μl of hexane according to the method of Napoli *et al.* [17]. The combined extracts were evaporated to dryness under a stream of nitrogen, reconstituted with 200 μl of acetonitrile and analytes isolated by reversed-phase HPLC. The HPLC system

consisted of a Model 6000 A solvent delivery system, a WISP Model 710B autosampler (both from Waters Assoc., Milford, MA, USA), an Applied Biosystems (Foster City, CA, USA) 783 programmable absorbance detector and a Soltec (San Fernando, CA, USA) 1242 chart recorder. A 195-μl aliquot of the sample was injected onto a Dupont (Wilmington, DE, USA) Zorbax-ODS (C_{18}) column (15 cm × 4.6 mm I.D.). The mobile phase was acetonitrile–water–acetic acid (75:24:1, v/v). The flow-rate was 1.5 ml/min. Acitretin and 13-*cis*-acitretin, which eluted as one peak at 6 min (detected at 365 nm, A.U.F.S. = 0.005), were collected. The retention time was determined by injecting external standards prior to the isolation of experimental samples.

Derivatization

The HPLC eluates were dried in a Savant (Farmingdale, NY, USA) Speed Vac and then were derivatized to their pentafluorobenzyl esters by adding 200 μl of the 0.5% PFBBr solution and a few crystals of potassium acetate according to the method of Rubio and Garland [18]. The derivatized samples were evaporated to dryness under a stream of nitrogen, solubilized in 1 ml of hexane and then centrifuged at 2560 *g* at 4°C for 10 min. The upper 900 μl were removed and filtered through a Cameo Nylon filter (0.45 μm) from MSI (Westboro, MA, USA). The samples were stored in the freezer at −20°C.

Normal-phase LC–NCI-MS

The derivatized samples were evaporated under a stream of nitrogen and then dissolved in 20 μl of mobile phase for LC–MS analysis. The chromatography was performed using an Applied Biosystems (Foster City, CA, USA) Microgradient System HPLC pump, which was interfaced to a 3200 Finnigan (San Jose, CA, USA) mass spectrometer modified for direct liquid introduction. The probe and mass spectrometer have been previously described [19,20]. The sample was injected using a microbore Rheodyne hand injector (Model number 8125, Cotati, CA, USA) onto a 25 cm × 1 mm I.D. diol column (obtained from E.S. Industries, Marlton, NJ, USA) and eluted isocratically using 35% toluene in hexane at a flow-rate of 50 μl/min. Eluate entered the source through 1 m of deactivated silica capillary tubing from SGE (Dallas, TX, USA) (I.D., 60 μm; O.D., 0.2 mm), which was connected to the microbore column and threaded through a 13-mm probe that was heated to 300°C. The Finnigan CI source was modified by the addition of two cartridge heaters. Excess eluate was removed from the source by a mechanical vacuum pump connected directly opposite the eluate entrance. The mass spectrometer was equipped with a Teknivent (St. Louis, MO, USA) data system for data analysis. Peak-height and peak-ratio data were calculated using a weighted linear analysis program [21].

RESULTS

Mass spectra

Fig. 2 illustrates the NCI mass spectrum of acitretin. The most intense ion is at m/z 325, which corresponds to the $[M-PFB]^-$ ion. A smaller fragment occurs at m/z 278, which is most likely due to $[M-PFB-CO_2]^-$.

Sensitivity

The limit of quantification of acitretin in human plasma samples is 1 ng/ml. A selected ion current profile for blank plasma fortified only with the reference standard (trideuterated acitretin) gave a signal only at the mass of the reference standard (Fig. 3A). Fig. 3B is a selected current ion profile of plasma fortified with 1 ng/ml of both acitretin and 13-*cis*-acitretin and 10 ng/ml trideuterated acitretin. Fig. 3C represents a selected ion current profile of a patient sample that contained 16 ng/ml acitretin.

Precision and reproducibility

The inter-assay precision for acitretin and 13-*cis*-acitretin, determined by the overall relative standard deviation of the difference between the amount found and added, was 5.3 and 3.9%, respectively (three calibration curves, Table I). The intra-assay precisions, estimated from the overall relative standard deviation of the ratio of duplicate analyses of the calibration standards and the quality assur-

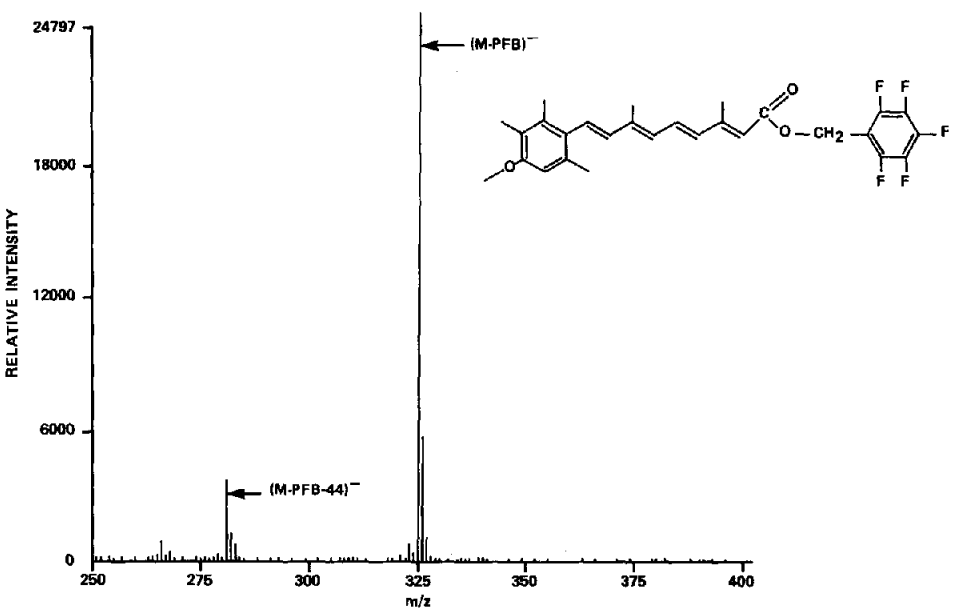


Fig. 2. NCI mass spectrum of the pentafluorobenzyl (PFB) derivative of acitretin.

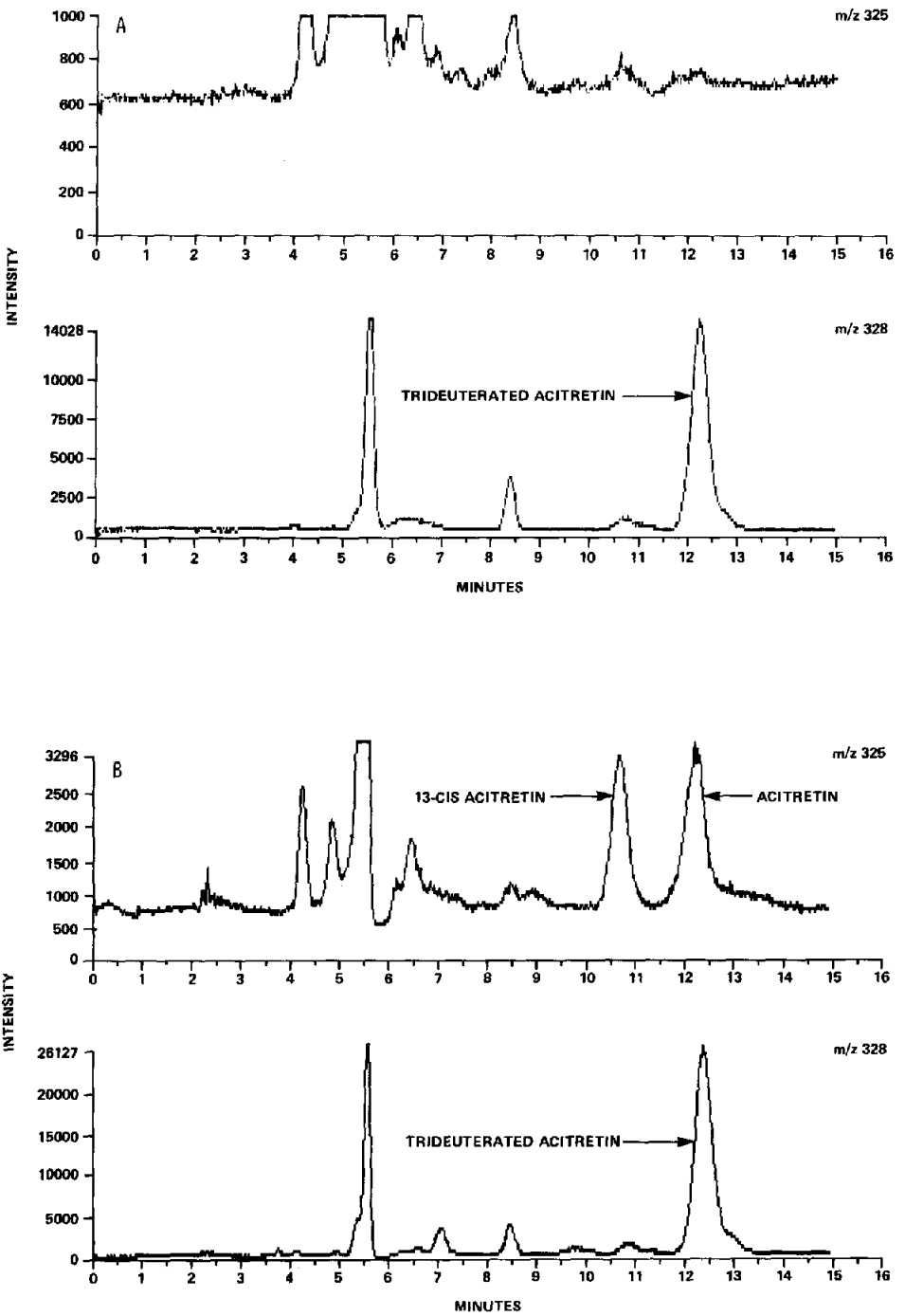

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Fig. 3.

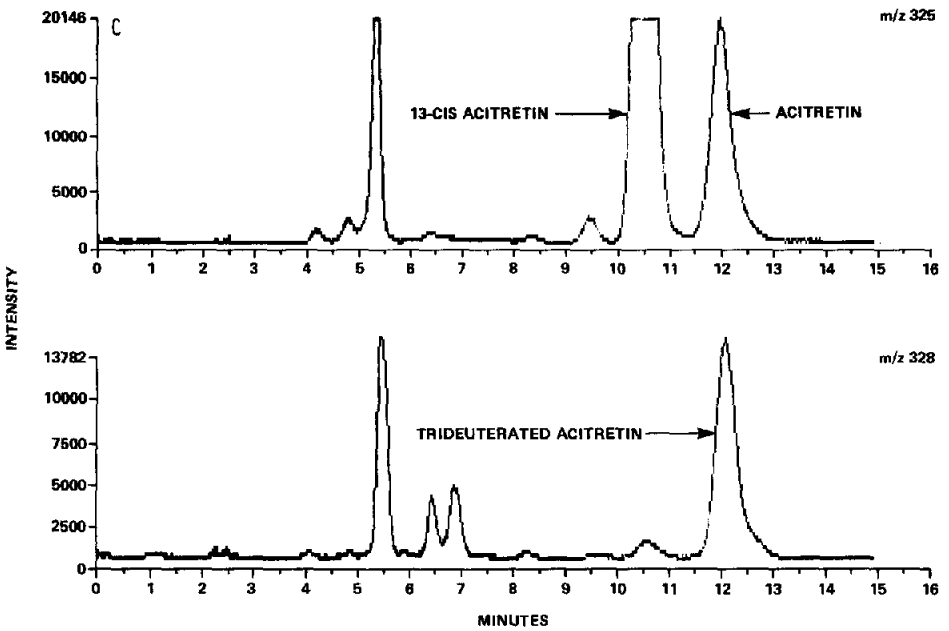

Fig. 3. Selected ion current profiles of (A) a drug-free plasma fortified with 10 ng/ml reference standard, trideuterated acitretin, (B) a low standard containing 1.0 ng/ml acitretin, 1.0 ng/ml 13-*cis*-acitretin and 10 ng/ml trideuterated acitretin and (C) a patient sample containing 16 ng/ml acitretin.

TABLE I

INTER-ASSAY PRECISION FOR ACITRETIN AND 13-*cis*-ACITRETIN

Concentration added (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	R.S.D. (%)
Acitretin		
1	1.10 ± 0.08	6.90
2	1.96 ± 0.13	6.80
5	4.78 ± 0.29	6.03
10	9.50 ± 0.35	3.70
20	20.99 ± 0.60	2.86
Overall R.S.D.[a]		5.26
13-cis-Acitretin		
1	1.00 ± 0.04	3.71
2	2.12 ± 0.09	4.21
5	4.70 ± 0.31	6.65
10	10.42 ± 0.32	3.08
20	19.99 ± 0.38	1.88
Overall R.S.D.[a]		3.91

[a] The overall R.S.D. was determined from three calibration curves. Each calibration standard on a calibration curve was assayed in duplicate.

ance samples, were 10.8 and 12.7% for acitretin and 13-*cis*-acitretin, respectively (data not shown). All calibration curves were linear over the range 1–20 ng/ml. The average of the correlation coefficients was >0.99. The reproducibilities of the assay for acitretin and 13-*cis*-acitretin, determined by the relative standard deviation of multiple analysis of the same quality assurance sample, were 5.9 and 8.1%, respectively.

Stability

As with most retinoids, acitretin is an ultraviolet light-sensitive compound [22]. Therefore, all sample handling was done under yellow lights and all the glassware was amberized. The results of the short-term stability study, which was accomplished by the method of Timm *et al.* [23], suggest that acitretin is stable on the benchtop at room temperature for 6 h. The results for the medium-term stability study suggest that acitretin is also stable when stored in the freezer at $-20°C$ for at least two months (see Table II).

Recovery

A recovery experiment was performed in which ^{14}C-labeled acitretin was added to human plasma containing 5 ng/ml of both acitretin and 13-*cis*-acitretin and the reference standard. The above extraction procedure was performed up to and including the HPLC purification step. No LC–MS analysis was performed. The HPLC radioactivity profile was obtained on-line using the FLO-One/Beta radioactive flow detector from Radiomatic, Camberra (Tampa, FL, USA) (Model A200). An average recovery of 79.7% was observed (see Table III).

Human plasma acitretin concentration–time profile

Psoriatic patients received 50 mg (two 25-mg capsules) of acitretin once daily

TABLE II

SHORT-TERM AND MEDIUM-TERM STABILITY STUDIES

Time[a] (h)	Concentration (ng/nl)	Difference	90% Confidence interval	
			Lower limit	Upper limit
Short-term: concentration of acitretin versus time at room temperature				
0	7.35	–	–	–
3	7.18	−2.34	−7.79	3.44
6	7.32	0.29	−8.25	8.36
Medium-term: samples stored in freezer (−20°C) for 67 days				
0	7.35	–	–	–
67 days	7.00	−4.79	−10.16	0.90

[a] Five stability samples were assayed at each time point.

TABLE III

RECOVERY OF [^{14}-C]ACITRETIN FROM CONTROL HUMAN PLASMA

Added 70 635 dpm per sample (specific activity 45 mCi/mmol) in 25 μl of methanol.

Sample No.	Total dpm in peak	Recovery (%)
1	51427	72.8
2	56965	80.6
3	61497	87.1
4	61059	86.4
5	50818	71.9
6	55958	79.2
Mean recovery		79.7 ± 6.5

for three weeks. Blood samples were collected on day 21 at 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 15, 18, 24, 36, 48, 72, 96, 120 and 144 h after the last dose. The plasma concentrations for the washout period were determined by an HPLC–UV method developed by Kneer [24]. In order to determine the terminal elimination half-life, the LC–MS assay was used to reanalyze patient samples that contained less than 20 ng/ml acitretin. There was no need to measure 13-*cis*-acitretin because all values exceeded 20 ng/ml. Fig. 4 shows the time-course of plasma concentrations below 20 ng/ml acitretin for one patient. The terminal elimination half-life for this patient was determined to be 55 h.

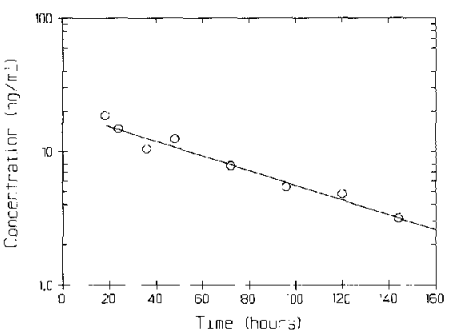

Fig. 4. Plasma concentration (below 20 ng/ml)–time profile for acitretin after the last dose of a multiple dosing regimen in a representative patient.

DISCUSSION

This paper describes a microbore LC–MS assay for the quantification of acitretin and its metabolite, 13-*cis*-acitretin. Studies conducted with radiolabeled acitretin indicate that the recovery is good. In addition, acitretin is quite stable when stored at −20°C for 60 days and for at least 6 h at room temperature.

Compared to many of the conventional HPLC – UV methods, this microbore LC–MS assay yields increased specificity and reproducibility with improved sensitivity. This increase in specificity and reproducibility is achieved by stable isotope dilution. Baseline separation of acitretin from its 13-*cis* metabolite is accomplished with the resolving power of normal-phase microbore HPLC.

Our experimental results indicate that this assay is suitable for defining the terminal pharmacokinetic behavior of acitretin in patients administered this antipsoriatic agent. An extensive evaluation of pharmacokinetic data obtained by this LC–MS technique will be the subject of a future publication.

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