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Short communication

Single-run analysis of isomers of retinoyl-β-D-glucuronide and retinoic acid by reversed-phase high-performance liquid chromatography

Jörn Oliver Sass*, Heinz Nau

Institut für Toxikologie und Embryopharmakologie, Freie Universität Berlin, Garystrasse 5, D-14195 Berlin, Germany

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Abstract

Reversed-phase HPLC methods capable of separating several retinoic acid isomers are generally not designed for simultaneous analysis of isomers of other classes of retinoids. A reversed-phase HPLC method is presented which allows the separation of at least four retinoic acid (RA) isomers (13-*cis*-RA, 9,13-di*cis*-RA, 9-*cis*-RA, all-*trans*-RA) and of all isomers of retinoyl-β-D-glucuronide (RAG), which have been observed in vivo (13-*cis*-RAG, 9-*cis*-RAG, all-*trans*-RAG). The recovery of retinoids was generally between 80 and 90%. Intra-day reproducibility (expressed as relative standard deviation) was ≤7.0%. As little as 0.25 ng of RA isomers and of all-*trans*-RAG could be detected. This method allowed the study of the metabolism of 9-*cis*-retinoids, where isomerization reactions play a predominant role.

1. Introduction

Retinoids, derivatives of vitamin A alcohol (retinol), are involved in several physiological processes such as embryonic development, growth promotion, differentiation and vision [1,2]. Some retinoids have also attracted attention as important agents in dermatology [3] and oncology [4]. Three geometric isomers of vitamin A acid (retinoic acid, RA) have been found to be endogenously present in blood or tissues of animals and humans: all-*trans*-RA [5–7], 13-*cis*-RA [6,7] and 9-*cis*-RA [8,9] (Fig. 1).

It has been suggested that the retinoid action is mediated by the interaction of retinoids with retinoid receptors and cellular retinoic acid binding proteins [1,8]. The various geometric isomers of the retinoic acid structure exhibit great differences towards the retinoic acid binding sites [10–12]. After administration of pharmacologic doses of all-*trans*-, 13-*cis*- and 9-*cis*-RA or 9-*cis*-retinaldehyde, the corresponding retinoyl-β-D-glucuronides (RAG) (Fig. 1) have been characterized as major plasma metabolites in monkeys, rats, mice and rabbits [13–20]. Recently, we have observed the in vivo formation of a further RA isomer: 9,13-di*cis*-RA (Fig. 1) was identified as a major metabolite after administration of 9-*cis*-RA and 9-*cis*-retinaldehyde to mice and rats [19,20]. Isomerization plays a major role in the metabolism of 9-*cis*-retinoids. Although some researchers have achieved separation of up

* Corresponding author.

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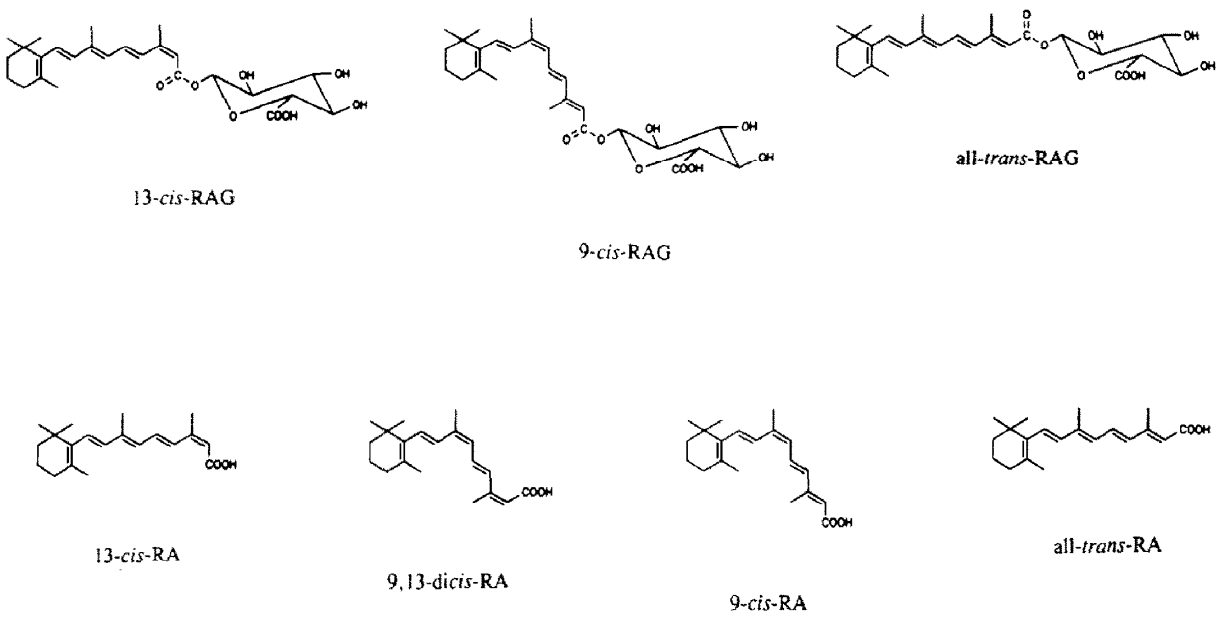

Fig. 1. Structures of isomers of retinoyl-β-D-glucuronide (RAG) and retinoic acid (RA).

to nine photoisomerization products of retinoic acid [21–23], the reversed-phase HPLC methods which are generally applied for analysis of biological samples, do not separate 9,13-di*cis*-RA from 13-*cis*-RA. Furthermore, the methods developed for separation of RA photoisomers are not designed for separation of isomers of retinoyl-β-D-glucuronides.

We have recently reported the separation of 9-*cis*-RAG from 13-*cis*- and all-*trans*-RAG using the HPLC method described by Collins et al. [24]. However, this method did not allow the separation of 13-*cis*-RA and 9,13-di*cis*-RA, and provided only limited separation of 13-*cis*-RA and 9-*cis*-RA as well as of 9-*cis*-RAG and all-*trans*-RAG.

We describe here a reversed-phase HPLC method, which allows the simultaneous analysis of all isomers of RAG and RA, which have been reported to be formed in mammalian species. This method has been used for analysis of photoisomers of RAG and RA and for examinations of the metabolism of retinoids, in particular of 9-*cis*-retinoids.

2. Experimental

2.1. Chemicals

Retinoic acid isomers as well as 4-oxoretinoic acids and all-*trans*-3,4-didehydroretinoic acid were provided by Hoffmann-La Roche (Basle, Switzerland; and Nutley, NJ, USA). All-*trans*-RAG was synthesized by Rühl [25] based on the method of Barua and Olson [26]. Isomers of RAG were prepared in a quartz cuvette by irradiation (30 min at room temperature) of a solution of all-*trans*-RAG in dimethyl sulfoxide or methanol–dimethyl sulfoxide, using a DESAGA duo-UV source (Desaga, Heidelberg, Germany) set to 366 nm. The RAG isomers were isolated using the newly developed HPLC method described herein and identified by enzymatic hydrolysis with β-glucuronidase (Boehringer Mannheim, Germany) [18], which yielded the corresponding retinoic acids. Bovine serum albumin (BSA; retinoid free) was obtained from Sigma (Deisenhofen, Germany).

Acetonitrile (HPLC grade) and all other chemicals (analytical grade) were purchased from Merck (Darmstadt, Germany). Water was purified with a Milli-Q system (Millipore, Eschborn, Germany).

Laboratory precautions. Due to the light sensitivity of retinoids, all work with these compounds was performed under dim amber light.

2.2. Animal experiment

Pregnant Wistar rats (gestational day 13) received a single intragastric dose of 100 mg 9-*cis*-RA per kg body mass. 9-*cis*-RA was suspended in peanut oil and a dosing volume of 5 ml/kg was administered. Two hours after treatment blood was collected and plasma was prepared from the heparinized blood by centrifugation at 4°C (1500 *g* for 10 min) [20].

2.3. Instrumentation and chromatographic conditions

The HPLC equipment used consisted of two type 64 pumps controlled by a gradient programmer 50B, a dynamic mixing chamber (all from Knauer, Berlin, Germany), a DEGASYS DG-1200 degasser (vds-optilab, Berlin, Germany), two Shimadzu UV detectors (SPD-6A and SPD 6-AV) for detection at 340 and 356 nm and a C-R4A integrator (Shimadzu, Duisburg, Germany). After the mixing chamber, the eluent mixture passed through a C-130B precolumn filled with Perisorb RP-18 material (Upchurch Scientific, Oak Harbour, WA, USA) and an online eluent filter (Knauer) before reaching an advanced automated sample processor (AASP; Varian, Darmstadt, Germany), which was followed by a second filter and a self-packed analytical column (120 × 4 mm), filled with Spherisorb ODS2 3 μm (silica modified with octadecyl groups, endcapped; Phase Separations, Deeside, UK). During chromatography, the analytical column was heated to 60°C. Analysis was performed with a multilinear gradient (Table 1) of solvent A [water with 0.2% (v/v) trifluoroacetic acid] and solvent B [acetonitrile with 0.2% (v/v) trifluoroacetic acid] at a flow-rate of 0.7 ml/min. Between the analyses (run time 36 min) the column was washed with 99% of solvent B and subsequently re-equilibrated (cycle time 42.5 min). Injection was performed by introducing an AASP C2 cartridge (silica modified with ethyl groups; ICT, Bad Homburg, Germany), which was loaded with analytes, into the stream of mobile phase. The cartridge remained the whole run time in the AASP system. Before and after analysis the cartridge was purged with 500 μl of water.

Table 1
Composition of mobile phase (multilinear binary gradient)

Time (min)	A (%)	B (%)
0	70	30
30	25	75
35.5	25	75
36	1	99
39	1	99
39.5	70	30
42.5	70	30

Solvent A: water with 0.2% (v/v) trifluoroacetic acid; solvent B: acetonitrile with 0.2% (v/v) trifluoroacetic acid.

2.4. Sample preparation

Sample enrichment was performed as recently described [24]. In brief, 125 μl of sample were mixed with 375 μl of isopropanol and shortly centrifuged; 400 μl of the supernatant were diluted with a threefold volume of 2% (w/v) aqueous ammonium acetate solution and poured with a nitrogen stream through a preconditioned AASP C2 cartridge. After washing with 1.5 ml of a mixture of 85% aqueous ammonium acetate solution (0.5%) and 15% acetonitrile the cartridge was loaded onto the AASP.

2.5. System validation

Standards for system validation as well as for calibration were prepared by spiking a filtered

5% (w/v) BSA solution with known amounts of reference retinoids (13-*cis*-RA, 9-*cis*-RA, all-*trans*-RA, all-*trans*-RAG). To determine recovery, peak areas of the standards were compared to those obtained after direct injection of respective amounts of retinoids. Reproducibility was tested on three subsequent days using standards with 15, 150 and 1500 ng/ml of 13-*cis*-RA, 9-*cis*-RA, all-*trans*-RA and all-*trans*-RAG. To examine linearity, further standards were analyzed which contained 2.5, 5, 50 and 500 ng/ml of each retinoid. Linear regression was performed by least squares analysis of the peak absorbance units and retinoid concentrations of the BSA standards. Quantification of retinoids in routine analysis was based on three standard concentrations, which covered the expected retinoid concentrations of the corresponding application. Concentrations of 9,13-di*cis*-RA were calculated using the standard values for 13-*cis*-RA at the 340 nm detection wavelength.

3. Results and discussion

The chromatogram in Fig. 2 demonstrates the separation of isomers of RAG and RA by the newly developed HPLC method. The corresponding k' values are given in Table 2. Fig. 3 shows a chromatogram of a plasma sample obtained from a pregnant rat on gestational day 13 2 h after oral treatment with 100 mg/kg 9-*cis*-RA. Here the different isomers of RA and RAG are also well separated. Details on the metabolism of 9-*cis*-RA will be presented elsewhere [20]. The HPLC method allows also the detection of 4-oxo-retinoic acids. 13-*cis*-4-Oxo-RA, which is endogenously present in human blood [7], elutes 3 to 3.5 min before all-*trans*-RAG, but no reliable separation of the different

Table 2
Retention factors (k') and resolution (R_s) of adjacent peaks (derived from Fig. 2)

Retinoid	k'	R_s
13-*cis*-RAG	11.78	
		1.22
9-*cis*-RAG	12.00	
		2.01
All-*trans*-RAG	12.33	
13-*cis*-RA	16.82	
		1.18
9,13-di*cis*-RA	17.05	
		1.51
9-*cis*-RA	17.35	
		2.43
All-*trans*-RA	17.89	

The resolution of two adjacent peaks (R_s) was calculated from the equation $R_s = 1.18(t_{R2} - t_{R1})/(w_{\frac{1}{2}1} + w_{\frac{1}{2}2})$ with $w_{\frac{1}{2}}$ being the peak width at half maximum peak height.

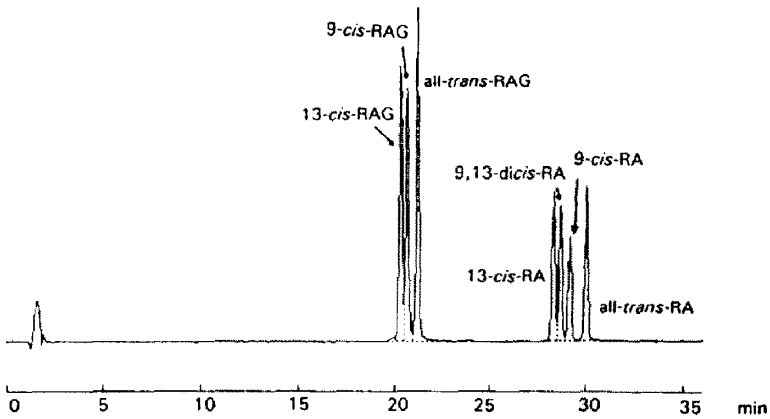

Fig. 2. Chromatogram of reference retinoids. Detection at 356 nm. A mixture of 13-*cis*-RAG, 9-*cis*-RAG, all-*trans*-RAG, 13-*cis*-RA, 9,13-di*cis*-RA, 9-*cis*-RA and all-*trans*-RA (total amount of each RAG isomer 40–50 ng, and of each RA isomer 14–18 ng) was submitted to solid-phase extraction and subsequently analyzed by reversed-phase HPLC, using the system described in the Experimental section.

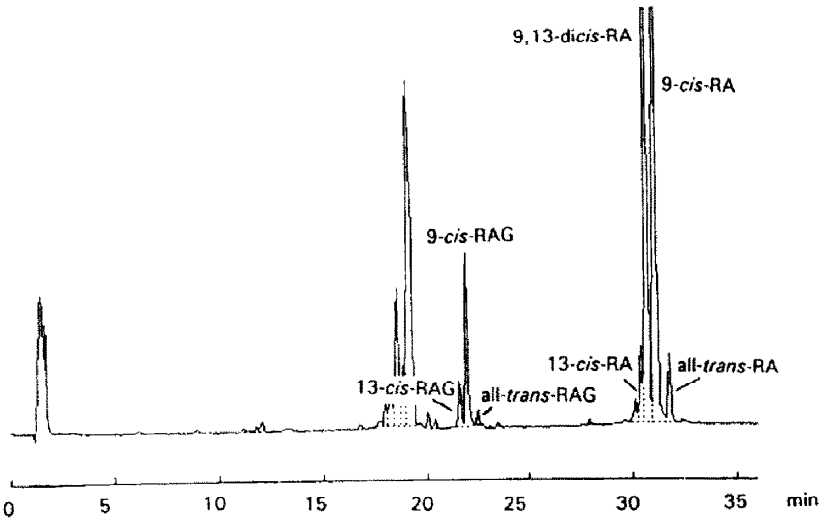

Fig. 3. Chromatogram of 100 μl rat plasma, obtained from a pregnant rat (gestational day 13) 2 h after oral treatment with 100 mg 9-*cis*-RA per kg body mass. Detection at 356 nm. Retinoid concentrations: 13-*cis*-RAG: 60.2 ng/ml; 9-*cis*-RAG: 210 ng/ml; all-*trans*-RAG: 15.3 ng/ml; 13-*cis*-RA: 53.2 ng/ml; 9,13-di*cis*-RA: 591 ng/ml; 9-*cis*-RA: 768 ng/ml; all-*trans*-RA: 43.5 ng/ml.

isomers was achieved for 4-oxo-retinoic acids. In addition to the three RAG isomers which were also found in vivo, photoisomerization of all-*trans*-RAG also resulted in a fourth β-glucuronide ($k' = 11.54$), as indicated by enzymatic hydrolysis with β-glucuronidase, which yielded a compound eluting slightly in front of 13-*cis*-RA. Our method can also resolve 13-*cis*-RA from the preceding peak of all-*trans*-3,4-didehydroretinoic acid ($R_s = 2.07$) (data not shown).

Examination of linearity based on standards with 15, 150 and 1500 ng/ml retinoids yielded a coefficient of regression ≥ 0.9999 for all four retinoids all-*trans*-RAG, 13-*cis*-, 9-*cis*- and all-*trans*-RA on three consecutive days. If the four further standard concentrations were included into the regression analysis (2.5 to 1500 ng/ml) the coefficient of regression was ≥ 0.999 for all-*trans*-RAG, 13-*cis*-, all-*trans*-RA and 0.9517 for 9-*cis*-RA. As little as 0.25 ng/100 μl of each retinoid could well be detected (signal-to-noise ratio > 10).

Table 3 demonstrates the recovery values obtained for the retinoid concentrations 15, 150 and 1500 ng/ml. Recovery was generally between 80 and 90%. The corresponding values for intra-day variation (relative standard deviation, R.S.D.) are presented in Table 4. In comparison, the R.S.D. for direct injection of respective amounts of retinoids is about 4%. Table 4 contains also values for inter-day variation. These values are below 3% for all RA isomers at the highest concentration level examined and reach 10% for the lowest RA concentrations.

The described HPLC system allows rapid and simple resolution of all four RA isomers, which have been detected in vivo, and furthermore provides single-run separation of several RAG isomers. This method is suitable for analysis of a

Table 3
Recovery (compared with direct injections of the respective amount of retinoids)

Retinoid	Recovery (%)		
	15 ng/ml	150 ng/ml	1500 ng/ml
13-*cis*-RA	81.7	87.5	83.0
9-*cis*-RA	82.4	85.6	84.3
All-*trans*-RA	79.0	86.3	84.0
All-*trans*-RAG	77.9	83.2	80.7

Analysis of BSA standard samples with 15, 150 and 1500 ng/ml of 13-*cis*-RA, 9-*cis*-RA, all-*trans*-RA and all-*trans*-RAG. (Values given are based on the mean peak area values of 6 standard samples and 6 direct injections.)

Table 4
Intra-day and inter-day reproducibility expressed as R.S.D.

Retinoid	Intra-day R.S.D. (%) ($n = 6$)			Inter-day R.S.D. (%) ($n = 3$)		
	15 ng/ml	150 ng/ml	1500 ng/ml	15 ng/ml	150 ng/ml	1500 ng/ml
13-*cis*-RA	6.4	1.6	3.4	7.9	5.3	2.3
9-*cis*-RA	3.9	1.7	2.6	9.7	3.8	2.4
All-*trans*-RA	7.0	1.6	2.4	10.4	2.3	1.7
All-*trans*-RAG	2.2	2.1	1.7	12.1	2.7	13.1

Data are given for three concentrations of retinoids: 15, 150 and 1500 ng/ml.

variety of samples and is applied in our laboratory to determine retinoid concentrations in plasma and tissues as well as in microsomal preparations used for examining in vitro glucuronidation of RA isomers.

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