ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
18 (1998) 761–776

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Determination of 13-*cis*-3-hydroxyretinoic acid, all-*trans*-3-hydroxyretinoic acid and their 4-oxo metabolites in human and animal plasma by high-performance liquid chromatography with automated column switching and UV detection

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Received 15 May 1998; accepted 6 July 1998

Abstract

A high-performance liquid chromatographic method with automated column switching was developed for the simultaneous determination of 13-*cis*-3-hydroxyretinoic acid, all-*trans*-3-hydroxyretinoic acid and their metabolites 13-*cis*-3-hydroxy-4-oxo-retinoic acid and all-*trans*-3-hydroxy-4-oxo-retinoic acid in plasma samples from man, rat, dog, rabbit and mouse. The method consists of deproteination of plasma (0.4 ml) with ethanol (1.5 ml), containing the internal standard Ro 12-7310. After centrifugation, 1.4 ml of the supernatant was directly injected onto the precolumn (PC) (4 × 4 mm) packed with LiChrospher 100 RP-18 (5 μm). Ammonium acetate (0.02%)-acetic acid-ethanol (100:3:4, v/v/v) was used as mobile phase M1A during injection, as well as to decrease the elution strength of the injection solution by on-line addition using a T-piece (M1B). After valve switching, the retained components were transferred to the analytical column (AC), separated by gradient elution and detected at 360 nm. Two coupled Purospher 100 RP-18 endcapped columns (both 250 × 4 mm) were used for the separation, together with a mobile phase consisting of acetonitrile-water-10% ammonium acetate-acetic acid, (A), 540:450:2:30 (v/v/v/v), (B), 600:350:2:30 (v/v/v/v), and (C), 950:40:2:30 (v/v/v/v). The method was linear in the range 1–500 ng ml^{-1}, at least, with a quantification limit of 1 ng ml^{-1}. The mean recoveries from human plasma were 100–107% and the mean inter-assay precision was 2.0–4.7% (range 1–500 ng ml^{-1}). Similar results were obtained for animal plasma. The analytes were stable in the plasma of all investigated species stored at −20°C for 3 months, at least. The method was successfully applied to clinical and toxicokinetic studies.

Keywords: 13-*cis*-3-Hydroxyretinoic acid; All-*trans*-3-hydroxyretinoic acid; Retinoids; Column switching

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0731-7085/98/$ - see front matter
PII: S0731-7085(98)00221-0

1. Introduction

The retinoids are a class of compounds which includes both naturally occurring substances with vitamin A activity and synthetic analogues used in dermatology and oncology. 13-*cis*-retinoic acid (13-*cis*-RA, isotretinoin) is used successfully in the treatment of severe cystic acne and related disorders [1]. Preclinical studies have shown that 13-*cis*-3-hydroxyretinoic acid (Ro 61-9538, **I**, Fig. 1), which is currently in development for the same indication, may be even better tolerated than 13-*cis*-RA. The 13-*cis* isomers of both compounds are now regarded to act as prodrugs for the biological active all-*trans* isomers. For the development programme of 13-*cis*-3-hydroxyretinoic acid, an analytical method was needed for the simultaneous determination of (**I**), all-*trans*-3-hydroxyretinoic acid (**II**), and their metabolites 13-*cis*-3-hydroxy-4-oxo-retinoic acid (**III**) and all-*trans*-3-hydroxy-4-oxo-retinoic acid (**IV**) in plasma samples from man, rat, dog, rabbit and mouse.

Several reviews on the determination of retinoids in biological samples have appeared during the last few years [2–6]. High-performance liquid chromatography (HPLC) with UV detection is the method of choice for the determination of retinoids in biological fluids. HPLC with automated column switching (on-line solid-phase extraction) is especially suitable for these labile compounds because of the sensitivity of the retinoids to photoisomerisation and oxidation. Therefore, a similar HPLC column-switching system as used for 13-*cis*-RA and metabolites [7], was used for the determination of 13-*cis*-3-hydroxyretinoic acid and its metabolites, and the method was validated for GLP requirements.

2. Experimental

2.1. Materials, reagents and solvents

Glacial acetic acid (100%), ammonium acetate (both p.a.), and ethanol (absolute p.a.), were obtained from E. Merck (Darmstadt, Germany), and acetonitrile (HPLC grade S) from Rathburn (Walkerburn, UK). Water was distilled twice from an all-glass apparatus. Argon was obtained from PanGas (Lucerne, Switzerland). 13-*cis*-3-hydroxyretinoic acid (**I**, Ro 61-9538/001), all-*trans*-3-hydroxyretinoic acid (**II**, Ro 14-7627/000), 13-*cis*-3-hydroxy-4-oxo-retinoic acid (**III**, Ro 18-5366/000), all-*trans*-3-hydroxy-4-oxo-retinoic acid (**IV**, Ro 61-6659/000) and (all-E)-9-(4-hydroxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoic acid (**V**, internal standard, Ro 12-7310/000) were provided by F. Hoffmann-La Roche (Basel, Switzerland) and were kept under argon at −20°C. Spiked plasma samples were prepared using fresh frozen plasma, either from sodium citrated human blood, obtained from a blood bank (Blutspendezentrum SRK, Basel, Switzerland), or from EDTA/NaF blood from our own laboratories.

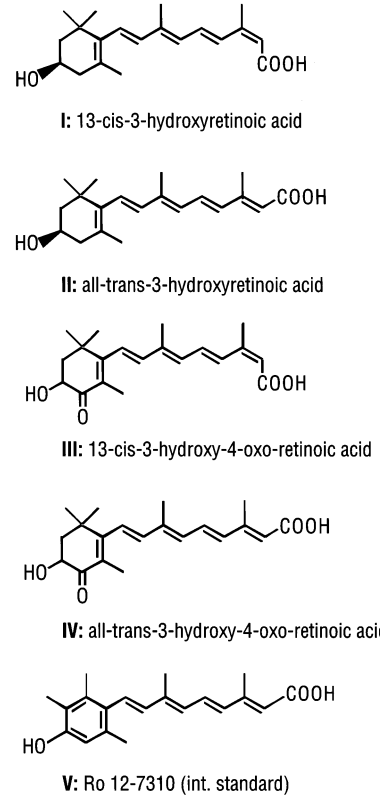

Fig. 1. Chemical structures of the compounds.

2.2. Solutions and standards

The weighing of the compounds, the preparation of calibration standards, and the handling of samples was performed under yellow light or diffuse light conditions.

A 10% (w/v) solution of ammonium acetate was made up in bidistilled water (100 g l^{-1}).

A stock solution of the internal standard was prepared in an amberised volumetric flask by dissolving 10 mg of **V** in 100 ml ethanol (HPLC grade; 100 μg ml^{-1}). An internal standard working solution was prepared by diluting 1 ml of the stock solution with ethanol to 2000 ml (50 ng ml^{-1}). This solution was stored in the dark at room temperature for several weeks. The stock solutions could be stored at 4°C for several months.

Four stock solutions of the analytes were prepared in amberised volumetric flasks by dissolving 10 mg of **I** in 20 ml of ethanol (HPLC grade) and 5 mg of **II**, **III**, and **IV**, respectively, in 10 ml of ethanol. Ultrasonication was used for complete dissolution. Appropriate amounts of each stock solution were combined and diluted with ethanol to give working solutions in the range 50–0.1 μg ml^{-1}. These working solutions were used as calibration standards by adding 0.5 to 49.5 ml of human plasma, yielding concentrations of 500, 200, 50, 10, 2 and 1 ng ml^{-1}. The calibration standards were stored at −20°C for several months.

Spiked plasma standards for validation or quality control samples were prepared by spiking blank plasma with a small volume (normally 1%) of an adequately prepared standard working solution.

2.3. Sample preparation

To 0.4 ml of plasma, 1.5 ml of the internal standard working solution were added for protein precipitation. After vortex-mixing and storing for 15 min in the deep freezer at −20°C, the sample was centrifuged (6 min at ca. 18 000 × *g* and 10°C), and the supernatant transferred to an autosampler vial (2.0 ml Safe-Lock, Eppendorf-Netheler-Hinz, Hamburg, Germany); 1.4 ml was subsequently injected.

When the expected concentration was higher than 500 ng ml^{-1} or less than 0.4 ml plasma were available, the following two procedures were used: When 0.2–0.4 ml plasma were analysed, the unknown samples were diluted with water ad 0.4 ml prior to protein precipitation. For plasma volumes < 0.2 ml, one volume of plasma was deproteinated with 3.75 volumes of ethanolic internal standard solution. An appropriate volume of the supernatant was further diluted with a solution containing the same ratio of water and ethanolic internal standard solution (1:3.75). 1.4 ml of this diluted solution was subsequently injected.

2.4. Chromatographic system and conditions

A schematic representation of the HPLC column-switching system is given in Fig. 2. A HPLC pump L-6000 (P1A; Merck), in combination with a solvent selector (SS; Labsource, Reinach BL, Switzerland), delivered mobile phase M1A (or alternatively M3). Aliquots (1.4 ml) were injected by the autosampler (AS; Model AS-4000A, Merck) onto one of the precolumns (PCs). In order to inject large sample volumes, the autosampler was used with two 5 ml syringes as dilutors 1 and 2 (solvents: water-ethanol (100:4, v/v), and ethanol, respectively), a 3 ml sample loop, and the slow needle-down-speed. The injected sample plug was diluted, on-line, with mobile phase M1B by HPLC-pump P1B (L-6000, Merck; flow-rate 3.2 ml min^{-1}) via a T-piece (Valco Instruments, Houston, USA; 1/16 in., bore 0.25 mm). The UV detector D1 (Spectroflow 773, Kratos, Ramsey, USA), operating at 230 nm, together with a W + W recorder 600 (Kontron, Zurich, Switzerland), was used during method validation to monitor the removal of polar components from the PC during the purge step; they were not needed for routine analysis. The gradient pump P2 (L-6200A, Merck) delivered mobile phase M2, which was degassed on-line (Solvent degaser SDU 2003, Labsource). A manual injector (MI; Model 7125 with a 200 μl loop, Rheodyne, Cotati, USA) was used for direct injection onto the analytical column (AC, e.g. for recovery experiments). Detection of the eluted compounds

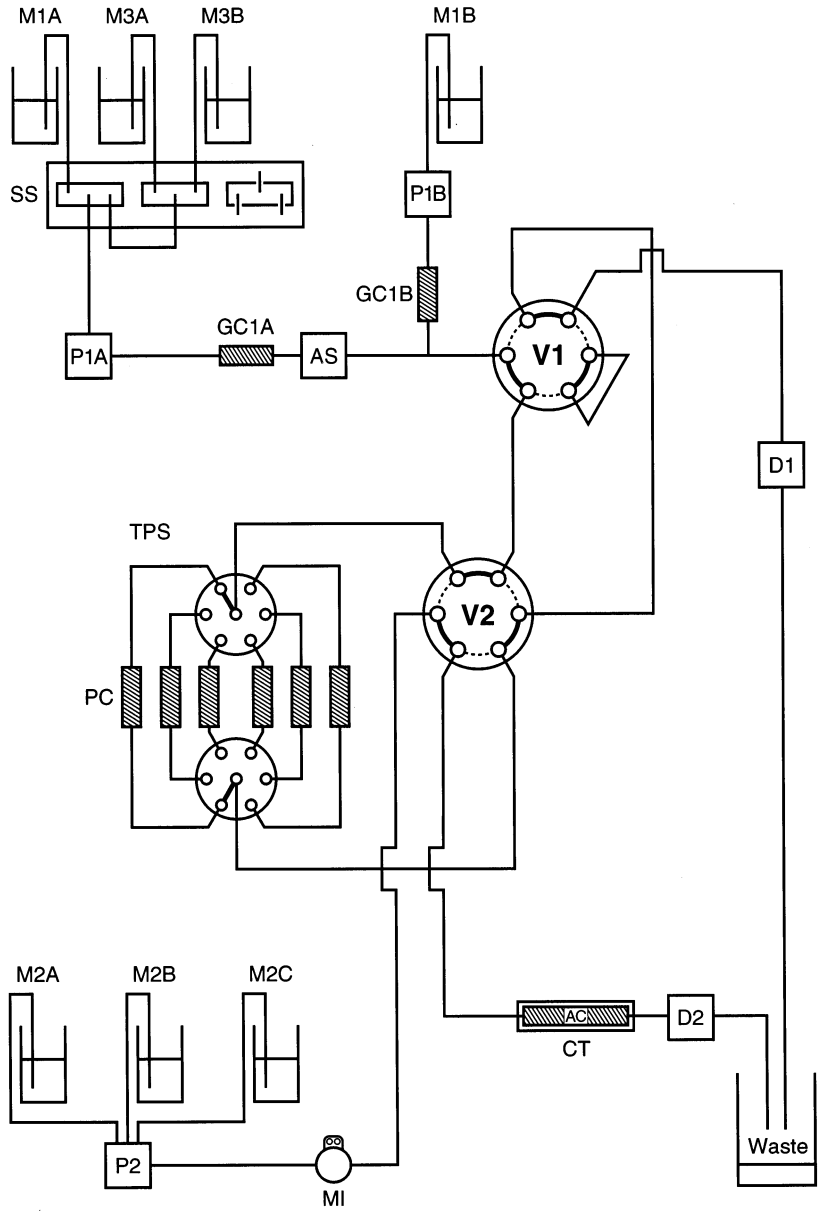

Fig. 2. Schematic representation of the HPLC column-switching system. See text for further details.

was carried out at 360 nm with an UV detector (D2; SPD-10A, Shimadzu, Kyoto, Japan; response value 1.0 s, aux range 3 AU V^{-1}). A HPLC ChemStation (Hewlett-Packard, Waldbronn, Germany), in connection with an in-house developed LIMS (UNICHROM and KIN-LIMS [8]), was used for data acquisition and processing.

The two electrically driven switching valves (V1 and V2; High speed valve 7000E, both Labsource) and the solvent selector were controlled by P2. PCs were automatically replaced by a tandem PC selector (TPS; EA6 Port valve 7066/CPR, Labsource), either when a pressure of 200 bar was reached, indicating PC clogging during the following injections, or after 300 injections.

2.4.1. Columns and mobile phases

LiChroCART HPLC cartridges (all 4 × 4 mm i.d.), packed with LiChrospher 100 RP-18 (5 μm), were used as guard columns, GC1A and GC1B, and as PCs. The AC consisted of two LiChroCART HPLC cartridges (250 × 4 mm i.d.) packed with Purospher RP-18 endcapped (5 μm) (all Merck) and was kept at 30°C by a column thermostat (CT; with Peltier controller unit PCU 1000, Labsource).

Mobile phases 1A (M1A) and 1B (M1B) were identical and were prepared by mixing 2 ml of 10% ammonium acetate with 1000 ml of water, 40 ml of ethanol and 30 ml of acetic acid. Mobile phase 2 (M2) consisted of three components: (M2A) 2 ml of 10% ammonium acetate, 450 ml of water, 30 ml of acetic acid, 540 ml of acetonitrile; (M2B) 2 ml of 10% ammonium acetate, 350 ml of water, 30 ml of acetic acid, 600 ml of acetonitrile; (M2C) 2 ml of 10% ammonium acetate, 40 ml of water, 30 ml of acetic acid, 950 ml of acetonitrile. Mobile phase 3A (M3A) and 3B (M3B) consisted of ethanol-water (8:2, v/v), and ethanol, respectively.

2.4.2. Procedure

The total sequence of automated sample analysis required 25 min. Details of the parameters used are shown in Table 1. The autosampler started the gradient programme of P2 and the HPLC ChemStation. P2 controlled the flow of P1A. In addition, the timer signals of P2 were also used for: (a) switching the valves (10/11 and 20/21 means switching of V1, and V2, respectively), (b) the solvent selector (30 = M1A, 31 = M3, 40 = M3A, 41 = M3B), and (c) to stop (62) P1B. Timer signal 72 activated the pulse counter of the TPS.

2.5. Calibration and calculations

Together with the unknown and quality control samples, six calibration standards, distributed over the whole set of samples, were processed as described above. The calibration curve ($y = a + bx$) was obtained by weighted linear least-squares regression (weighting factor $1/x^2$) of the measured peak-height ratios **I**–**IV**/**V** (y) versus the concentration of **I**–**IV** (x).

3. Results and discussion

3.1. Sample preparation

An automated HPLC column-switching system, which had been used successfully for the determination of 13-*cis*-RA and metabolites [7] and also, with modifications, for other retinoids in biological samples [9–13], was adapted to the determination of 13-*cis*-3-hydroxyretinoic acid and its metabolites. The only off-line step was deproteination of 0.4 ml of plasma by adding 1.5 ml of ethanol containing the internal standard. A high volume of ethanol was preferred compared to other solvents (e.g. acetonitrile), because it resulted in the most efficient protein precipitation and solubility of the analytes. After centrifugation, 1.4 ml of the supernatant was injected. This large volume of ethanol would prevent pre-concentration of the analytes on the PC. Therefore, ammonium acetate was added, on-line (M1B in Fig. 2), to reduce the elution strength of the injection solution.

At the beginning, when samples were analysed with an expected concentration higher than 500 ng ml^{-1} or when less than 0.4 ml plasma was available, unknown samples were diluted with water ad 0.4 ml prior to protein precipitation. As the dilution of plasma samples with a large volume of water resulted in an increase of the pressure in the PC after several days of analysis, dilution with water was only used for plasma volumes > 0.2 ml. For plasma volumes < 0.2 ml, one volume of plasma was deproteinated with 3.75 volumes of ethanolic internal standard solution. An appropriate volume of the supernatant was further diluted with a solution containing the same ratio of water and ethanolic internal standard solution (1:3.75). Both procedures gave the same result. However, it is recommended to use the second procedure also for plasma volumes > 0.2 ml. An appropriate alternative is dilution with blank plasma. However, this is often not possible for small animals for which it is difficult to obtain large amounts of blank plasma.

Table 1
Column-switching parameters and gradient programme

Time (min)	P2 gradient			P2 flow (ml min^{-1})	P1A flow (ml min^{-1})	P1B flow (ml min^{-1})	P2 timer				Comment
	A(%)	B(%)	C(%)				V1	V2	SS	Others	
0	100	0	0	0.05	0.05	3.2	10[a]	20			Injection of the sample onto PC using M1A+M1B for pre-concentration of the analytes. PC was purged using M1A only.
0.1				1.0	1.8				30, 40		
2.5					1.8	0				62	
2.6					4.5						
5	100	0	0		4.5			21			Transfer of the retained components from PC to AC. In the meantime, the capillaries between AS and D1 were purged with M3.
5.1					1.0		11		31		
5.5								20			
6.0										72	
6.5									41		
18	0	100	0						40		
19					1.0				30		Re-equilibration of PC with M1.
19.1					2.0						
23.0	0	100	0								
23.1	0	0	100								
25.9	0	0	100				10				
26.0	100	0	0								
26.1				1.0	2.0						

[a] The first digit of the timer signal represents the address and the second one the activation.

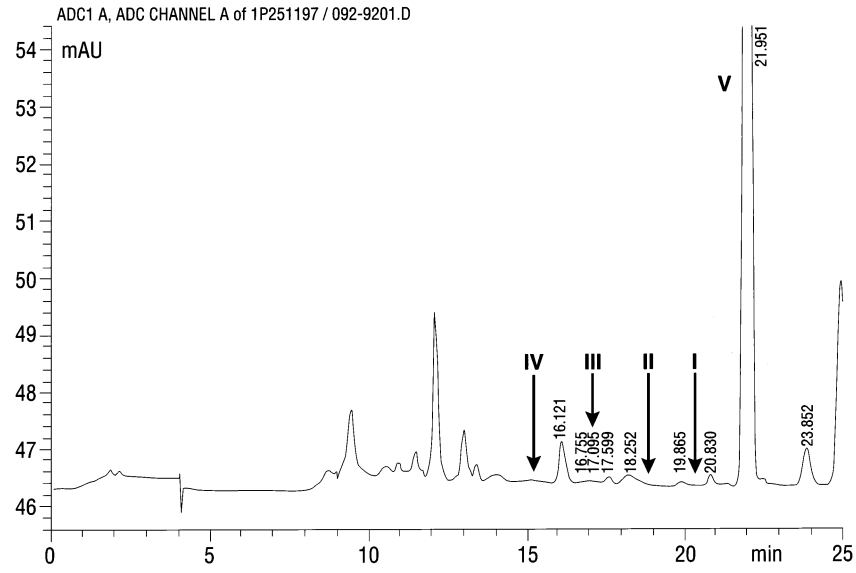

Fig. 3. Chromatogram of a human blank plasma sample containing the internal standard **V**.

3.2. Chromatographic system

The column-switching system used for the determination of retinoids in our laboratory has been continuously improved over the last ten years [10,11,14–16]. The advantages have been discussed recently in the report on the isotretinoin method [7]. The main differences of the method for 3-hydroxyretinoic acids compared to the latter are a mobile phase 1A containing only, approximately, 4% ethanol instead of 20%. This reduction in the content of organic modifier in M1A was necessary to enable pre-concentration of all analytes and to avoid breakthrough of the 4-oxo metabolites. Accordingly, the content of acetonitrile in mobile phase 2 was also lowered. For the final separation step, two coupled columns of 250 mm length each were used, packed with Purospher RP-18 endcapped. This stationary phase had some advantages in the separation of the analytes from plasma interferences compared to Superspher 100 RP-18 endcapped. The number of injections onto one PC was about 300 before it was replaced.

3.3. Selectivity

To reach the required selectivity for 3-hydroxyretinoic acids and their metabolites at the low ng ml^{-1} level is a demanding task. The reason is not only that geometrical isomers of 3-hydroxyretinoic acids and 4-oxo metabolites have to be separated, but also endogenous interferences with similar retention times. Because the 3-hydroxyretinoic acids are more polar than the retinoic acids, even more peaks from endogenous interferences had to be separated.

Typical chromatograms for human plasma samples are shown in Figs. 3–6. Chromatograms from animal plasma samples were similar.

3.4. Recovery

The recovery from plasma was determined during replicate analysis, by comparison of peak heights of spiked plasma samples, processed as described above, with ethanol-water (3.75:1, v/v) solutions which contained the same amount of analytes as the spiked plasma. The five injections of spiked plasma samples were all obtained from an inter-assay study, i.e. one injection of each specimen was made per day. The 100% values obtained by injection of ethanol-water (3.75:1, v/v) solutions onto the PC were also compared with solutions of the same amount of analytes

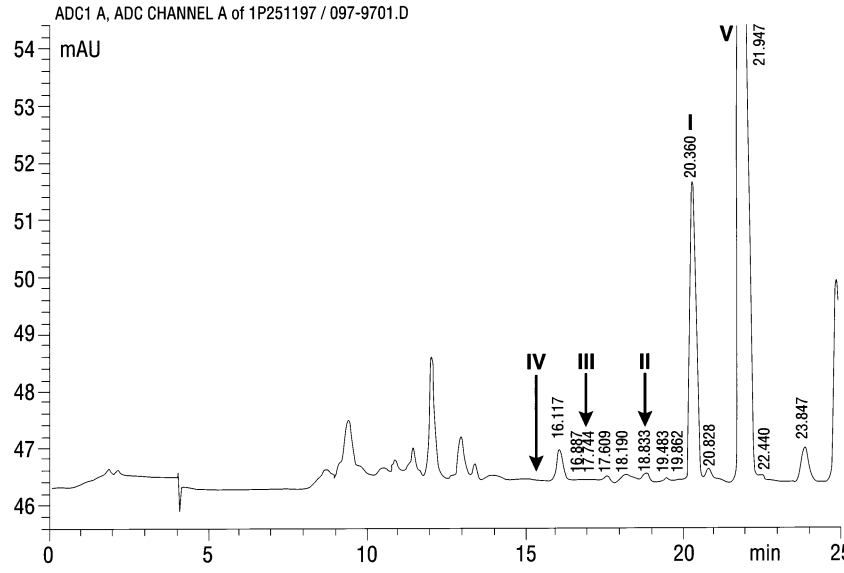

Fig. 4. Chromatogram of a human plasma sample taken 1.5 h after administration of a single oral dose of 25 mg of 13-*cis*-3-hydroxyretinoic acid. Measured concentrations of 13-*cis*-3-hydroxyretinoic acid (**I**) 35.1 ng ml^{-1}, all-*trans*-3-hydroxyretinoic acid (**II**) < 1 ng ml^{-1}. 13-*cis*-3-hydroxy-4-oxo-retinoic acid (**III**) and all-*trans*-3-hydroxy-4-oxo-retinoic acid (**IV**) were not found.

directly injected onto the AC. For the latter experiment, the analytes were dissolved in mobile phase 2A, and 100 μl were injected ($n = 3$). The peak heights were similar (110.8 for **I**, 111.8 for **II**, 111.9 for **III** and 110.3% for **IV**) as in the experiment with PC injection, when the latter are defined as 100%. The recoveries of the analytes and the internal standard are presented in Table 2.

3.5. Linearity

The method was linear in the range 1–1000 ng ml^{-1}. However, a calibration range 1–500 ng ml^{-1} was used to ensure sufficient separation of the analyte peaks. For plasma samples with concentrations > 500 ng ml^{-1}, dilution of the injection solution with internal standard solution-water (3.75:1, v/v) is recommended.

3.6. Limit of quantification

The limit of quantification was 1 ng ml^{-1} using 0.4 ml of plasma. The mean inter-assay ($n = 6$–18) relative standard deviations (RSD) at this concentration were 10.7, 9.9, 9.1 and 8.4% and the mean accuracy was 103.4, 103.8, 97.8 and 98.4% for **I**–**IV**, respectively, (see Table 3).

3.7. Precision and accuracy

The inter-assay precision (defined as the RSD of replicate analyses) and the accuracy (defined as the deviation between found and added concentration) of the method were evaluated in human, rat, dog, rabbit and mouse plasma, by analysing spiked samples on several days, using a separate calibration set on each day. The results are compiled in Table 3. The mean inter-assay precision in human plasma was 2.0–4.7%, and the mean accuracy 99.8–102.0% (range 1–500 ng ml^{-1}). For animal plasma the imprecision was slightly higher (3.9–8.7%). The mean inter-assay accuracy for animal plasma was in the range 93.9–111.5%.

Table 2
Recoveries of **I**–**V** ($n = 4$–6)

Species	Concentration added (ng ml^{-1})	**I**		**II**		**III**		**IV**		Internal standard (**V**)	
		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Man	1	114.5	6.9	105.4	6.5	120.7	7.3	96.8	6.9	104.8	8.1
	50	99.3	2.6	99.9	2.6	100.0	2.7	100.4	3.4	105.0	8.5
	500	100.8	2.0	102.2	2.1	101.3	2.8	104.1	2.9	104.5	8.1
	Mean	104.9		102.5		107.3		100.4		104.7	
Rat	1	93.2	9.7	93.2	14.3	98.9	9.8	93.7	15.5	103.4	9.5
	50	96.9	2.9	101.8	3.7	98.3	3.8	101.4	4.4	104.8	7.7
	500	95.6	5.0	100.6	5.1	97.5	6.6	101.6	7.0	103.1	7.2
	Mean	95.2		98.5		98.2		98.9		103.8	
Dog	1	107.3	16.9	95.3	4.0	106.7	5.5	88.5	7.5	95.6	10.4
	50	96.0	3.5	100.3	3.6	96.4	4.6	101.1	4.4	96.8	9.6
	500	93.4	5.1	98.5	5.5	92.1	6.7	101.0	5.5	94.2	10.5
	Mean	98.9		98.0		98.4		96.9		95.5	
Rabbit	1	94.1	2.4	103.8	2.8	96.1	4.7	nd[a]	nd[a]	100.8	0.7
	50	99.1	2.2	102.4	2.1	97.9	2.4	99.9	2.9	102.8	0.7
	500	101.5	1.5	104.5	1.4	99.3	1.5	99.3	1.5	99.8	0.6
	Mean	98.2		103.6		97.8		99.6		101.1	
Mouse	1	113.9	1.9	103.2	3.4	116.1	2.9	97.2	3.7	104.1	0.3
	50	107.2	0.5	106.9	0.5	103.4	0.5	103.9	0.8	106.0	0.2
	500	99.1	0.5	98.9	0.6	97.4	0.5	97.6	0.5	105.5	0.3
	Mean	106.7		103.0		105.6		99.6		105.2	

[a] Not determined.

Table 3
Inter-assay precision and accuracy

Species	Concentration added (ng ml^{-1})	n	I			II			III			IV		
			Concentration found (ng ml^{-1})	RSD (%)	Accuracy	Concentration found (ng ml^{-1})	RSD (%)	Accuracy	Concentration found (ng ml^{-1})	RSD (%)	Accuracy (%)	Concentration found (ng ml^{-1})	RSD (%)	Accuracy (%)
Man	1	6	1.01	6.2	101.0	1.00	11.6	100.0	1.04	7.7	104.0	1.06	4.2	106.0
	50	5	49.8	1.3	99.6	50.0	1.6	100.0	49.3	1.7	98.6	49.9	0.9	99.8
	500	6	493	0.4	98.6	496	1.0	99.2	487	0.6	97.4	504	0.9	100.8
	Mean			2.6	99.8		4.7	99.9		3.3	100.1		2.0	102.0
Rat	1	8	0.89	8.7	89.0	0.96	11.6	96.0	0.85	8.7	85.0	0.87	18.1	87.0
	50	6	50.3	1.7	100.6	52.7	3.5	105.4	49.8	2.2	99.6	51.5	3.2	103.0
	500	4	490	3.8	98.0	513	5.1	102.6	486	5.4	97.2	509	4.9	101.8
	Mean			4.7	96.0		6.7	101.2		5.4	93.9		8.7	97.3
Dog	1	6	1.12	14.8	112.0	0.98	8.0	98.0	0.99	7.0	99.0	1.06	6.2	106.0
	50	6	53.0	2.5	106.0	55.2	3.0	110.4	52.1	3.0	104.2	55.1	3.2	110.2
	500	6	508	6.2	101.6	534	7.1	106.8	493	6.9	98.6	545	8.1	109.0
	Mean			7.8	106.6		6.0	105.1		5.6	100.6		5.8	108.4
Rabbit	1	6	1.08	13.4	108.0	1.23	11.9	123.0	0.93	12.4	93.0	0.87	6.6	87.0
	50	6	51.7	2.9	103.4	53.2	2.9	106.4	50.8	3.2	101.6	51.4	3.3	102.8
	500	6	513	1.9	102.6	525	2.2	105.0	508	2.5	101.6	514	2.5	102.8
	Mean			6.1	104.7		5.7	111.5		6.0	98.7		4.1	97.4
Mouse	1	18	1.07	10.5	107.0	1.02	6.4	102.0	1.08	9.9	108.0	1.06	6.7	106.0
	50	19	53.3	3.4	106.6	51.3	3.5	102.6	54.3	3.5	108.6	50.6	4.1	101.2
	500	19	509	1.7	101.8	493	1.7	98.6	524	1.8	104.8	486	2.2	97.2
	Mean			5.2	105.1		3.9	101.1		5.1	107.1		4.3	101.5

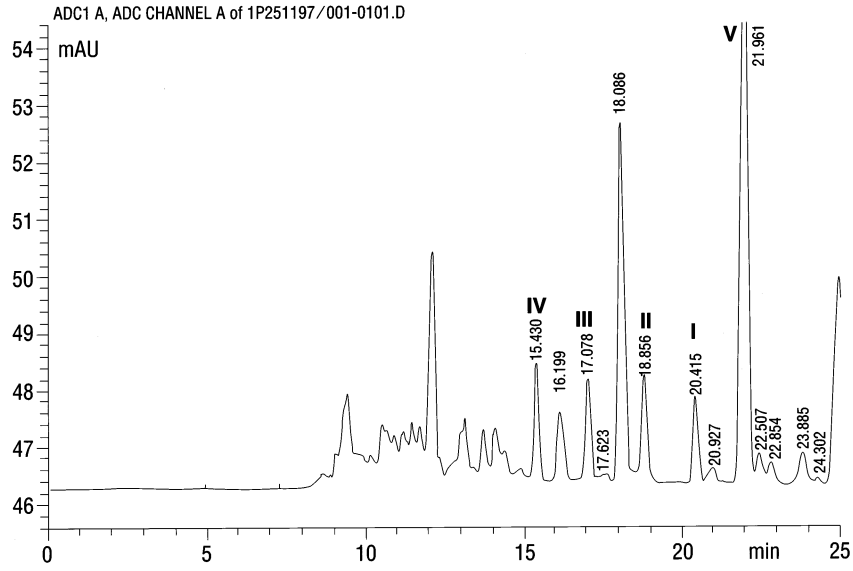

Fig. 5. Chromatogram of a human blank plasma sample spiked with 10 ng ml^{-1} of **I**–**IV**.

3.8. Stability

The stability of retinoids was recently reviewed [2]. Retinoids are sensitive to light, oxygen and heat, but apart from light protection, no special precautions, such as addition of antioxidants, are needed for plasma samples.

Stability tests for compounds **I**–**IV** were conducted according to our internal guidelines, which are based on a published procedure [17]. However, a decrease of 15% is now considered relevant, in contrast to 10% in the published procedure [17]. The results of several storage experiments are presented in Tables 4–6. Storage of plasma samples at room temperature for 24 h or at −20°C for 3 months did not show a relevant decrease (see Tables 4 and 5). When only the parent drug was added to the plasma, similar results were obtained, assuming that there was no interconversion of *cis-trans* isomers.

The influence of repeated thawing and freezing of plasma samples on the stability of the analytes was also investigated. Spiked plasma samples were submitted to 1–3 freezing (3 h at −20°C) and thawing cycles. No relevant difference could be observed (see Table 6).

3.9. Application of the method to biological samples

The method described was successfully applied to pharmacokinetic and toxicokinetic studies in four animal species, as well as a human volunteer study. Figs. 3 and 4 show typical chromatograms from a human volunteer, demonstrating the validity of the assay. Although 3-hydroxyretinoic acid could be expected to be a metabolite of retinoic acid, no endogenous concentrations could be found using the described method. However, the plasma volumes investigated were rather low (0.4 ml).

4. Conclusions

For a development programme for 13-*cis*-3-hydroxyretinoic acid in acne, a sensitive HPLC method with automated column switching and UV detection was developed and validated for the simultaneous determination of 13-*cis*-3-hydroxyretinoic acid (**I**), all-*trans*-3-hydroxyretinoic acid (**II**), and their metabolites 13-*cis*-3-hydroxy-4-oxo-retinoic acid (**III**) and all-*trans*-3-hydroxy-4-oxo-retinoic acid (**IV**) in plasma samples from

Table 4
Stability of **I**–**IV** in plasma after storage for 24 h at 22°C

Species	Concentration (ng ml^{-1})	n	**I**		**II**		**III**		**IV**	
			Change after storage (%)	90% Confidence interval (%)	Change after storage (%)	90% Confidence interval (%)	Change after storage (%)	90% Confidence interval (%)	Change after storage (%)	90% Confidence interval (%)
Man	5	5	+0.1	−3.0 to +3.4	−0.7	−1.2 to −0.2	−3.8	−4.8 to −2.7	−1.2	−2.6 to +0.3
	500	5	−2.8	−4.7 to −0.9	−2.2	−2.9 to −1.5	−4.3	−5.9 to −0.3	−1.3	−4.3 to +1.9
	500[a]	5	−1.0	−1.5 to +1.9						
Rat	5	5	+0.1	−0.6 to +0.7	−4.4	−6.8 to −1.9	+2.2	−1.2 to +5.6	−7.9	−11.4 to −4.3
	500	5	−0.5	−1.6 to +0.6	−3.6	−5.1 to −2.0	+1.2	−0.4 to +2.9	−6.1	−7.5 to −0.5
	500[a]	5	−2.5	−2.8 to −2.1						
Dog	5	5	−8.2	−13.2 to −2.9	−1.2	−4.2 to +1.8	−8.1	−12.5 to −3.6	−2.3	−5.5 to +1.1
	500	5	−4.6	−7.9 to −1.1	+1.4	−1.8 to +4.7	−6.4	−10.9 to −1.8	−2.2	−5.5 to +1.1
	500[a]	5	−3.1	−4.2 to +1.9						
Rabbit	5	5	−1.2	−2.7 to +0.4	−2.9	−4.6 to −1.2	−2.0	−2.8 to −1.1	−2.5	−5.1 to +0.3
	500	5	−0.7	−1.4 to +0.1	−3.1	−4.5 to −1.8	−1.8	−2.4 to −1.2	−3.3	−5.4 to −1.1
	500[a]	5	−1.6	−2.9 to −0.3						
Mouse	500	3	+2.9	+0.6 to +5.3	−2.4	−4.7 to +0.1	+2.7	+0.4 to +5.1	−2.1	−4.6 to +0.6

[a] Plasma spiked with compound **I** only.

Table 5
Stability of **I**–**IV** in plasma after storage for 3 months at −20°C

Species	Concentration (ng ml^{-1})	n	**I**		**II**		**III**		**IV**	
			Change after storage (%)	90% Confidence interval (%)	Change after storage (%)	90% Confidence interval (%)	Change after storage (%)	90% Confidence interval (%)	Change after storage (%)	90% Confidence interval (%)
Man	5	5	−3.0	−3.9 to −2.2	−4.2	−5.1 to −3.3	+5.4	+3.0 to +7.9	−3.7	−5.9 to −1.5
	500	5	−2.9	−3.7 to −2.1	−2.6	−3.6 to −1.6	−3.8	−4.9 to −2.7	−2.4	−3.5 to −1.2
	500[a]	5	−3.8	−5.5 to −2.2						
Rat	500	5	−5.2	−7.0 to −3.5	−0.3	−4.0 to +3.5	−6.9	−8.7 to −5.0	−2.9	−6.6 to +0.9
Dog	500	5	−2.2	−7.3 to +3.3	−1.2	−5.1 to +2.8	−4.0	−10.6 to +3.0	−1.0	−6.9 to +5.3
Rabbit	500	5	−5.7	−6.5 to −4.4	−2.7	−4.0 to −1.3	−5.5	−6.7 to −4.4	−4.1	−5.7 to −2.5
Mouse	500	5	−5.3	−6.0 to −4.5	−4.1	−5.0 to −3.2	−6.0	−6.7 to −5.2	−6.3	−7.2 to −5.4

[a] Plasma spiked with compound **I** only.

Table 6
Stability of **I**–**IV** in plasma after 1–3 cycles of freezing and thawing for 3 h at −20°C (concentration 500 ng ml^{-1})

Species	Cycles	*n*	**I**		**II**		**III**		**IV**	
			Change after storage (%)	90% Confidence interval (%)	Change after storage (%)	90% Confidence interval (%)	Change after storage (%)	90% Confidence interval (%)	Change after storage (%)	90% Confidence interval (%)
Man	1	3	−0.2	−2.1 to +1.8	−0.1	−1.9 to +1.8	−0.4	−2.5 to +1.8	−0.5	−3.2 to +2.3
	2	3	+0.9	−1.8 to +3.6	+2.0	−0.7 to +4.8	+0.1	−2.6 to +2.8	+1.8	−1.4 to +5.1
	3	3	−1.4	−2.5 to −0.3	−0.7	−1.8 to +0.5	−1.7	−3.0 to −0.3	−1.4	−2.8 to +0.2
Man[a]	1	3	−0.8	−1.9 to +0.2						
	2	3	−0.2	−1.9 to +1.6						
	3	3	−0.9	−2.2 to +0.5						

[a] Plasma spiked with compound **I** only.

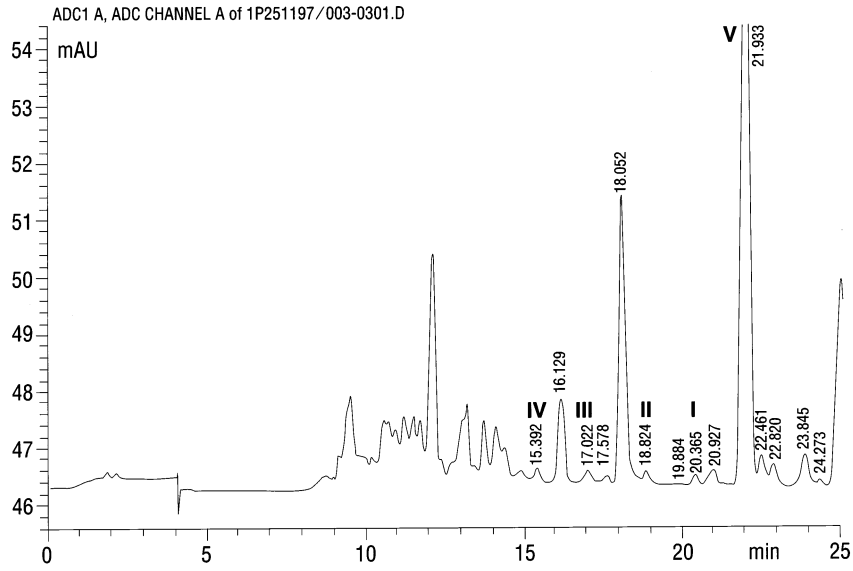

Fig. 6. Chromatogram showing the quantification limit of the method. Human blank plasma sample spiked with 1 ng ml^{-1} of **I**–**IV**.

man, rat, dog, rabbit and mouse. The main advantages of this technique are a high degree of automation, protection from light during analysis, and a higher sensitivity compared to off-line HPLC methods. As direct injection of plasma samples without special measures is not possible due to low recoveries, protein precipitation with ethanol and direct injection of the supernatant onto the PC was performed. As used in a previous method for 13-*cis*-RA (isotretinoin) and metabolites, an additional pump and a T-piece allowed a decrease of the elution strength of the injection solution, and, therefore, a concentration of the analytes on the PC.

Using these conditions, mean recoveries from human plasma were 100–107% and the mean inter-assay precision was 2.0–4.7% (range 1–500 ng ml^{-1}). Similar results were obtained for animal plasma. The analytes were stable in the plasma of all investigated species stored at −20°C for 3 months, at least. The method was successfully applied to clinical and toxicokinetic studies.

Acknowledgements

The authors thank Dr D. Dell for reading the manuscript and Hp. Suter for the drawings.

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