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Quantitative analysis of retinoids in biological fluids by high-performance liquid chromatography using column switching

III. Determination of the arotinoid sumarotene and its *Z*-isomer in human and animal plasma

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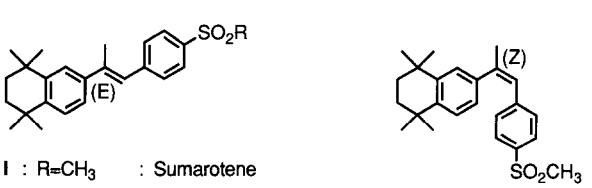
ABSTRACT

A fully automated and sensitive high-performance liquid chromatographic method, using on-line solid-phase extraction, automated column switching and ultraviolet detection, was developed for the third-generation retinoid (arotinoid) sumarotene {methyl *p*-[(*E*)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)propenyl]phenyl sulphone; Ro 14-9706} and its *Z*-isomer. Nearly quantitative recoveries for human, rat and dog plasma were obtained by addition of acetonitrile (final content *ca.* 17%) to the plasma sample prior to injection. No isomerization was observed when the samples were stored in the autosampler for more than 20 h. The injection volume was 0.5 ml, resulting in quantification limits of 1 ng/ml for sumarotene and 2 ng/ml for the *Z*-isomer. More than 40 injections could be made on to one precolumn, allowing routine overnight injections. Using a 1-ml injection volume, the limit of quantification for sumarotene could be improved to 0.5 ng/ml. The method was applied to toxicokinetic studies in rats and dogs, and was used to monitor human plasma samples after repeated topical application. The method could also be adapted to etarotene (Ro 15-1570), which was used as an internal standard, and which is at present in clinical development.

INTRODUCTION

Sumarotene {methyl *p*-[(*E*)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)propenyl]-phenyl sulphone; Ro 14-9706, I, Fig. 1} is a third-generation retinoid, which has been in clinical development for the treatment of photodamaged skin and has been tested as a topical agent against psoriasis, ichthyosis and acne. A sensitive assay was needed for its pharmacokinetic evaluation in laboratory animals and man. The many methods that had been developed for the determination of first-, second- and third-generation retinoids in biological samples were recently reviewed [1]. Only a few of these deal with third-generation retinoids, the so-called arotinoids. High sensitivity was obtained using gas chroma-



I : R=CH_3 : Sumarotene

III: R=CH_2CH_3 : Etarotene (int. standard)

II: Ro 18-6776

Fig. 1. Structures of the compounds.

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tography–mass spectrometry (GC–MS) for the arotinoid ethyl ester Ro 13-6298 {ethyl *p*-[(*E*)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)propenyl]benzoate} [2], the arotinoid free acid Ro 13-7410 {*p*-[(*E*)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)propenyl]-benzoic acid} [3] and the arotinoid ethyl sulphone Ro 15-1570 {ethyl *p*-[(*E*)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)propenyl]phenyl sulphone} [4]. Only one of these methods [3] was published in detail. For the latter, the remarkable quantification limit of 50 pg/ml in plasma samples could only be achieved after extensive sample clean-up, a derivatization step, sacrificing geometric isomer distinction and two-dimensional gas chromatography (GC). High-performance liquid chromatographic (HPLC) methods reported for temarotene were much simpler and more rapid. However, the quantification limits were much higher, namely 20 ng/ml using 1 ml of plasma [5] and 100 ng/ml using 0.2 ml of plasma [6]. For sumarotene (I), a quantification limit of 50 ng/ml using 0.5 ml of plasma was briefly reported [7].

In contrast, HPLC with on-line solid-phase extraction combines high sensitivity with minimum sample handling. As demonstrated for first-generation [8–11] and second-generation [9,12] retinoids, this technique also resulted in better precision. Using direct injection of large plasma volumes (1 ml), the sensitivity could be improved to the subnanogram per millilitre range [13,14]. This paper describes the successful application of the latter technique to sumarotene (I) and its-*Z*-isomer (II).

EXPERIMENTAL

Materials, reagents and solvents

Tetrahydrofuran (puriss. p.a. grade) was obtained from Fluka (Buchs, Switzerland), ethanol (HPLC grade) and ammonium acetate (analytical-reagent grade) from E. Merck (Darmstadt, Germany) and acetonitrile (HPLC grade S) from Rathburn (Walkerburn, UK). Water was distilled twice from an all-glas apparatus. Argon and helium were obtained from PanGas (Lucerne, Switzerland). Compounds I and II and the internal standard (etarotene, Ro 15-1570, III, Fig. 1) were provided by F. Hoffmann-La Roche (Basle, Switzerland) and were kept under argon at −20°C. Plasma standards were prepared using fresh frozen plasma either from sodium citrated human blood, which was obtained from a blood bank (Blutspendezentrum SRK, Basle, Switzerland) or from potassium oxalated rat and dog blood from our own laboratories.

Preparation of standards

The preparation of plasma standards and the dilution of the samples were performed under diffuse light conditions.

Two stock solutions were prepared in amber-coloured volumetric flasks by dissolving 20 mg of I and II in 2 ml of tetrahydrofuran and diluting to 20 ml with ethanol. Appropriate amounts of the two stock solutions were combined and diluted with ethanol to give working solutions in the range 100–0.1 μg/ml. Plasma standards were prepared from these working solutions by adding, *e.g.*, 0.1 ml to 10 ml of blank plasma, yielding concentrations of 1000, 500, 100, 20, 5, 2 and 1 ng/ml of plasma. The plasma standards were stored at −20°C.

A stock solution of the internal standard was prepared in an amber-coloured volumetric flask by dissolving 10 mg of III in 5 ml of tetrahydrofuran and diluting to 100 ml with ethanol. An internal standard working solution was prepared by diluting 0.5 ml of the stock solution with acetonitrile to 100 ml (500 ng/ml). This working solution was freshly prepared prior to use. The stock solution could be stored at 4°C for several months.

Chromatographic system and conditions

A schematic representation of the modular HPLC column-switching system is given in Fig. 2. A Model 420 LC pump (P1) (Kontron, Zurich, Switzerland) delivered mobile phase M1, which was used as the purge solvent at a flow-rate of 1.5 ml/min. Diluted plasma samples (0.5 ml) were injected with a WISP 712 automatic sample injector with cooling module (I1) (Waters, Milford,

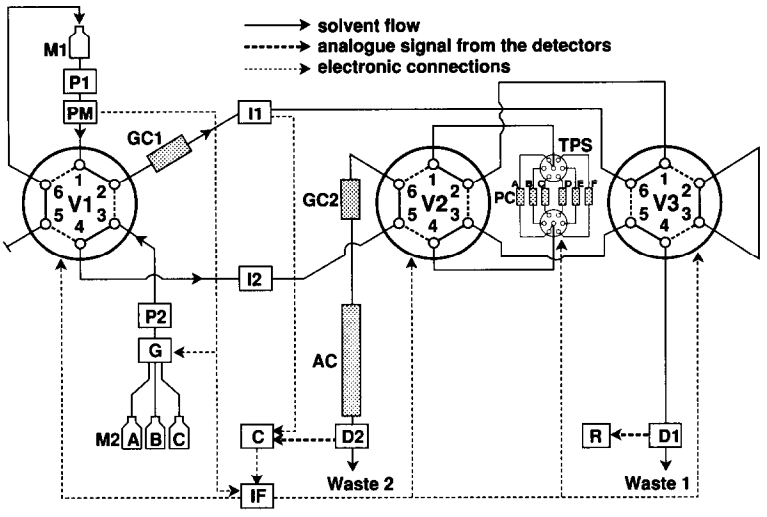

Fig. 2. Schematic representation of the HPLC column-switching system. Position of the valves: V1 = T5, V2 = T4 and V3 = T8. After valve switching the positions are defined as V1 = T6, V2 = T3 and V3 = T7 (see text for further details).

MA, USA) (10°C) on to one of the precolumns (PC). In order to inject sample volumes larger than 200 μl, the autosampler was used with a 1-ml syringe, the 2-ml auxiliary sample loop and a syringe motor rate of 1.85 μl/s. The UV detector D1 (Spectroflow 773; Kratos, Ramsey, NJ, USA), operating at 230 nm, together with a W+W 320 recorder (R) (Kontron) were used to monitor the removal of plasma components from the precolumn during the purge step; they were not needed for routine analysis. Pump P2 with a low-pressure gradient system (G) (Spectroflow 400 solvent-delivery system and 430 gradient former; Kratos) delivered mobile phase M2 (flow-rate 1.0 ml/min). A manual injector (I2) Model 7125 with a 500-μl loop; Rheodyne, Cotati, CA, USA) was used for direct injection on to the analytical column (*e.g.*, for recovery experiments). Detection of the eluted compounds was carried out at 303 nm with a UV detector (D2) (Spectroflow 783; Kratos) (rise time 1 s, range 0.01 a.u.f.s.), and integration was performed by means of a computing integrator (C) (Model SP 4200; Spectra-Physics, San Jose, CA, USA) (sensitivity 8 mV, chart speed 0.5 cm/min).

The gradient former (G) and the three pneumatically operated switching valves (V1–V3) (Model 7000P; Rheodyne), the latter connected to three solenoid valves (Model 7163; Rheodyne), were controlled by the external time events (T3–T8) of the integrator. To achieve compatibility, an interface (IF), produced in the electronic workshop at Hoffmann-La Roche, was placed between the integrator output and the solenoid valve input. The positions in Fig. 2 are V1 = T5 (alternative flow T6), V2 = T4 (T3) and V3 = T8 (T7). During injection and purging of the PC, the pressure was measured by a pressure monitor (PM) (Bischoff-Analysentechnik, Leonberg, Germany). When a pressure of 80 bar was reached, forecasting PC clogging during the next injections, a signal was sent to a second interface (also represented as IF in Fig. 2) which, after obtaining an end-of-run-signal from the gradient former, effected replacement of the PC by the tandem precolumn selector (TPS) (Model 7066; Rheodyne).

Columns and mobile phases

The guard column GC1 and the PC (all 14 mm × 4.6 mm I.D.) (Bischoff) were packed with Bondapak C_{18} Corasil, 37–50 μm (Waters). The analytical column (AC) (three coupled columns, each 125 mm × 4 mm I.D.) and the guard column GC2 (30 mm × 4 mm I.D.) (all from E. Merck) were packed with Spherisorb ODS-1, 5

μm (Phase Separations, Queensferry, UK) using a slurry packing technique.

Mobile phase 1 (M1) consisted of 1% ammonium acetate–acetonitrile (85:15, v/v), and the gradient mobile phase 2 (M2) contained three acetonitrile–water components: (A) 70:30, (B) 95:5 and (C) 99:1 (v/v). All mobile phases were degassed with helium prior to use.

Procedure

A 0.2-ml aliquot of the internal standard working solution was added to 1.0 ml of plasma. After vortex-mixing, centrifugation (6 min at 3400 *g*) and transfer to the injection vial (Model 3810 microtubes; Eppendorf Gerätebau, Hamburg, Germany), 0.5 ml was injected by the autosampler. The total sequence of automated analysis required 34 min, and included the following steps:

Step A (0–7 min, V1 = T5, V2 = T4, V3 = T8). Injection and pre-concentration of the sample on to PC. Proteins and polar compounds were washed out to waste 1 with M1. AC was equilibrated with M2 (100% A).

Step B (7–10 min, V1 = T5, V2 = T4, V3 = T7). PC was purged in the backflush mode by M1.

Step C (10–27 min, V1 = T5, V2 = T3, V3 = T7). M1 passed directly to waste 1. The retained components were transferred from PC to AC in the backflush mode by the gradient M2: from 100% A to 100% B (10–20 min), 100% B (20–24 min), 100% B to 100% C (24–24.1 min), 100% C (24.1–27 min).

Step D (27–30 min, V1 = T6, V2 = T3, V3 = T7; 30–30.9 min, V1 = T6, V2 = T4, V3 = T8). While M1 was running in a recycling mode, the capillaries between V1 and D1 were purged with M2 (100% C) to prevent any memory effects during the next injection.

Step E (30.9–34 min, V1 = T5, V2 = T4, V3 = T8). After 31.1 min, M2 was changed from 100% C to 100% A within 0.1 min. AC and PC were re-equilibrated with M2 and M1, respectively.

Calibration and calculations

Together with the unknown and quality control samples, seven plasma standards, distributed over the whole set of samples, were processed as described above. The calibration graph ($y = a + bx$) was obtained by weighted linear least-squares regression (weighting factor $1/y^2$) of the measured I/III and II/III peak-height ratios (y) *versus* the concentrations of I and II (x). The calibration graph was used to interpolate unknown concentrations in the biological samples from measured peak-height ratios.

RESULTS AND DISCUSSION

Sample pretreatment

HPLC with on-line solid-phase extraction has been successfully applied to the determination of first-generation [8–11,13] and second-generation [9,12–14] retinoids. The column-switching technique is particularly useful for the labile retinoids, not only because of the high degree of automation but also because of total protection from light during analysis. In this paper, this technique has been described in detail for the first time for a third-generation retinoid. It has been shown that the recoveries of the highly protein bound retinoids are low when plasma samples are injected directly on to a precolumn using water as mobile phase 1 [15]. Two different methods of sample injection can be used to overcome these problems: (a) addition of a water-miscible organic solvent (*e.g.*, acetonitrile) to the plasma sample and to M1 [8,9,11,13,14]; using a final proportion of less than 20% of acetonitrile, plasma proteins are not precipitated, but the mass transfer of the retinoids from the protein to the stationary phase of the precolumn is improved; (b) protein precipitation with an organic solvent (*e.g.*, ethanol) and injection of the supernatant [9,10,12]. Whereas the latter conditions are very robust and avoid any unintentional transfer of proteins to the analytical column, direct injection of plasma results in higher sensitivity when large plasma volumes are injected [13,14].

For the determination of sumarotene in plasma samples, a highly sensitive method was needed, which should also allow the pharmacokinetic profile to be investigated after topical application

to man. Therefore, addition of acetonitrile was used without protein precipitation. However, only limited plasma volumes were available from toxicokinetic studies in rat and dog after oral administration. Therefore, the method was validated for a final injection volume of 0.5 ml. For studies in man, it was planned to increase the injected plasma volume. In a preliminary study it was shown that 1 ml could be injected, resulting in a quantification limit for sumarotene of 0.5 ng/ml [13].

The addition of acetonitrile is the only off-line manipulation step, which makes the method especially suitable for routine determinations. Addition of 0.2 ml of acetonitrile, instead of 0.25 ml, to 1 ml of plasma was preferred, as the latter impaired the consistency of the plasma after several hours of storage in the autosampler and resulted in a pressure increase in the analytical column. No difference in recovery or precision could be observed from the addition of the internal standard in a 200-μl volume compared with the more usual 5-μl volume. In this way, an additional step could be avoided. In contrast to first- and second-generation retinoids, no isomerization was found when the plasma sample containing the acetonitrile was stored in the autosampler. The same injection conditions, when used for acitretin and 13-*cis*-acitretin, sometimes resulted in *cis–trans* isomerization after storage for more than 10 h. Finally, for these compounds, acetonitrile was added, on-line, by means of an additional pump and a T-piece [14].

Analytical system and chromatography

The column-switching system (see Fig. 2) used for sumarotene offered several valve-switching possibilities which were already used for other retinoid methods. V2 was the central valve connected with the precolumn PC and the analytical column AC, which was protected by a guard column GC2. Another guard column (GC1) prevented pre-concentration of interferences from M1 on the PC during the equilibration step. V1 allowed the purge of the capillaries between the autosampler (I1) and the UV detector D1 with M2. In this way, memory effects by adsorption of the retinoids on the steel capillaries could be prevented. These memory effects could occur because of the low solubility of the retinoids in aqueous mobile phase 1. The installation of a third valve (V3) allowed forward- and backflush purging of the precolumn. Proteins and solid particles, which would have been partially adsorbed on the top of the precolumn, were transferred to waste instead of to the analytical column, thereby prolonging the lifetime of the analytical column.

The tandem precolumn selector (TPS) and the pressure monitor (PM), which were introduced for the fully automated injection of 1.0-ml plasma volumes [13,14], were also used for sumarotene, although they were not essential. Normally, about 40 injections (corresponding to about 17 ml of injected plasma) were made within a 24-h period before a precolumn was replaced. No precolumn clogging was observed under these conditions. However, the tandem precolumn selector increased the safety of the system during overnight injection. Further, it may be especially advantageous in routine analysis when the maximum number of injections on to one precolumn using the TPS is compared with the expensive use of disposable cartridges in off-line solid-phase extraction.

Several stationary phases were tested as analytical columns (Spherisorb ODS-2, Hypersil ODS, Superspher 100 RP-18). Three coupled columns (each 125 mm long) of Spherisorb ODS-1 (5 μm) gave the best separation of geometrical isomers and interferences. As I–III are neutral compounds, the use of ammonium acetate in M2 was of no advantage. Therefore, only water and acetonitrile were used in M2. In contrast, the selectivity and peak shape of retinoids with a carboxylic group were strongly influenced by ammonium acetate in M2. Etarotene (III), which is also in clinical development for the same indications, proved to be an ideal internal standard owing to its structural similarity. The *Z*-isomer of III was also separated under the actual assay conditions. Therefore, the method could also be applied to the determination of etarotene using sumarotene as internal standard.

Fig. 3 shows chromatograms of a blank plas-

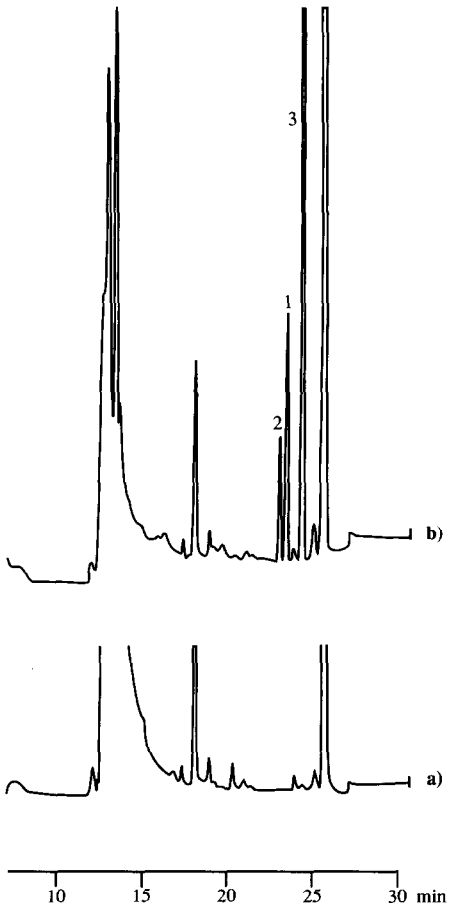

Fig. 3. Chromatograms of human plasma samples. (a) Blank plasma sample; (b) blank plasma sample spiked with 20 ng/ml sumarotene (1) and its Z-isomer (2), and 100 ng/ml internal standard etarotene (3).

ma and a spiked human plasma sample, demonstrating the good selectivity of the chromatographic system. More than twenty human blank plasma samples and several blank plasma samples from rat, dog and pig showed no interference. The peak height of II was only about half of that of I owing to a lower absorption of the Z-isomer in the mobile phase (data not shown). During 24 h of replicate injections of a plasma standard on to the same precolumn, the peak heights of I and II decreased by less than 5%. This could be due to an alteration of the precolumn. However, the decrease in the peak heights was fully compensated for by the internal standard.

Recovery

The recovery from plasma was determined in replicate analyses by comparison of peak heights of spiked plasma samples (equilibrated for *ca.* 30 min at 22°C) with 200-μl injections of standard solutions in mobile phase 2A. These solutions, injected directly on to the analytical column using I2, provided the 100% values. By adding acetonitrile to the plasma sample and to mobile phase 1, excellent recoveries were obtained, as shown in Table I. Recoveries in rat and dog plasma were higher than in human plasma (see Table I). Therefore, calibration standards were prepared in the plasma of the corresponding species.

Linearity

The method was linear over the range 1–1000 ng/ml, at least. Calibration graphs for I (1–1000 ng/ml) and II (2–1000 ng/ml) were calculated by means of weighted least-squares regression using $1/y^2$ as weighting factor.

Limit of quantification

The limit of quantification was 1 ng/ml for I and 2 ng/ml for II, using 0.5-ml injection volumes. The inter-assay (n = 6) relative standard deviations (R.S.D.) at these concentrations were 7.2% for both compounds (see Table II). A chromatogram of a human plasma sample spiked at 1 ng/ml is shown in Fig. 4. Using 1.0-ml injection volumes, the limit of quantification of I could be improved to 0.5 ng/ml. This is comparable to that obtained with GC–MS methods for similar arotinoids [2,4]. The higher sensitivity for the arotinoid free acid Ro 13-7410 [3] is attributable mainly to the use of a derivatization step. This would not be possible for compounds I–III, which have no functional group for derivatization. In addition, the large differences in sample handling and clean-up between GC–MS and HPLC with on-line solid-phase extraction have to be considered.

Precision and accuracy

Inter-assay precision and accuracy were evaluated during method validation by analysing one series of human plasma standards over six days

TABLE I

RECOVERIES OF I–III (n = 5)

Concentration (ng/ml)	I		II		III	
	Recovery (%)	R.S.D (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)
Man						
5	93.4	3.0	101	2.4	82.2	3.5
100	93.6	1.9	104	1.1	83.6	2.9
500	93.3	2.1	104	1.2	82.8	3.2
Rat						
100	105	0.7	111	0.5	98.2	0.6
Dog						
100	99.3	1.6	110	0.9	87.3	2.7

against an independent calibration set. The results are given in Table II. In addition, quality control samples in human, rat and dog plasma were analysed alongside unknown samples from these species. These data are given in Table III.

TABLE II

INTER-ASSAY PRECISION AND ACCURACY FROM HUMAN PLASMA STANDARDS (n = 6)

Concentration (ng/ml)		R.S.D. (%)	Difference between found and added (%)
Added	Found		
Compound I			
1	1.02	7.2	+2.0
2	2.04	2.4	+2.0
5	5.02	2.7	+0.4
20	20.0	3.6	0
100	101	2.3	+1.0
500	517	3.1	+3.4
1000	1021	2.4	+2.1
Compound II			
2	2.02	7.2	+1.0
5	5.01	3.6	+0.2
20	20.2	5.9	+1.0
100	101	2.4	+1.0
500	524	3.5	+4.8
1000	1040	4.9	+4.0

Stability

The stability of I and II in plasma was investigated by storing spiked blank plasma samples at different concentrations. The results of these sta-

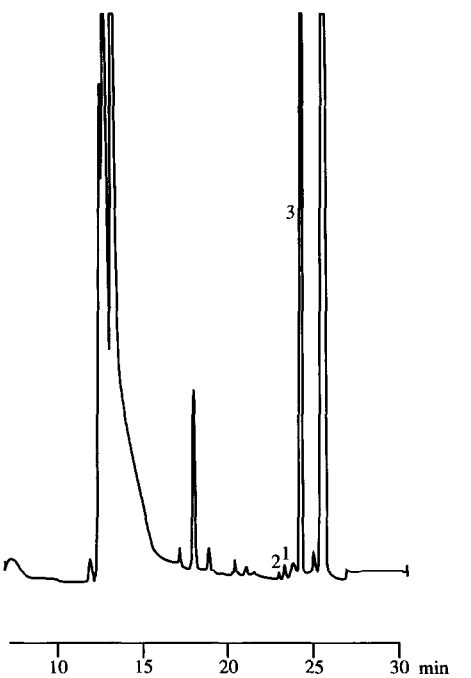

Fig. 4. Chromatogram showing the limit of quantification for sumarotene (1): human blank plasma spiked with 1 ng/ml sumarotene (1) and the Z-isomer (2). Peak 3 is the internal standard; the injection volume was 0.5 ml.

TABLE III

INTER-ASSAY PRECISION AND ACCURACY FROM QUALITY CONTROL SAMPLES

Compound	Concentration (ng/ml)		R.S.D. (%)	*n*	Difference between found and added (%)
	Added	Found			
Man					
I	2.81	2.71	5.2	5	−3.6
	195	187	2.1	4	−4.1
II	6.10	5.82	5.5	5	−4.6
	412	370	3.0	4	−10.2
Rat					
I	4.57	4.45	1.8	18	−2.6
	458	448	2.1	18	−2.2
II	4.64	4.16	4.0	18	−10.3
	444	433	2.9	18	−2.5
Dog					
I	5	4.77	5.9	9	−4.6
	100	98.4	3.3	9	−1.6
	500	501	2.8	9	+0.2

TABLE IV

STABILITY OF I AND II IN PLASMA (n = 5)

Compound	Storage conditions	Concentration (ng/ml)	Change in concentration after storage (%)	90% confidence interval (%)
Man				
I	24 h at 22°C	10	−0.3	−2.3 to +1.6
		100	−4.2	−6.5 to −2.0
		500	+1.0	−1.0 to +3.1
	3 months at −20°C	10	−2.1	−3.7 to −0.5
		100	+1.0	+0.4 to +1.5
		500	−1.4	−3.2 to +0.4
II	24 h at 22°C	10	+3.0	−1.4 to +7.5
		100	−5.7	−8.7 to −2.6
		500	+1.5	−1.9 to +5.0
	3 months at −20°C	10	−2.1	−6.6 to +2.7
		100	+1.2	+0.5 to +1.8
		500	−1.0	−4.0 to +2.1
Rat				
I	24 h at 22°C	100	−0.5	−1.5 to +0.5
II	24 h at 22°C	100	+0.2	−1.7 to +2.1
Dog				
I	24 h at 22°C	100	−1.1	−2.6 to +0.3
II	24 h at 22°C	100	−1.3	−3.4 to +0.9

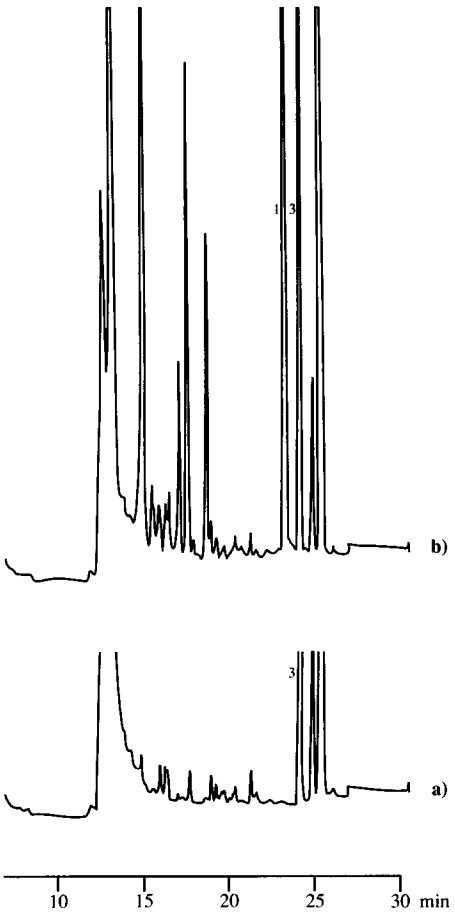

Fig. 5. Chromatograms of rat plasma samples from a toxicokinetic study: (a) predose sample; (b) sample taken on day 9 after an oral daily treatment of 10 mg/kg. Measured concentration of sumarotene (1), 161 ng/ml. Peak 3 is the internal standard.

bility tests, which were carried out according to our established method [16], are presented in Table IV. The data indicate that I and II are stable in human, rat and dog plasma.

Application to biological samples

The method was successfully applied to toxicokinetic studies in rats and dogs after oral administration of sumarotene. Fig. 5 shows chromatograms of rat plasma samples from one of these studies. The *Z*-isomer II could not be found in any of the samples from these rat and dog studies, indicating that isomerization is not a metabolic pathway for I. In addition, sumarotene (10 g as a 0.5% gel) was topically applied to a total area of *ca.* 2500 cm^2 on the face, neck, back, chest and upper extremity of healthy volunteers, once daily for 30 days, and plasma samples were collected before and after application of the drug. Neither I nor II could be detected in the plasma samples (quantification limit 1 ng/ml).

CONCLUSIONS

A fully automated and sensitive HPLC method, using on-line solid-phase extraction and automated column switching, was developed and used for the first time for third-generation retinoids, namely sumarotene (I) and its *Z*-isomer (II). Good recoveries were obtained by addition of acetonitrile to the plasma sample prior to injection. The final acetonitrile content of *ca.* 17% avoided protein precipitation. No isomerization in the autosampler could be observed, as was sometimes found for acitretin and 13-*cis*-acitretin using the same injection conditions [14]. The injection of 0.5 ml of diluted plasma resulted in a limit of quantification of 1 ng/ml for sumarotene (I) and 2 ng/ml for II. More than 40 injections could be made on to one precolumn, allowing routine overnight injections. Using a 1.0-ml injection volume, the limit of quantification for sumarotene could be improved to 0.5 ng/ml. However, this was not needed for the analysis of plasma samples from animal studies. The method was applied to toxicokinetic studies in rats and dogs, and was used to monitor human plasma samples after repeated topical application to healthy volunteers.

Owing to the minimum sample handling and the high sensitivity, the direct plasma injection technique is superior to conventional HPLC methods for arotinoids [5–7]. It can even compete with the sensitivity of GC–MS methods [2,4] for arotinoids, which have no functional group for derivatization. The method described could also be adapted to etarotene (III), which was used as internal standard, and which is at present in clinical development.

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