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Determination of endogenous levels of 13-*cis*-retinoic acid (isotretinoin), all-*trans*-retinoic acid (tretinoin) and their 4-oxo metabolites in human and animal plasma by high-performance liquid chromatography with automated column switching and ultraviolet detection

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Abstract

A highly sensitive HPLC method with automated column switching was developed for the simultaneous determination of endogenous levels of 13-*cis*-retinoic acid (isotretinoin), all-*trans*-retinoic acid (tretinoin) and their 4-oxo metabolites in plasma samples from man, Cynomolgus monkey, rabbit, rat and mouse. Plasma (0.4 ml) was deproteinated by adding ethanol (1.5 ml) containing the internal standard acitretin. After centrifugation, 1.4 ml of the supernatant were directly injected onto the precolumn packed with LiChrospher 100 RP-18 (5 μm). 1.25% ammonium acetate and acetic acid–ethanol (8:2, v/v) was used as mobile phase during injection and 1% ammonium acetate and 2% acetic acid–ethanol (102:4, v/v) was added, on-line, to decrease the elution strength of the injection solution. After backflush purging of the precolumn, the retained components were transferred to the analytical column in the backflush mode, separated by gradient elution and detected at 360 nm. Two coupled Superspher 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) 600:300:60:10 (v/v/v/v), (B) 950:20:5:20 (v/v/v/v), and (C) 990:5:0:5 (v/v/v/v). The method was linear in the range 0.3–100 ng/ml, at least, with a quantification limit of 0.3 ng/ml. The mean recoveries from human plasma were 93.2%–94.4% and the mean inter-assay precision was 2.8%–3.2% (range 0.3–100 ng/ml). Similar results were obtained for animal plasma. The analytes were found to be stable in the plasma of all investigated species stored at −20°C for 4.3 months and at −80°C for 9 months, at least. At this temperature, human plasma samples were even stable for 2 years. The method was successfully applied to more than 6000 human and 1000 animal plasma samples from clinical and toxicokinetic studies. Endogenous levels determined in control patients and pregnant women were similar to published data from volunteers. © 1997 Elsevier Science B.V.

Keywords: Retinoic acid; 4-Oxoretinoic acid; Isotretinoin

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1. Introduction

The retinoids (vitamin A derivatives) have elicited great interest in recent years because of their importance in development and cell biology, including cell differentiation and cell proliferation, morphogenesis, reproduction, vision, immunology and hematopoiesis [1]. Although three generation of retinoids are now under investigation, the first-generation agents, the retinoic acids, are still the most successful in disease treatment. In oncology, all-*trans*-retinoic acid (all-*trans*-RA, tretinoin, II, Fig. 1) showed promising results in the treatment of acute promyelocytic leukemia [2]. Other retinoids, as single agents or in combination, generated interesting preliminary results in the prevention of, or in the therapy of various precancerous and cancerous lesions [3]. In dermatology, 13-*cis*-retinoic acid (13-*cis*-RA, isotretinoin, I, Fig. 1) proved to be very successful in the oral treatment of severe cystic acne and related disorders [4]. All-*trans*-RA, due to a low oral therapeutic index, has found limited use in topical application for acne, but was recently introduced, additionally, for the treatment of photodamaged skin. A drawback of topical all-*trans*-RA in clinical use has been local skin irritation. Therefore, 13-*cis*-RA was also tested for topical application in the same indication.

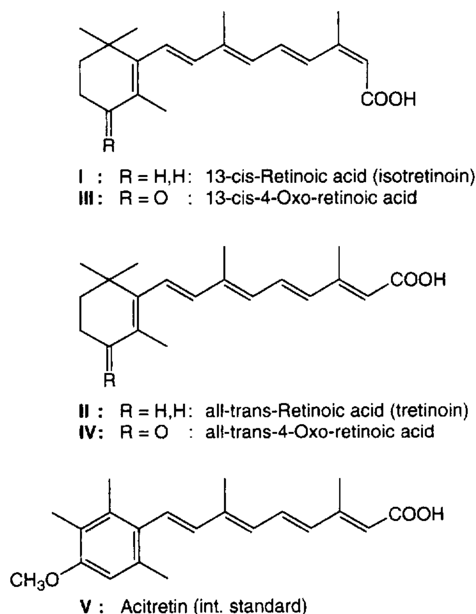

Fig. 1. Chemical structures of the compounds.

The goal of this study was to develop and validate an analytical method for the development program of isotretinoin (13-*cis*-RA) in photodamage. This included the determination of endogenous levels of 13-*cis*-RA (I), its isomer all-*trans*-RA (II), and their metabolites 13-*cis*-4-oxo-RA (III, Fig. 1) and all-*trans*-4-oxo-RA (IV) in plasma, as well as the investigation of the influence of food and of topical application of isotretinoin on the plasma levels of the four compounds.

Several reviews [5–9] concerning the determination of retinoids in biological samples have appeared over the last few years. Dozens of methods were discussed and cited. However, only a few of these methods deal with the determination of endogenous levels of retinoic acids. The first methods reported could only measure all-*trans*-RA [10–13]. They were designed for RA identification and not for routine measurements of large sample numbers. Two of these methods [10,12] used gas chromatography–mass spectrometry. Tang and Russell [14] determined 13-*cis*-RA and all-*trans*-RA by high-performance liquid chromatography (HPLC), and Eckhoff and Nau [15] determined all-*trans*- and 13-*cis*-RA and their 4-oxo metabolites. The latter method used the HPLC column-switching technique and needed only 0.35 ml of plasma. However, both reports concentrated on identification of the analytes in human plasma and only limited validation data were presented. The latter is also true for our own feasibility test, using the column-switching technique and direct injection of plasma samples [16]. Huselton et al. [17] developed an interesting HPLC–MS method for all-*trans*- and 13-*cis*-RA. This method was later improved by Ranalder et al. [18], enabling also the determination of the two 4-oxo metabolites. However, only a simultaneous clean-up of retinoic acids and 4-oxo metabolites was possible, but not simultaneous separation and detection. In spite of the high sensitivity and selectivity of this method, the sample throughput was very low and not suitable for large studies. Very recently, Lehman and Franz [19] reported an HPLC–MS method using the particle beam interface. The HPLC–UV method of Periquet et al. [20] could only determine all-*trans*- and 13-*cis*-RA and needed a large plasma volume (3.5 ml),

whereas two other methods [21,22] concentrated on the simultaneous determinatiom of all-*trans*- and 13-*cis*-RA and retinol. Another recently published investigation [23] on reversed-phase capillary liquid chromatography with amperometric electrochemical detection reported pg/ml detection limits for pure solutions of 13-*cis*- and all-*trans*-RA, retinol and retinaldehyde. This enabled the determination of endogenous plasma levels of retinoic acids. However, the presented method was not validated and not suitable for routine determinations which are required for pharmacokinetic studies.

Therefore, the use of HPLC–UV with automated column switching (on-line solid-phase extraction) appeared the most promising, and our first method for the determination of exogenous levels of I–IV at the 2 ng/ml level [24,25] was improved, and the advances of the column-switching technique which were achieved for second- [26–28] and third-generation retinoids [29,30] were incorporated.

2. Experimental

2.1. Materials, reagents and solvents

Glacial acetic acid (100%) and ammonium acetate (both p.a.) and ethanol (absolute p.a. and HPLC grade), 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). Isotretinoin (13-*cis*-retinoic acid, I), tretinoin (all-*trans*-retinoic acid, II), 13-*cis*-4-oxo-retinoic acid (III) and all-*trans*-4-oxo-retinoic acid (IV) as well as acitretin (I.S., V) were provided by F. Hoffmann–La Roche Ltd. (Basel, Switzerland) and were kept under argon at −20°C. Reference compounds (see legend of Fig. 2) were also provided by F. Hoffmann–La Roche, whereas all-*trans*-retinoyl β-glucuronide was a gift from J.A. Olson and A.B. Barua, Iowa State University (Ames, IA, USA). Spiked plasma samples were prepared using fresh frozen plasma, either from sodium citrated human blood, which was obtained from a blood bank (Blutspendezentrum SRK, Basel, Switzerland), or from EDTA rat, mouse and rabbit blood from our own laboratories, or from EDTA/NaF Cynomolgus monkey blood obtained from Corning Hazleton (Münster, Germany).

2.2. Preparation of standards

The weighing of the compounds and samples, the preparation of calibration standards, as well as the

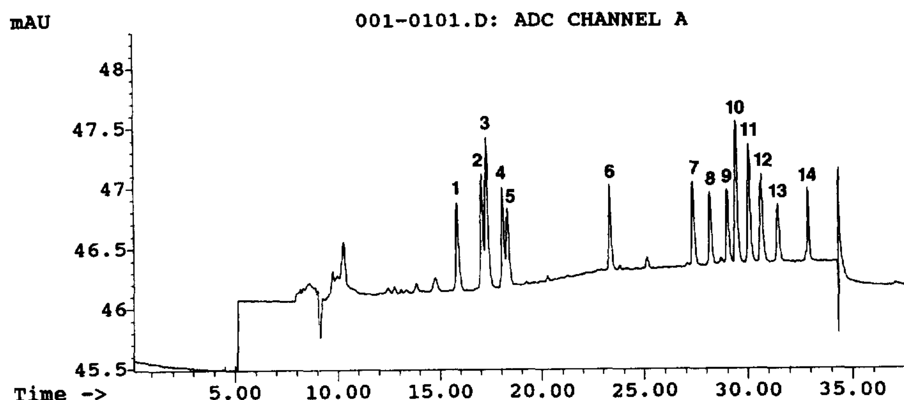

Fig. 2. Chromatogram of reference compounds. Peaks: 1=all-*trans*-4-hydroxy-RA, 2=all-*trans*-4-oxo-RA, 3=13-*cis*-4-hydroxy-RA, 4=13-*cis*-4-oxo-RA, 5=9-*cis*-4-oxo-RA, 6=all-*trans*-5,6-epoxy-RA, 7=all-*trans*-3,4-didehydro-RA, 8=all-*trans*-3,4-didehydroretinol, 9=13-*cis*-RA, 10=9,13-di*cis*-RA, 11=9-*cis*-RA, 12=all-*trans*-RA, 13=all-*trans*-retinol, 14=all-*trans*-retinal.

handling of the samples were performed under diffuse light conditions or under yellow light.

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

A stock solution of the internal standard was prepared in an amberized volumetric flask by dissolving 10 mg of V in 100 ml of ethanol (HPLC grade; 100 μg/ml). An internal standard working solution was prepared by diluting 0.4 ml of the stock solution with ethanol to 2000 ml (20 ng/ml). 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 amberized volumetric flasks by dissolving 10 mg of I and II and 5 mg of III and IV, respectively, in 10 ml of ethanol (HPLC grade). 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 10–0.03 μg/ml. These working solutions were used as calibration standards by adding 0.2 ml to 75 ml of internal standard solution followed by the addition of 20 ml of water, yielding concentrations of 100, 50, 10, 2, 0.5 and 0.3 ng/ml. The calibration standards were stored at 4°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 procedure

To 0.4 ml of plasma, 1.5 ml of the internal standard working solution were added for protein precipitation. After vortex-mixing and standing for 30 min in the deep freezer at −20°C, the sample was centrifuged (6 min at 3400 *g* and 10°C) and the supernatant transferred to the autosampler vial (1.5-ml Safe-Lock, Eppendorf–Netheler–Hinz, Hamburg, Germany), and 1.4 ml were injected. For calibration, 1.4 ml of the calibration standards [ethanol–water (3.75:1, v/v)] were directly injected. The samples were kept at ambient temperature and 4°C prior to injection for the analysis of human and animal plasma samples, respectively.

2.4. Chromatographic system and conditions

A schematic representation of the HPLC column-switching system is given in Fig. 3. An 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 (PC). In order to inject large sample volumes, the autosampler was used with two 5-ml syringes as dilutors 1 and 2 [solvents: water–ethanol (p.a.) (100:2, v/v) and ethanol (p.a.), respectively], a 3-ml sample loop, and the slow needle-down-speed. Animal plasma samples were cooled by a Peltier cooling system (PCU 1000, Labsource; 4°C). The

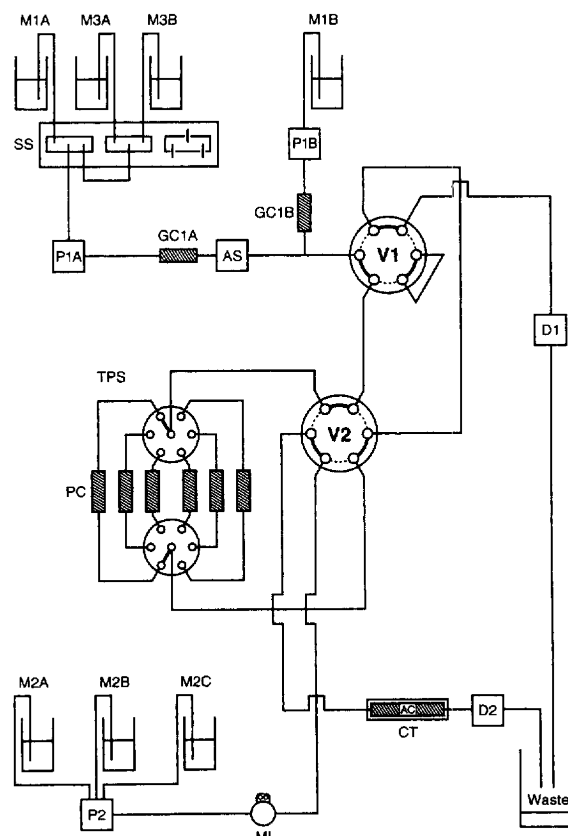

Fig. 3. Schematic representation of the HPLC column-switching system showing the configuration during the injection of the sample (step A). See Section 2.4 for further details.

injected sample plug was diluted, on-line, with mobile phase M1B by HPLC-pump P1B (L-6000, Merck; flow-rate 2.6 ml/min) via a T-piece (Valco Instruments, Houston, TX, USA; 1/16 in., bore 0.25 mm). The UV detector D1 (Spectroflow 773, Kratos, Ramsey, NJ, 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 precolumn 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, CA, USA) was used for direct injection onto the analytical column (e.g. for recovery experiments). Detection of the eluted compounds 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). An HPLC ChemStation (Hewlett–Packard, Waldbronn, Germany), in connection with an in-house developed LIMS (UNICHROM and KINLIMS [31]), 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. Precolumns were automatically replaced by a tandem precolumn selector (TPS; EA6 Port valve 7066/CPR, Labsource), either when a pressure of 150 bar was reached, indicating PC clogging during the following injections, or after 200 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 precolumns PC. The analytical column (AC) consisted of two LiChroCART HPLC cartridges (250×4 mm I.D.) packed with Superspher 100 RP-18 (end-capped), linked together by a manu-CART coupling unit (all Merck) and kept at 26°C by a column thermostat (CT; with Peltier controller unit PCU 1000, Labsource).

Mobile phase 1A (M1A) was prepared by mixing 100 ml of 10% ammonium acetate with 700 ml of water, 200 ml of ethanol (p.a.) and 10 ml of acetic acid. Mobile phase 1B (M1B) was prepared by mixing 100 ml of 10% ammonium acetate with 900 ml of water, 40 ml of ethanol (p.a.) and 20 ml of acetic acid. Mobile phase 2 (M2) consisted of three components: (M2A) 60 ml of 10% ammonium acetate, 300 ml of water, 10 ml of acetic acid, 600 ml of acetonitrile; (M2B) 5 ml of 10% ammonium acetate, 20 ml of water, 20 ml of acetic acid, 950 ml of acetonitrile; (M2C) 5 ml of water, 5 ml of acetic acid, 990 ml of acetonitrile. Mobile phase 3A (M3A) and 3B (M3B) consisted of ethanol (p.a.)–water (8:2, v/v) and ethanol (p.a.), respectively.

2.4.2. Procedure

The total sequence of automated sample analysis required 38 min. Details of the parameters used are shown in Table 1. The autosampler started pump 1B and the gradient program of P2. The latter started the HPLC ChemStation and 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 (either M3A or M3B), 40=M3A, 41=M3B), and (c) to start (52) or 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 plasma samples, six ethanolic calibration standards, distributed over the whole set of samples, were processed as described above. The calibration curves ($y=a+bx$) were obtained by weighted linear least-squares regression (weighting factor $1/y^2$) of the measured peak-height ratios I–IV/V (y) versus the concentrations of I–IV (x).

3. Results and discussion

3.1. Sample preparation procedure

The ideal way of using the column-switching technique for plasma sample analysis is direct injection of untreated plasma onto the precolumn, as

Table 1
Column-switching parameters and gradient program

Step	Time (min)	P2 gradient A (%)	B (%)	C (%)	P2 flow (ml/min)	P1A flow (ml/min)	P1B flow (ml/min)	P2 timer V1	V2	SS	others	Comment
A	0	100	0	0	0.05	0.05	2.6	10[a]	20			Injection of the sample onto PC using M1A+
	0.1				1.0	1.8				30,40		M1B for pre-concentration of the anaytes.
	2.5					1.8	0				62	PC was purged using M1A only.
	2.6					4.5						
B	4.5					4.5		11				PC was purged in the backflush mode.
C	5	100	0	0		2.0			21			Transfer of the retained components from
	5.1					1.0				31		PC to AC in the backflush mode. In the
	11					1.0			20			meantime, the capillaries between AS and
	12					0.5					72	D1 were purged with M3.
	13	80	20	0						41		
	19	40	60	0	1.0							
	19.1				1.2							
	27							10				
	30									40		
D	32	0	100	0	1.2	0.5				30		Re-equilibrationof PC with M1.
	32.1	0	0	100	2.0	1.8	0					
	35						2.6				52	
	37.9						0				62	
	38.9	0	0	100								
	39	100	0	0	2.0							
	39.1				1.0	1.8						

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

demonstrated first by Roth et al. [32]. Many other applications have been reported in the meantime and were reviewed recently [33]. For retinoids, this technique has the following advantages: a high degree of automation, no evaporation of extraction solvents, no problems with redissolving of extracts before injection into the HPLC system, protection from light during analysis and higher sensitivity compared to conventional extraction methods [5].

However, direct injection of plasma samples containing retinoids resulted in low recoveries [24,27]. This phenomenon, a result of high protein binding, is not only observed in the column-switching technique, but also in off-line solid-phase extraction or liquid–liquid extraction using hexane for direct extraction of plasma. The reason is a low mass transfer of the lipophilic analytes from the plasma protein to the stationary phase (or organic phase in the case of liquid–liquid extraction) through an aqueous phase.

These problems may be dealt with as follows: (a) addition of a water-miscible organic solvent (e.g. acetonitrile) to the plasma sample to improve the transfer of the retinoid from the protein to the stationary phase; when the final acetonitrile content is below 20%, plasma proteins are not precipitated; (b) protein precipitation with an organic solvent (e.g. ethanol) and injection of the supernatant. Procedure (a) was used for retinoic acids [15,16,24,34,35] and other retinoids [29]. However, isomerization of *cis*/*trans*-isomers was sometimes observed in the autosampler vials [28]. Therefore, plasma was injected and acetonitrile added, on-line, during injection onto the precolumn by means of an additional pump (P1B in Fig. 3) and a T-piece. This prevented any isomerization [28]. Even more robust conditions could be obtained with procedure (b) [25,26,36–38]. However, an injection containing a relatively high amount of ethanol is sometimes limiting because of breakthrough generated by the high elution strength of the ethanol.

To obtain the high sensitivity needed to determine endogenous concentrations of retinoic acids, the following approach was chosen in the present method: 0.4 ml of plasma was deproteinated by adding 1.5 ml of ethanol, and 1.4 ml of the supernatant were injected after centrifugation. This large volume of ethanol would prevent pre-concentration of the analytes on the precolumn. Therefore, ammonium acetate was added, on-line (M1B in Fig. 3), to reduce the elution strength of the injection solution. In an intermediate version of the method, using a less sensitive UV detector, 1.33 ml plasma were deproteinated with 4 ml of ethanol and, finally, 4 ml of the supernatant were injected. As long as the ratios of plasma (or water)–ethanol were not changed (or compensated by M1B), even higher volumes could be injected, e.g. up to 24 ml for in vitro metabolism studies [38]. 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.

3.2. Analytical system and chromatography

The column-switching system used for the determination of retinoic acids and their 4-oxo metabolites at the 2-ng/ml level in our laboratory [24] has been continuously improved over the last years. The addition of a third valve (V1 in the actual configuration, see Fig. 3) allowed forward- and backflush purging of the precolumn [25,27]. In this way, proteins and solid particles which can be adsorbed on the sieves of the precolumn were removed by backflush purging to prevent a transfer to the analytical column, thereby avoiding a long-term deterioration of the analytical column. A further extension of the system was the incorporation of the precolumn selector (TPS in Fig. 3), which automatically replaced a clogged precolumn with a new one, improving routine operation during the night or over the week-end [16]. The precolumn selector is now commercially available and allows the definition of the number of injections onto a precolumn before switching to a new one. In addition, guard columns (GC1A and GC1B [28]) were introduced to avoid the pre-concentration of impurities in the mobile phase [16]. The use of a low-pressure valve enabled purging of the capillaries between the autosampler and the precolumn with a mobile phase 3 (80% acetonitrile) instead of the gradient M2. This resulted in an overall reduced runtime [28]. In the final method, the low-pressure valve was replaced by a commercial solvent selector (SS in Fig. 3) which enabled the use of two (or more) mobile phases 3 (M3A and M3B) [28]. Finally, the T-piece and an

additional pump P1B (see Fig. 3), which were introduced to dilute injected plasma samples, on-line, with acetonitrile [28], were used to add 1% ammonium acetate and 2% acetic acid–ethanol (102:4, v/v) to the ethanolic injection solution to decrease the elution strength.

In all previous methods, Bondapak C_{18} Corasil was used as precolumn material. Its large particle size (37–50 μm) prevented clogging of the precolumn during injection of plasma samples or solutions containing residual proteins. In the present method, using a large volume of ethanol for protein precipitation, LiChrospher 100 RP-18, a 5-μm material, could be successfully used without premature clogging of the precolumn. The advantage of the LiChrospher material was the absence of isomerization of retinoic acids in monkey or rat plasma, which sometimes occured with the Corasil precolumn. The number of injections onto one precolumn was about 200 before it was replaced. This could be carried out automatically by the precolumn selector (TPS in Fig. 3), although manual replacement every few days is also possible. The potential of isomerization of the analytes and the internal standard in monkey and rat plasma was reduced by cooling animal plasma samples in the autosampler before injection. This was not necessary for human plasma.

The use of ethanol instead of acetonitrile as an organic modifier in mobile phase 1A resulted in a better removal of co-injected lipids from the precolumn, less isomerization during pre-concentration and better recovery of the 4-oxo metabolites. In addition, ethanol is cheaper than acetonitrile. Mobile phase 1B, consisting of 1% ammonium acetate and 2% acetic acid–ethanol (102:4, v/v), was needed to prevent a breakthrough of the analytes on the precolumn. A small amount of ethanol was added to avoid bacterial growth in pure ammonium acetate buffer. Ethanol was also preferred for purging of capillaries (M3A and M3B), as well as diluters of the autosampler to prevent memory effects.

Acitretin is an ideal internal standard because it is similar in structure, also having a tetraene side chain, and because it elutes between the retinoic acids and the 4-oxo metabolites. Thermostating of the analytical column to 26°C did not improve the separation of the analytes, but resulted in more reproducible retention times, which avoided time-consuming manual corrections of peak identification in the data system. Using the precolumn replacement as described above, more than 3000 injections could be made onto the analytical column before a deterioration was observed.

The relatively long total run time of 38 min for one sample is a consequence of the difficult separation of two pairs of geometrical isomers and additional endogenous interferences which needed gradient elution. The pre-concentration on the precolumn including backflush purging needed only 5 min per sample. The gradient pump (P2) was used for controlling the time functions shown in Table 1. Additional control signals were used to stop sample injection when the previous run was not successfully completed (not shown in Table 1).

Although the same matrix for calibration and unknown samples should be used whenever possible, this may give rise to problems with endogenous concentrations in control plasma. Procedures for removing the endogenous retinoids from control serum or plasma by photochemical degradation [39] or treatment with charcoal [40] have some drawbacks and have rarely been used routinely. A standard addition procedure, using control plasma with endogenous concentrations is only feasible for the measurement of unknown concentrations above the endogenous levels [21,25,41]. Some irreproducible isomerization was observed just after addition of very small amounts of retinoids (<2 ng/ml) to control plasma, making it, sometimes, impossible to prepare a plasma standard of low concentration with a defined amount of analyte. However, after spiking, these plasma levels were quite constant over several months. As the measured unknown concentrations are often below the lowest concentration of a calibration curve with standard addition, plasma as calibration matrix was often replaced by phosphate-buffered saline [17,18] or 5% serum albumin in phosphate-buffered saline [15]. In the present method ethanol–water solutions were used which had the same ratio as plasma samples after addition of the ethanolic internal standard solution for protein precipitation. As the recoveries of the analytes in plasma, as well as in ethanol–water, were about 90% or higher (see Table 2 and Section 3.4), this procedure is considered acceptable. For the determination of unknowns, quality control samples (spiked

Table 2
Recoveries of I–V (n=6)

Species	Concentration added (ng/ml)	13-*cis*-RA (I)		All-*trans*-RA (II)		13-*cis*-4-oxo RA (III)		All-*trans*-4-oxo-RA (IV)		I.S. (V)	
		Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)
Man	0.3	92.4	3.4	92.6	1.5	95.0	4.3	94.7	5.0	99.3	1.9
	2	92.7	1.7	92.7	1.9	92.7	4.5	92.2	3.7	100.1	1.1
	100	95.2	2.0	94.3	1.8	95.6	3.9	96.2	3.8	99.7	0.7
	Mean	93.4		93.2		94.4		94.4		99.7	
Cynomolgus monkey	0.3	95.6	3.3	90.7	3.4	94.2	6.9	92.9	6.1	95.9	2.1
	2	90.5	3.0	92.2	3.2	91.1	6.0	92.8	6.0	95.5	2.4
	100	88.1	2.1	92.7	2.2	88.9	4.6	93.1	4.7	96.5	2.2
	Mean	91.4		91.9		91.4		92.9		96.0	
Rabbit	0.3	98.3	1.8	95.1	2.4	98.2	2.6	94.6	8.3	94.8	2.5
	2	93.1	3.7	90.8	3.3	91.8	4.9	89.2	6.5	94.8	2.5
	100	91.9	4.9	91.0	4.8	91.7	6.3	93.1	6.0	95.0	2.7
	Mean	94.4		92.3		93.9		92.3		94.9	
Rat	0.3	83.8	4.2	93.5	7.9	101.5	23.0	68.2	9.1	91.1	4.8
	2	81.1	1.6	90.6	3.8	86.1	4.7	84.9	7.3	89.5	1.5
	100	85.3	1.5	92.7	4.4	84.1	3.8	94.0	7.1	90.6	1.1
	Mean	83.4		92.3		90.6		82.4		90.4	
Mouse	0.3	108.7	12.8	88.1	7.2	98.5	10.1	124.2	9.0	98.9	1.4
	2	96.7	2.5	91.7	2.1	94.8	3.9	96.3	3.4	98.1	0.3
	100	94.7	1.6	92.8	1.8	94.5	3.6	95.4	3.5	97.6	0.6
	Mean	100.0		90.9		95.9		105.3		98.2	

plasma of the same species) were always used for verification of the calibration procedure.

3.3. Selectivity

To reach the required selectivity for retinoic acids and their metabolites is a demanding task, especially at concentrations <2 ng/ml. The reason is not only that geometrical isomers of retinoic acids and 4-oxo metabolites have to be separated, but also endogenous interferences with similar retention times. Our first separation system consisted of two coupled 125 mm columns packed with Spherisorb ODS (5 μm) and a gradient mobile phase of acetonitrile and ammonium acetate [24]. Subsequently, three columns of 125 mm each (or one 250 mm and one 125 mm) of the same stationary phase were used in series [16]. Non-endcapped C_{18} material, in combination with acetonitrile in the mobile phase, resulted in the best separation of retinoic acids, but was less favourable for the separation of 4-oxo-RA isomers and endogenous peaks. Therefore, one endcapped coupled to one non-endcapped Superspher 100 RP-18 column (both 250 mm) were used in an intermediate method, showing improved separation for 4-oxo metabolites, but still not optimal separation from plasma interferences in that region. Finally, two endcapped Superspher 100 RP-18 columns (both 250 mm) were used. Retinoic acids were still sufficiently separated, whereas plasma interferences were better separated than in the previous system. A chromatogram showing the separation of 14 retinoids is presented in Fig. 2. Using endcapped Superspher, all-*trans*-3,4-didehydroretinol and all-*trans*-3,4-didehydro-RA, which were the subject of recent investigations [42,43], are also well separated. It would have been possible to improve the separation of 9-*cis*- from 13-*cis*-4-oxo-RA and of all-*trans*-4-oxo-RA from 13-*cis*-4-hydroxy-RA using specifically tailored conditions. However, this would have impaired the separation of the analytes from endogenous interferences. Fig. 4 shows a human blank plasma sample and the same plasma spiked with an additional 2 ng/ml of the four analytes, demonstrating the importance of finding a space for all-*trans*-4-oxo-RA in the chromatogram. Endogenous levels and interferences were generally lower in animal plasma. A typical example of a rat plasma sample, obtained after repeated topical application of isotretinoin (13-*cis*-RA), is shown in Fig. 5.

The goal of this study was to validate a method for the determination of 13-*cis*- and all-*trans*-RA and their metabolites 13-*cis*- and all-*trans*-4-oxo-RA. However, in another preliminary investigation, 9-*cis*-RA was also measured successfully [44]. The method may also be suitable to determine 9,13-di*cis*-RA, a major plasma metabolite of 9-*cis*-RA [45,46], which proved to be difficult to separate from 13-*cis*- and 9-*cis*-RA by other methods [45,46]. Moreover, additional retinoids shown in Fig. 2 may also be determined using the same conditions. Retinoyl β-glucuronides are also under investigation as important metabolites of retinoic acid [37,47]. However, all-*trans*-retinoyl β-glucuronide proved to be difficult to obtain in good purity and was, therefore, not included in Fig. 2. A preliminary investigation showed that this compound was eluted after approximately 13.5 min. An improved separation from plasma interferences is possible by a slight modification of the mobile phase and a prolongation of the gradient [40].

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 the sum of peak heights obtained from the endogenous levels of the same plasma ($n=6$) and the 100% values. The latter were identical to the calibration standards in ethanol/water and contained the same amount of analyte as the spiked plasma. The 6 injections of spiked plasma, blank plasma and 100% value 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 onto the precolumn were also compared with solutions of the same amount of analyte directly injected onto the analytical column. For this study, the analytes were dissolved in mobile phase 2A, and 100 μl were injected ($n=3$). The peak heights were similar (102.6% for I, 102.0% for II, 91.7% for III and 94.4% for IV) as in the experiment with precolumn injection, when the latter are defined as 100%.

The recoveries of the analytes and the internal standard are presented in Table 2, and they appear

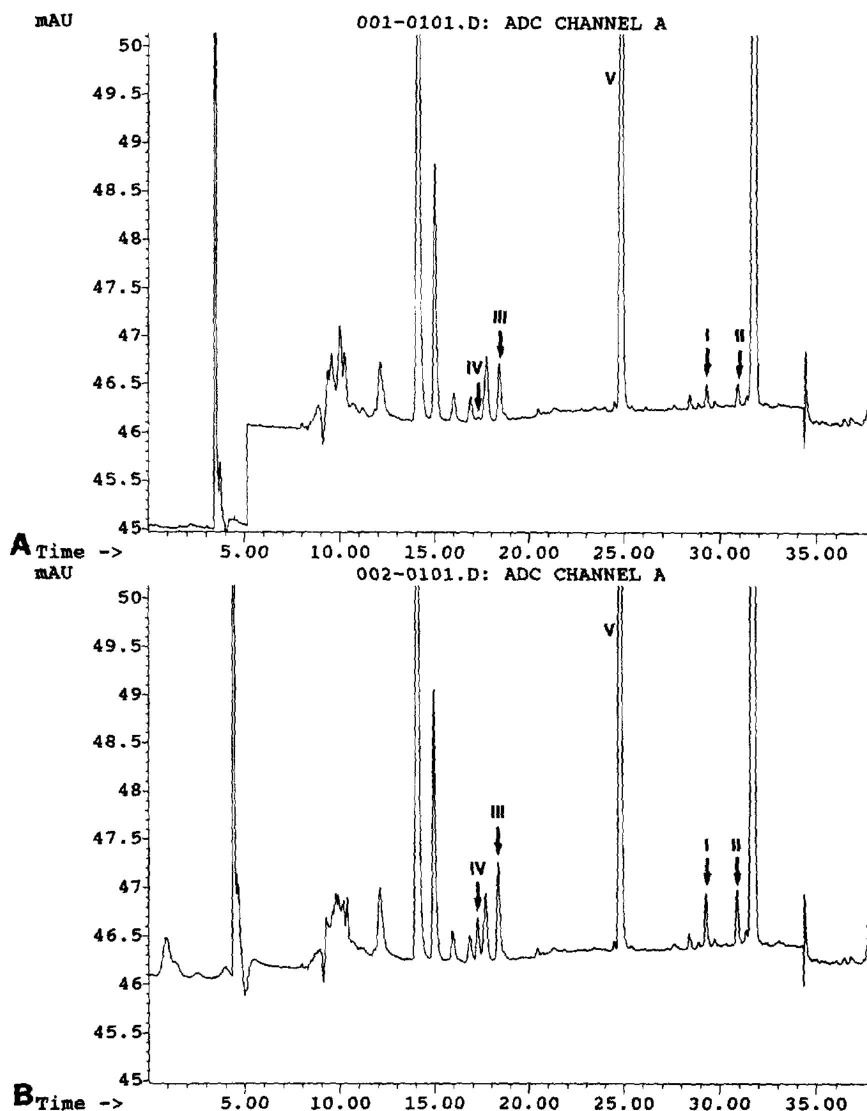

Fig. 4. Chromatograms of human plasma samples. (A) Volunteer plasma sample with endogenous levels of 1.36 ng/ml of 13-*cis*-RA (I), 1.20 ng/ml of all-*trans*-RA (II), 2.58 ng/ml of 13-*cis*-4-oxo-RA (III) and <0.3 ng/ml of all-*trans*-4-oxo-RA (IV). (B) Same plasma as in (A) but spiked with an additional 2 ng/ml of each of the four compounds. Peak V is the I.S. (acitretin).

quite consistent for all species. Only near the limit of quantification of the assay (endogenous concentration plus 0.3 ng/ml) do a few recovery values from rat and mouse plasma show some deviation. This may be a result of endogenous concentrations below or at the limit of quantification which were difficult to estimate and which showed some variability. The mean recoveries over all species are 92.5% for I (C.V.=6.5%), 92.1% for II (C.V.=0.9%), 93.2% for III (C.V.=2.4%) and 93.5% for IV (C.V.=8.7%).

3.5. Linearity

The method was linear in the range 0.3–100 ng/ml, at least. For plasma samples with concentrations >100 ng/ml (possible after oral administration),

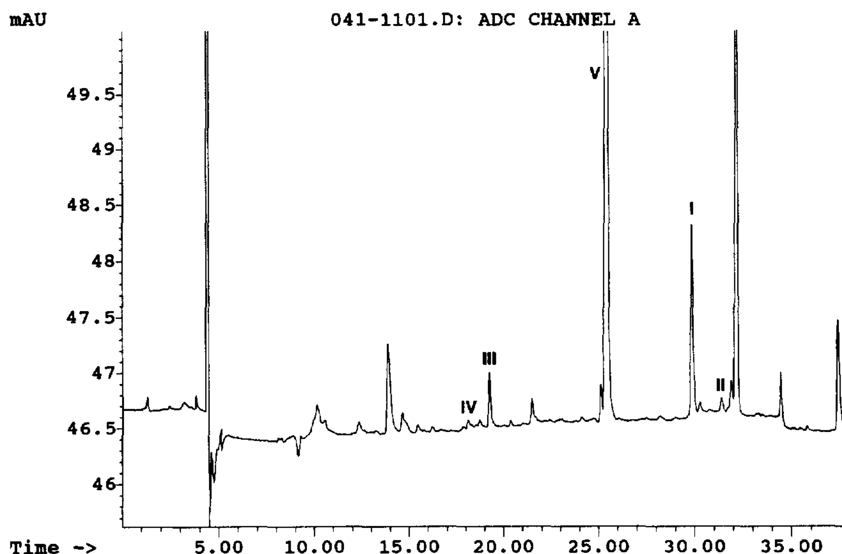

Fig. 5. Chromatogram of a rat plasma sample, collected on day 17, 1 h after a daily topical application of 0.03 mg/day of isotretinoin (13-*cis*-RA). Measured concentrations of 13-*cis*-RA (I) 9.30 ng/ml, all-*trans*-RA (II) 0.61 ng/ml, 13-*cis*-4-oxo-RA (III) 2.09 ng/ml and all-*trans*-4-oxo-RA (IV) 0.37 ng/ml.

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 0.3 ng/ml, using 0.4 ml of plasma. The inter-assay relative standard deviations (R.S.D.) and the accuracies for calibration samples ($n=6$) at this concentration were 1.2%–2.4% and 100.4%–102.1%, respectively.

3.7. Precision and accuracy

Inter-assay precision and accuracy were evaluated by analysing one series of plasma standards over six days. The results are compiled in Table 3. The mean R.S.D. for human plasma are 2.8%–3.2%, and the mean accuracies 92.5%–96.7% (range of added concentration 0.3–100 ng/ml). Similar results were obtained for monkey and rabbit plasma. For rat and mouse plasma the imprecision was slightly higher. In a few species, the accuracy at the +0.3 ng/ml level did not fulfil our normal acceptability criteria because the endogenous levels could not be determined exactly. These values are shown in brackets in Table 3.

3.8. Stability

The stability of retinoids was recently reviewed [5]. 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 [48]. However, a decrease of 15% is now considered relevant, in contrast to 10% in the published procedure [48]. The results of several storage experiments at −20°C and −80°C for human, monkey, rabbit, rat and mouse plasma are presented in Table 4. Whereas storage of plasma samples at −20°C for 4.3 months did not show any relevant degradation (except 13-*cis*-4-oxo-RA (III) in Cynomolgus monkey plasma), storage for 9 months resulted in a considerable decrease in most species. Therefore, plasma samples were stored at −80°C and found to be stable in all species for 9 months. Human plasma samples were even stable for two years when stored at −80°C (see Table 4).

The influence of the size of the air volume in the storage tube may be of importance. This was discussed in the past, including suggestions to minimize

Table 3
Inter-assay precison and accuracy ($n=6$)

Species	Concentration added (ng/ml)	13-*cis*-RA (I)			All-*trans*-RA (II)			13-*cis*-4-oxo-RA (III)			All-*trans*-4-oxo-RA (IV)		
		Conc. found (ng/ml)	R.S.D. (%)	Accuracy (%)	Conc. found (ng/ml)	R.S.D. (%)	Accuracy (%)	Conc. found (ng/ml)	R.S.D. (%)	Accuracy (%)	Conc. found (ng/ml)	R.S.D. (%)	Accuracy (%)
Man	0	1.36	4.9		1.20	4.2		2.58	3.4		<0.3		
	0.3	1.54	4.4	92.8	1.39	2.3	92.7	2.72	2.8	94.4	0.43	3.5	(143.3)
	2	3.09	2.6	92.0	2.89	3.5	90.3	4.18	3.1	91.3	1.95	2.7	97.5
	100	97.7	2.5	96.4	95.6	3.1	94.4	98.5	2.6	96.1	95.9	3.0	95.9
	Mean (0.3–100)		3.2	93.7		3.0	92.5		2.8	93.9		3.1	96.7
Cynomolgus monkey	0	0.50	8.8		0.84	6.5		<0.3			0.27	3.7	
	0.3	0.80	3.4	100.0	1.04	3.0	91.2	0.49	5.7	(163.3)	0.52	3.9	91.2
	2	2.35	2.7	94.0	2.63	2.6	92.6	2.07	3.4	103.5	2.14	2.9	94.3
	100	92.7	1.8	92.3	96.7	1.5	95.9	92.5	1.7	92.5	96.1	1.8	95.9
	Mean (0.3–100)		2.6	95.4		2.4	93.2		3.6	98.0		2.9	93.8
Rabbit	0	0.78	6.7		0.72	6.0		0.48	6.9		0.27	1.3	
	0.3	1.09	1.4	100.9	0.99	2.6	97.1	0.78	2.6	100.0	0.50	7.3	87.7
	2	2.68	2.1	96.4	2.51	2.3	92.3	2.34	1.8	94.4	2.04	3.5	89.9
	100	98.4	2.6	97.6	96.3	4.7	95.7	97.2	4.2	96.7	97.5	4.3	97.2
	Mean (0.3–100)		2.0	98.3		3.2	95.0		2.9	97.0		5.0	91.6
Rat	0	<0.3			0.35	11.8		<0.3			0.45	20.3	
	0.3	0.37	11.0	(123.3)	0.64	6.4	98.5	0.36	17.5	(120.0)	0.54	7.8	(72.0)
	2	1.93	1.5	96.5	2.28	3.6	97.0	1.89	2.9	94.5	2.26	5.3	92.2
	100	95.2	1.5	95.2	103	5.2	102.2	93.1	1.5	93.1	103	5.2	102.9
	Mean (0.3–100)		4.7	95.9		5.1	99.2		7.3	93.8		6.1	97.6
Mouse	0	<0.3			0.42	16.7		<0.3			0.29	5.4	
	0.3	0.35	12.2	(116.7)	0.63	7.2	87.5	0.30	9.9	100.0	0.38	9.1	(64.4)
	2	1.95	3.1	97.5	2.19	3.1	90.5	1.90	2.0	95.0	1.94	2.5	84.7
	100	98.0	2.3	98.0	95.3	1.7	94.9	97.0	1.8	97.0	97.1	1.9	96.8
	Mean (0.3–100)		5.9	97.8		4.0	91.0		4.6	97.3		4.5	90.8

Table 4
Stability of I–IV in plasma (blank plasma spiked with 5 ng/ml)

Species	Storage conditions	*n*	13-*cis*-RA (I)		All-*trans*-RA (II)		13-*cis*-4-oxo-RA (III)		All-*trans*-4-oxo-RA (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	4.3 months at −20°C	5	−3.6	−4.5 to −2.6	−4.0	−5.1 to −2.9	−5.3	−6.2 to −4.4	−4.0	−5.0 to −3.0
	9 months at −20°C	2	−24.5	−26.0 to −23.0	−0.6	−3.1 to +1.9	−4.7	−6.8 to −2.5	+7.2	+4.6 to +10.0
	9 months at −80°C	5	−5.2	−6.6 to −3.9	+0.3	−1.2 to +1.8	+2.8	+1.3 to +4.3	+5.4	+3.7 to +7.1
	2 years at −80°C	6	+2.9	+1.5 to +4.2	+1.5	+0.3 to +2.7	+9.5	+8.2 to +10.8	+5.7	+4.6 to +6.9
Cynomolgus monkey	4.3 months at −20°C	5	−11.3	−13.6 to −8.9	−5.8	−7.7 to −4.0	−16.0	−18.0 to −13.9	−4.3	−6.9 to −1.6
	9 months at −20°C	2	−37.1	−46.5 to −26.1	−25.7	−35.2 to −14.9	−30.9	−40.5 to −19.7	−12.2	−23.7 to +1.1
	4.7 months at −80°C	5	−5.8	−7.2 to −4.3	+0.4	−0.8 to +1.6	−8.1	−9.4 to −6.7	−1.5	−3.1 to +0.2
	9 months at −80°C	7	−14.3	−15.4 to −13.1	−4.9	−6.4 to −3.4	−7.3	−8.7 to −5.8	+2.2	+0.6 to +3.9
Rabbit	4.3 months at −20°C	4	−5.7	−7.0 to −4.3	−7.0	−8.2 to −5.8	−8.0	−9.2 to −6.7	−7.7	−8.9 to −6.4
	9 months at −20°C	2	−22.9	−27.9 to −17.6	−20.3	−25.1 to −15.3	−12.7	−18.2 to −7.0	−15.9	−21.3 to −10.2
	4.7 months at −80°C	6	+1.8	−3.5 to +7.2	+0.1	−4.1 to +4.5	−1.6	−4.9 to +2.0	−0.4	−3.3 to +2.6
	9 months at −80°C	7	−2.5	−3.8 to −1.2	+1.8	+0.6 to +2.9	+6.4	+4.9 to +8.0	+6.1	+4.4 to +7.8
Rat	4.3 months at −20°C	5	+1.4	−2.8 to +5.7	−2.5	−6.1 to +1.3	−6.2	−12.2 to +0.3	−2.2	−7.1 to +2.9
	9 months at −20°C	2	−21.3	−30.1 to −11.2	−21.3	−30.2 to −11.2	−19.9	−29.4 to −9.0	−18.2	−28.6 to −6.1
	4.7 months at −80°C	5	−1.8	+3.9 to +0.4	−3.5	−5.9 to −1.0	−5.1	−7.7 to −2.4	−5.2	−7.7 to −2.5
	9 months at −80°C	7	+1.2	−4.4 to +7.0	−2.2	−4.7 to +0.3	+5.1	+3.3 to +7.0	+2.6	+0.9 to +4.3
Mouse	4.3 months at −20°C	5	−0.4	−2.0 to +1.2	−4.5	−6.1 to −2.9	−6.8	−9.1 to −4.5	−3.1	−5.1 to −1.0
	9 months at −20°C	2	−10.5	−11.9 to −9.0	−7.7	−9.6 to −5.8	−2.3	−4.7 to +0.2	+2.1	0 to +4.2
	4.7 months at −80°C	3	−1.8	−8.5 to +5.4	−3.7	−5.7 to −1.6	−6.3	−12.0 to −0.2	−4.0	−6.7 to −1.2
	9 months at −80°C	7	−6.8	−8.2 to −5.3	−2.1	−3.8 to −0.3	+1.9	+0.2 to 3.6	+1.6	−0.2 to +3.5

Table 5
Endogenous concentratons of I–IV in human plasma. Comparison with data from the literature

Source	Subjects	*n*	13-*cis*-RA (I) Mean±S.D. (ng/ml) (Range)	All-*trans*-RA (II) Mean±S.D. (ng/ml) (Range)	13-*cis*-4-oxo-RA (III) Mean±S.D. (ng/ml) (Range)	All-*trans*-4-oxo-RA (IV) Mean±S.D. (ng/ml) (Range)
R. Theiler, University Hospital, Zurich[a]	Control patients[b]	16	1.85±1.13 (0.99–5.27)	1.39±0.33 (0.89–2.20)	3.25±2.17 (1.45–9.10)	<0.3
U.-W. Wiegand, F. Hoffmann-La Roche, Basel[a]	Pregnant women (1st trimester, 8 centers)	53	1.41±0.63 (0.72–4.72)	1.33±0.28 (0.68–2.18)	2.44±1.34 (0.84–7.72)	0.34±0.10 (<0.26–0.54)
Tang and Russell [14]	Fasting volunteers	26	1.4±0.3 (1.0–2.2)	1.4+0.2 (1.1–1.9)	n.d.[c]	n.d.
	Non-fasting volunteers	10	1.9±0.6 (1.1–2.8)	1.9±0.7 (1.0–2.9)	n.d.	n.d.
Eckhoff and Nau [15]	Male volunteers	10	1.63±0.85	1.32±0.46	3.68±0.99	<0.5
	Volunteers dosed with 833 I.U. of vitamin A/kg	5	9.75±2.18	3.92±1.40	7.60±1.45	<0.5 (*n*=4)
Huselton et al. [15]	Volunteers	13	1.6±0.4	0.9±0.2	n.d.	n.d.
Ranalder et al. [18]	Volunteers	8	(1.19-1.99)	(1.4–1.76)	(1.92–3.03)	(<0.3–0.7)
Lehman and Franz [19]	Male volunteers	19	0.93±0.39	1.28±0.32	n.d.	n.d.
	Female volunteers	9	0.92±0.25	1.69±0.53	n.d.	n.d.
Periquet et al. [20]	Fasting volunteers	16	1.59±0.73	3.54±0.99	n.d.	n.d.
Meyer et al. [21]	Volunteers	22	1.79±0.56	1.35±0.42	n.d.	n.d.
Takeda and Yamamoto [22]	Volunteers	20	1.80±0.62) (1.21–3.46)	1.77±0.75 (0.94–3.42)	n.d.	n.d.

[a] Samples analysed by this method.
[b] 13 male and 3 female, aged 18–79.
[c] Not determined.

the headspace above the plasma, or to flush the tubes with nitrogen or argon [9]. To further investigate this question, spiked human plasma samples were not only stored in 4-ml vials (these are the results presented in Table 4), but also in 12-ml glass tubes for 9 months at −20°C and −80°C. In both cases, the plasma volume was 3 ml. However, no significant difference in the decrease of the concentrations of the four analytes could be found (data for 12-ml tubes not shown). Even when the plasma was stored in a 50-ml vessel, which was about half full with plasma, at −20°C for 9 months, the decreases were very similar. The only minor differences were a slightly higher decrease of 9% for all-*trans*-RA (II) and a slightly lower decrease of 16% for 13-*cis*-RA (I) (data not shown). From these data, it can be concluded that the headspace volume of the storage tube, at least for human plasma, is of less importance for retinoids than previously assumed [9].

3.9. Application to biological samples

The method was successfully applied to clinical and toxicokinetic studies. Fig. 4A and Fig. 5 show representative chromatograms from a human volunteer and a rat, treated topically with 0.03 mg 13-*cis*-RA (I) per day. Overall, more than 6000 human plasma samples and more than 1000 plasma samples from rats, rabbits, Cynomolgus monkeys and mice were analysed. Several laboratories were involved in these analyses, demonstrating the robustness of the method.

Endogenous concentrations of the four analytes determined in human plasma from two studies are shown in Table 5. The results concur with most of the published data from other laboratories which are also listed in Table 5. Final data of the study with pregnant women will be reported elsewhere. The presented method, using only 0.4 ml of plasma, may be well suited for further investigations on the variability of retinoic acids in the plasma of volunteers and patients.

4. Conclusions

Retinoids are well known to be difficult to determine in biological samples because of their sensitivity to light and oxygen, their high protein binding, insolubility in aqueous solutions, the problem of separation of geometrical isomers, and the determination of low endogenous levels. For a development program for isotretinoin (13-*cis*-RA) in photodamage, a highly sensitive HPLC method with automated column switching and UV detection was developed and validated for the simultaneous determination of endogenous levels of 13-*cis*- and all-*trans*-RA and their 4-oxo metabolites in plasma samples from man, Cynomolgus monkey, rabbit, rat 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, a protein precipitation with ethanol and direct injection of the supernatant onto the precolumn was performed. The use of an additional pump and a T-piece allowed a decrease of the elution strength of the injection solution, and, therefore, a pre-concentration of the analytes on the precolumn.

In spite of the complexity of the column-switching system, the method had an excellent repeatability with an inter-assay precision of only about 3% for human plasma. This confirms the general observation that the column-switching technique usually provides better precision than off-line techniques. In some animal species the imprecision was sometimes higher, especially near the quantification limit of the assay. However, this may be not a result of the analytical method, but of the isomerization or degradation potential of the plasma samples. The presented method was also robust and could be successfully transferred to several other laboratories. Within a run time of 38 min for one sample, not only could the retinoic acid isomers and metabolites be separated from interferences, but the method may also be used for the determination of other endogenous retinoids, such as retinol, retinal, 3,4-didehydroretinol, 9-*cis*- or 9,13-di*cis*-retinoic acid or all-*trans*-3,4-didehydroretinoic acid.

The method was successfully applied to the determination of more than 6000 human plasma samples and more than 1000 samples from rats, rabbits, Cynomolgus monkeys and mice. The measured endogenous levels in control patients and pregnant

women were in good agreement with published data from volunteers. Due to the high sensitivity of the method (only 0.4 ml of plasma are needed) and the high degree of automation, this method will be well suited for the determination of endogenous levels of retinoids in large series of study samples.

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References

[1] M.B. Sporn, A.B. Roberts, D.S. Goodman (Eds.), The Retinoids: Biology, Chemistry and Medicine, 2nd ed., Raven Press, New York, NY, 1994.
[2] S. Castaigne, C. Chomienne, M.T. Daniel, P. Ballerini, R. Berger, P. Fenaux, L. Degos, Blood 76 (1990) 1704.
[3] E.E. Holdener, W. Bollag, Curr. Opin. Oncol. 6 (1993) 1059.
[4] G.L. Peck, J.J. DiGiovanna, in: M.B. Sporn, A.B. Roberts, D.S. Goodman (Eds.), The Retinoids: Biology, Chemistry and Medicine, 2nd ed., Raven Press, New York, NY, 1994, p. 631.
[5] R. Wyss, J. Chromatogr. B 671 (1995) 381.
[6] H.C. Furr, A.B. Barua, J.A. Olson, in: M.B. Sporn, A.B. Roberts, D.S. Goodman (Eds.), The Retinoids: Biology, Chemistry and Medicine, 2nd ed., Raven Press, New York, NY, 1994, p. 179.
[7] A.P. De Leenheer, W.E. Lambert, E. Meyer, in: M.A. Livrea, L. Packer (Eds.), Retinoids, Progress in Research and Clinical Applications, M. Dekker, New York, NY, 1993, p. 551.
[8] H.C. Furr, A.B. Barua, J.A. Olson, in: A.P. De Leenheer, W.E. Lambert, H.J. Nelis (Eds.), Modern Chromatographic Analysis of Vitamins, 2nd ed., M. Dekker, New York, NY, 1992, p. 1.
[9] R. Wyss, J. Chromatogr. 531 (1990) 481.
[10] M.G. De Ruyter, W.E. Lambert, A.P. De Leenheer, Anal. Biochem. 98 (1979) 402.
[11] A.P. De Leenheer, W.E. Lambert, I. Claeys, J. Lipid Res. 23 (1982) 1362.
[12] J.L. Napoli, B.C. Pramanik, J.B. Williams, M.I. Dawson, P.D. Hobbs, J. Lipid Res. 26 (1985) 387.
[13] A.B. Barua, J.A. Olson, Am. J. Clin. Nutr. 43 (1986) 481.
[14] G. Tang, R.M. Russell, J. Lipid Res. 31 (1990) 175.
[15] C. Eckhoff, H. Nau, J. Lipid Res. 31 (1990) 1445.
[16] R. Wyss, F. Bucheli, J. Pharm. Biomed. Anal. 8 (1990) 1033.
[17] C.A. Huselton, B.E. Fayer, W.A. Garland, D.J. Liberato, in: M.A. Brown (Ed.), Liquid Chromatography/Mass Spectrometry: Applications in Agricultural, Pharmaceutical, and Environmental Chemistry, ACS Symposium Series, vol. 420, American Chemical Society, Washington, DC, 1990, p. 166.
[18] U.B. Ranalder, B.B. Lausecker, C. Huselton, J. Chromatogr. 617 (1993) 129.
[19] P.A. Lehman, T.J. Franz, J. Pharm. Sci. 85 (1996) 287.
[20] B. Periquet, W. Lambert, J. Garcia, G. Lecomte, A.P. De Leenheer, B. Mazieres, J.P. Thouvenot, J. Arlet, Clin. Chim. Acta 203 (1991) 57.
[21] E. Meyer, W.E. Lambert, A.P. De Leenheer, Clin. Chem. 40 (1994) 48.
[22] N. Takeda, A. Yamamoto, J. Chromatogr. B 657 (1994) 53.
[23] J.J. Hagen, K.A. Washco, C.A. Monning, J. Chromatogr. B 677 (1996) 225.
[24] R. Wyss, F. Bucheli, J. Chromatogr. 424 (1988) 303.
[25] R. Wyss, Meth. Enzymol. 189 (1990) 146.
[26] R. Wyss, F. Bucheli, J. Chromatogr. 431 (1988) 297.
[27] R. Wyss, F. Bucheli, J. Chromatogr. 456 (1988) 33.
[28] R. Wyss, F. Bucheli, J. Chromatogr. 593 (1992) 55.
[29] R. Wyss, F. Bucheli, J. Chromatogr. 576 (1992) 111.
[30] R. Wyss, F. Bucheli, B. Hess, J. Chromatogr. A 729 (1996) 315.
[31] U. Timm, B. Hirth, in: E.J. Karjalainen (Ed.), Scientific Computing and Automation (Europe) 1990, Elsevier, Amsterdam, 1990, p. 329.
[32] W. Roth, K. Beschke, R. Jauch, A. Zimmer, F.W. Koss, J. Chromatogr. 222 (1981) 13.
[33] P. Campins-Falco, R. Herraez-Hernandez, A. Sevillano-Cabeza, J. Chromatogr. 619 (1993) 177.
[34] C. Eckhoff, W. Wittfoht, H. Nau, W. Slikker, Biomed. Environ. Mass Spectrom. 19 (1990) 428.
[35] G. Tzimas, H. Bürgin, M.D. Collins, H. Hummler, H. Nau, Arch. Toxicol. 68 (1994) 119.
[36] J. Creech Kraft, C. Eckhoff, W. Kuhnz, B. Löfberg, J. Nau, Liq. Chromatogr. 11 (1988) 2051.
[37] J.O. Sass, H. Nau, J. Chromatogr. A 685 (1994) 182.
[38] R.C. Chou, R. Wyss, C.A. Huselton, U.-W. Wiegand, Xenobiotica 22 (1992) 993.
[39] J.L. Napoli, Meth. Enzymol. 123 (1986) 112.
[40] G. Fex, personal communication.
[41] T.A.G. Noctor, J. Burnett, T. Thompson, personal communication.
[42] C. Thaller, G. Eichele, Nature 345 (1990) 815.
[43] J. Creech Kraft, D. Kimelman, M.R. Juchau, Drug Metab. Dispos. 23 (1995) 83.
[44] R. Wyss, F. Bucheli, unpublished data.
[45] G. Tzimas, J.O. Sass, W. Wittfoht, M.M.A. Elmazar, K. Ehlers, H. Nau, Drug Metab. Dispos. 22 (1994) 928.
[46] R.L. Horst, T.A. Reinhardt, J.P. Goff, B.J. Nonnecke, V.K. Gambhir, P.D. Fiorella, J.L. Napoli, Biochemistry 34 (1995) 1203.
[47] A.B. Barua, J.A. Olson, Am. J. Clin. Nutr. 43 (1986) 481.
[48] U. Timm, M. Wall, D. Dell, J. Pharm. Sci. 74 (1985) 972.