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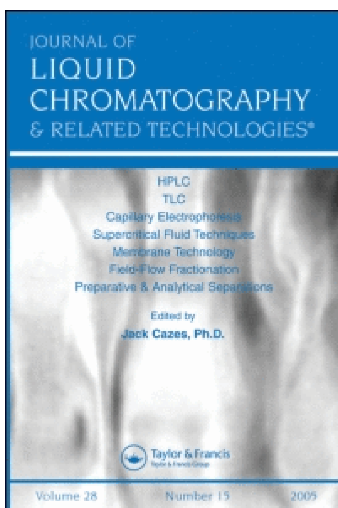

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Automated Determination of 13-Cis-and All-Trans-Retinoic Acid, Their 4-Oxo-Metabolites and Retinol in Plasma, Amniotic Fluid and Embryo by Reversed-Phase High-Performance Liquid Chromatography with a Precolumn Switching Technique

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To cite this Article Kraft, J. Creech , Echoff, Ch. , Kuhnz, W. , Löfberg, B. and Nau, H.(1988) 'Automated Determination of 13-Cis-and All-Trans-Retinoic Acid, Their 4-Oxo-Metabolites and Retinol in Plasma, Amniotic Fluid and Embryo by Reversed-Phase High-Performance Liquid Chromatography with a Precolumn Switching Technique', Journal of Liquid Chromatography & Related Technologies, 11: 9, 2051 — 2069

To link to this Article: DOI: 10.1080/01483918808069040

URL: http://dx.doi.org/10.1080/01483918808069040

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JOURNAL OF LIQUID CHROMATOGRAPHY, 11(9&10), 2051-2069 (1988)

AUTOMATED DETERMINATION OF 13-CIS- AND ALL-TRANS-RETINOIC ACID, THEIR 4-OXO METABOLITES AND RETINOL IN PLASMA, AMNIOTIC FLUID AND EMBRYO BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH A PRECOLUMN SWITCHING TECHNIQUE

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ABSTRACT

A fully automated reversed-phase high-performance liquid chromatography (HPLC) procedure with precolumn switching is presented which allows quantitative assay of 13-cis-retinoic acid and all-trans-retinoic acid, their 4-oxo-metabolites and retinol in plasma, amniotic fluid and tissue homogenates. A binary gradient system allowed baseline separation of the retinoids within 15 minutes. Sample preparation was kept simple in order to minimize degradation and isomerization of the unstable substances and required only the addition of isopropanol, freezing in liquid nitrogen, and centrifugation. Overall recovery was quantitative allowing for external standardization. Calibration curves were linear in mouse plasma, amniotic fluid and human serum albumin solution ($r > 0.99$) and in mouse embryo homogenate ($r > 0.98$) over a concentration range of 4 orders of magnitude. The detection limit was 2 ng/ml or g with a sample size of only 0.1 ml

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or 0.1 g. Coefficients of variation of the assay were less than 5% (within day) and 8% (day to day). The small sample size required, their simple preparation, the rapid analysis, and the high degree of automation make this method well suited for experimental or clinical pharmacokinetic studies following administration of therapeutic or toxic doses. This method was applied in a study on the transplacental pharmacokinetics of 13-cis and all-trans-retinoic acid during mouse embryo organogenesis.

INTRODUCTION

Retinol and its physiological metabolites are designated as natural retinoids [1]. These compounds are essential for normal growth and are needed for the division and differentiation of epithelial cells [2]. In spite of the fact that considerable research has shown that high-affinity binding proteins exist for retinol and retinoic acid and may mediate their cellular effects [3], little is known about the regulation of their blood and tissue concentrations and their interactions on the cellular level.

Therapeutically, retinoids are highly interesting compounds, particularly because of their effects on epithelial tumors and for their use in dermatology. All-trans-retinoic acid (all-trans-R.A.) has led to complete or partial regressions of basal cell carcinomas of the skin [4] and papillomas of the urinary bladder [5]. 13-cis-retinoic acid (13-cis-R.A.) is a very effective drug for the therapy of cystic acne [6]. Other retinoids including synthetic derivatives have been screened for their anti-tumor activity and suitability for clinical application.

Unfortunately, many retinoids were shown to be potent teratogens in animals and humans. It is therefore important to be able to measure the levels of retinoids in serum, plasma, amniotic fluid and tissues, particularly embryo and placenta, which are a result of additional intake either in animal experiments in teratology or in clinical investigations. Previous studies, for example, showed that 13-cis-R.A. (a potent human teratogen) [7] was a much less potent teratogen in the mouse than all-trans-R.A. [8]. Quantitative measurements of mouse embryo concentrations of these compounds showed that all-trans-R.A. but not 13-cis-R.A. was able to reach the embryo in large amounts [9,10]. Such pharmacokinetic studies are of great importance for the

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rational interpretation of animal experiments as well as for extrapolation of the results obtained to the human therapeutic situation.

High-performance liquid chromatography (HPLC) is the method of choice for determining retinoids in biological samples, and consequently, a number of assays have been published to date [11-21]. However, all these require a more or less elaborate procedure for sample purification. In this communication, we present a fully automated reversed-phase HPLC procedure with a column switching facility for the quantitative assay of the natural retinoids 13-cis R.A., all-trans-R.A., 4-oxo 13-cis-retinoic acid (4-oxo-13-cis-R.A.), 4-oxo-all-trans-retinoic acid (4-oxo-all-trans-R.A.) and retinol by direct injection of plasma and amniotic fluid samples after addition of isopropanol, precipitation in liquid nitrogen and subsequent centrifugation. With a minor modification this method can also be applied for the determination of the five compounds in tissue , e.g. mouse embryo.

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MATERIALS AND METHODS

Laboratory Precautions

All manipulations were performed in a darkened room with amber light to prevent photoisomerization. The autosampler was equipped with a thermostated sample tray (temperature 7°C) and was covered with aluminium foil for protection from light.

Chemicals and Reagents

4-oxo-13-cis-R.A. (RO 22 6595), 4-oxo-all-trans-R.A. (RO 12-4824) and 13-cis-R.A. (isotretinoin, Accutane^{R}, Roaccutan^{R}) were gifts from Hoffmann-La Roche (Basel, Switzerland). All-trans R.A. (tretinoin) and retinol were purchased from Sigma (Munich, FRG), methanol (HPLC grade), ethanol, isopropanol, acetic acid, ammonia and ammonium acetate (all of analytical grade) were obtained from Merck (Darmstadt, FRG). Human serum albumin (5%) was obtained from the Blutspendedienst DRK Landesverbände Nordrhein und Westfalen-Lippe, Institut Hagen. Amniotic

fluid (bovine) was purchased from Sigma (Munich, FRG). Water for HPLC was purified using a Milli Q water purification system.

Preparation of Standards

Stock solutions of all retinoids were prepared by dissolving 10 mg of the compound in 100 ml of ethanol. These solutions were kept in glassware at -20°C and freshly prepared every 6 weeks. Standard samples of 0.2 ml plasma, amniotic fluid or human serum albumin solution or 0.2 g embryo homogenate were prepared by adding 10 μl of each retinoid from the described stock solutions, to give a concentration of 5000 ng retinoid per ml or g in the biological matrix. The lower concentrated standard samples were made in the same manner by the use of appropriate dilutions of the stock solution.

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Sample Preparation

0.1 ml - 0.2 ml plasma or amniotic fluid were treated with an equal volume of isopropanol, frozen in liquid nitrogen over night and then centrifuged at 4°C for 20 minutes at 4000 g (Heraeus Christ Minifuge 2) (modification of method from Löfberg et al. [22]). The supernatant could then be injected directly (0.1 ml - 0.2 ml). Mouse embryos (day 11 of gestation, 0.1 - 0.2 g) were suspended in one volume of ethanol and two volumes of isopropanol, vortexed one minute and frozen in liquid nitrogen over night. They were then homogenized at 4°C using a sonic L converter for 10 seconds at setting 2 (Branson Sonic Power Co.). The homogenate was vortexed one minute and centrifuged at 4°C for 20 minutes at 4000 g. 0.1 ml - 0.2 ml of clear supernatant were injected into the HPLC system. A 5% human serum albumin solution spiked with known amounts of 13-cis- and all-trans-retinoic acid, the corresponding 4-oxo compounds and retinol could be injected directly into the HPLC system without sample pretreatment.

Calibration

Calibration was performed by analysing duplicate standard samples of plasma, amniotic fluid, human serum albumin solution or mouse embryo

homogenate with concentrations of 5, 50, 500 and 5000 ng/ml or g of the retinoids. Automatic calculation of the response factors and coefficients of correlation was performed online by a BASIC program loaded into the integrator. Calibration graphs were only accepted if $r > 0.99$ (plasma, amniotic fluid, human serum albumin solution) or $r > 0.98$ (embryo homogenate).

Animal Experiments

NMRI mice were treated on day 11 of gestation (plug day = day 0) with a single oral dose of 10 mg all-trans-R.A/kg or 10 mg 13-cis-R.A./kg, dissolved in soybean oil containing 8% ethanol. For the pharmacokinetic studies mice were sacrificed at different time intervals after drug administration. Embryos and maternal plasma were collected and analysed for retinoids by HPLC.

Storage of Samples

Biological samples were stored at -80°C in polypropylene tubes until analysis.

HPLC-System

The configuration of the column switching system is modified from the system described by Kuhnz et al. [23] and shown in Fig.1. In brief, two precolumns are used which enable sample enrichment, sample clean-up and analysis on the one, by backflushing the sample onto the analytical column, and on the other, a conditioning procedure in preparation for the next analysis (Löfberg et al. [22]). The apparatus consisted of two HPLC pumps type 64 (**pump C** and **D**, Fig.1) controlled by an HPLC -programmer 5O B in order to form the analytical gradient, a dynamic mixing chamber (**DMC**) (all from Knauer, Berlin, FRG,), a Perkin Elmer ISS-100 autoinjector (**AI**) (Überlingen, FRG) with refrigerated sample tray, a Gynkothek SE-2 column switching module (**CSM**) (Munich, FRG) and a SPD-6A UV-detector (**D**) and a C-R3A integrator from Shimadzu. Additionally two Knauer HPLC

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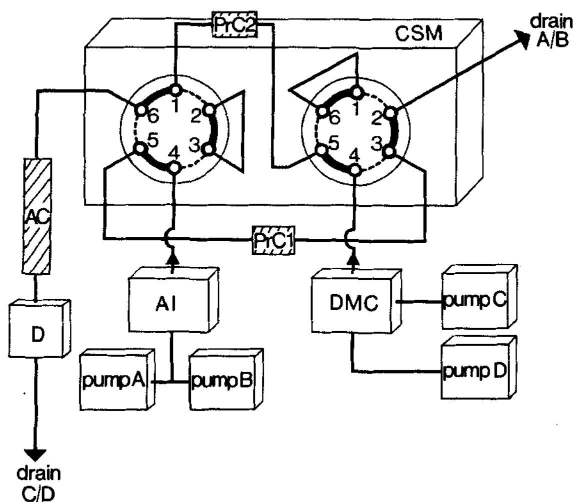

FIGURE 1. Configuration of the column switching system. The abbreviations are explained in the Materials and Methods section. The position of the valves corresponds to the time - 1.oo given in Table 1.

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pumps type 52 were used for the sample enrichment and cleanup procedure (**pump A** and **B**). The analytical column (**AC**) (125 x 4.6 mm) was slurry packed with Spherisorb 3 ODS II (3 μm; Phase Sep.) in our laboratory. As precolumns (**PrC 1** and **2**) we used 20 x 4.6 mm cartridges prepacked with Lichrosorb RP 18, 10 μm (VDS-Säulentechnik, Berlin, FRG).

The analytical eluents were : solvent C: 40 mM NH_4Ac (adjusted to pH 7.3 with 25% NH_3) - MeOH (50 : 50 v/v); solvent D: methanol. Gradient and flow conditions are given in Fig. 1.

Pump A was run at a flow rate of 2ml/min with 40 mM NH_4Ac (adjusted to pH 5.8 with acetic acid), pump B with methanol at the same flow rate. All solvents were degassed by water stream jet vacuum prior to use.

To ensure the clean separation seen in the chromatograms, the precolumns were replaced after about 40 injections. The time course of one analysis is described in Table 1.

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TABLE 1

HPLC Procedure of the Column Switching System

Time (min)	Operation	Effect
- 1.oo	Sample injection. Triggering of a 60 sec time relay in the CSM	Sample enrichment and cleaning on Prc 1 with buffer delivered by pump A
0.oo	Switching of valves. Delivery of start signal by CSM	Start of analytical gradient by back-flushing sample from Prc 1 onto AC. Start of integrator. Pump A stops. Prc 2 and capillaries cleaned from residues of the previous analysis with methanol from pump B.
10.oo	Relais signal of HPLC programmer	Pump B stops. Prc 2 is equilibrated for the next analysis with buffer from pump A
17.oo	End of analytical gradient and return to starting conditions within 2 min	Equilibration of AC for next analysis
19.oo	Stopping of integrator	Calculation of results
21.oo = -1.oo	Next sample introduction by autoinjector	Next analysis. Sample is now injected on Prc 2

RESULTS

Chromatographic Separation

Fig. 2 shows a typical chromatogram of serum albumin solution spiked with retinol, its naturally occuring metabolite, all-trans-R.A. and its isomer 13-cis-R.A., as well as their main metabolites, 4-oxo-13-cis-R.A. and 4-oxo-all-trans-R.A.. The retention times of the compounds are listed in Table 2. There are no carry-over effects because the column switching technique allows for the simultaneous methanol clean-up and subsequent equilibrium with aqueous buffer (40 mM NH_4Ac, pH 5.8) of the precolumn not in use for the analysis.

Precision of Chromatographic Assay

The reproducibility of the assay has been evaluated with human serum albumin solution at different concentrations of all retinoids. The within-day coefficients of variation were less than 5% and the day to day coefficients of variations were less than 7.6% (Table 3).

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TABLE 2

Retention Times of Retinoids

Peak Number in Chromatograms	Compound	Retention Time (min)
1	4-Oxo-All-Trans-R.A.	6.1
2	4-Oxo-13-Cis-R.A.	6.9
3	13-Cis-R.A.	11.5
4	All-Trans-R.A.	12.2
5	Retinol	14.5

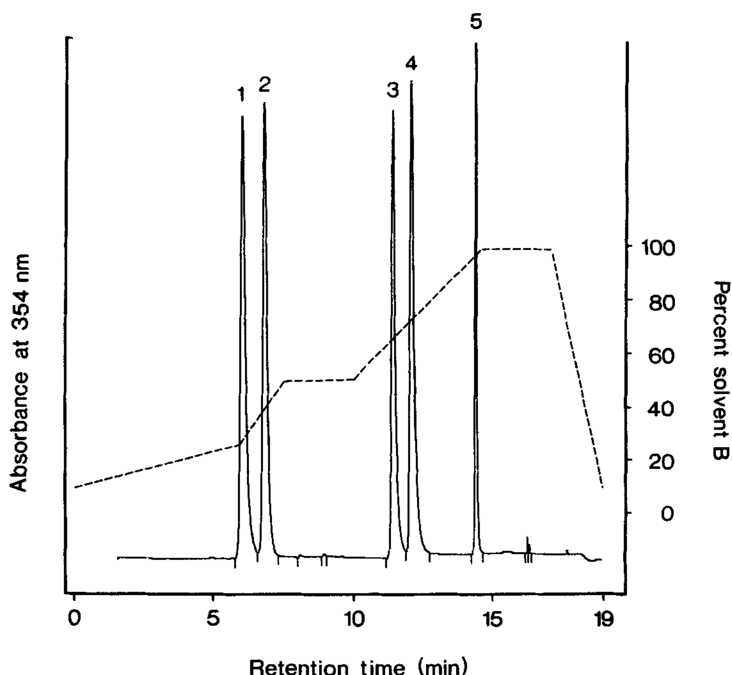

FIGURE 2. Separation of retinoids and gradient profile (dashed line). A mixture of 4-oxo-all-trans R.A. (1), 4-oxo-13-cis R.A. (2), 13-cis R.A. (3), all-trans R.A. (4) and retinol (5) at a concentration of 800 ng/ml in serum albumin was analyzed as described in the text. The compounds were detected by their absorbance at 354 nm, sensitivity: 0.02 a.u.f.s. The separation was achieved at room temperature with a flow rate of 2.0 ml/min producing a back pressure of approximately 260 bar at the beginning of the gradient.

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Recovery

After the precipitation procedure, the overall recovery from spiked mouse plasma and mouse embryo homogenate was determined by comparing the peak areas with those obtained by direct injection of a respective amount of drug dissolved in ethanol. The recovery was about 90 - 100% for all substances at the different concentrations (Table 4). Human serum albumin solution can be injected directly without isopropanol precipitation and recovery was found comparable for all compounds at the concentration tested (Table 5).

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TABLE 3

Reproducibility of Chromatographic Assay for 5 Retinoids in Serum Albumin Solution

Compound	Conc.[a] ng/ml	Within Day n[b]	Within Day C.V.%[c]	Day to Day n[b]	Day to Day C.V.%[c]
4-Oxo-	50	5	2.3		
All-Trans-	300			5	4.6
R.A.	500	5	1.6		
	1000	10	5.0		
	3000	4	5.0	6	5.6
	5000	5	3.0		
4-Oxo-	50	6	0.9		
13-Cis-	300			5	4.4
R.A.	500	5	2.7		
	1000	10	1.5		
	3000	4	2.5	6	7.6
	5000	5	1.6		
13-Cis-	50	5	1.5		
R.A.	300			5	5.2
	500	5	2.0		
	1000	10	2.2		
	3000	4	2.6	6	5.6
	5000	5	2.7		
All-Trans-	50	4	0.5		
R.A.	300			5	5.0
	500	5	2.6		
	1000	10	1.7		
	3000	4	2.9	6	5.0
	5000	5	1.6		
Retinol	1000	10	1.9		

a) concentration added
b) number of samples analyzed
c) coefficient of variation

TABLE 4

Analytical Recovery of 4-Oxo-All-Trans-R.A., 4-Oxo-13-Cis-R.A., 13-Cis-R.A. and All-Trans-R.A. From Mouse Plasma and Mouse Embryo Homogenate

Compound	Conc.[a] ng/ml or g	%Recovery (Mean ± S.D.[b])			
		n[c]	Mouse Plasma	n[c]	Mouse Embryo Homogenate
4-Oxo-All-Trans-R.A.	50	4	97 ± 6.8	4	95 ± 7.0
	500	5	87 ± 5.9	7	96 ± 5.1
	5000	5	87 ± 12.0	6	104 ± 9.9
4-Oxo-13-Cis-R.A.	50	4	101 ± 4.0	5	93 ± 6.6
	500	5	90 ± 2.2	6	100 ± 10.0
	5000	5	84 ± 1.5	3	101 ± 2.1
13-Cis-R.A.	50	4	98 ± 8.9	5	101 ± 11.0
	500	5	114 ± 1.9	6	106 ± 9.0
	5000	5	97 ± 3.8	5	107 ± 9.6
All-Trans-R.A.	50	4	99 ± 7.6	5	107 ± 8.2
	500	5	106 ± 4.0	5	109 ± 4.9
	5000	5	100 ± 3.6	5	104 ± 7.8

a) concentration added
b) standard deviation
c) number of samples analyzed

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TABLE 5

Analytical Recovery of 4-Oxo-All-Trans-R.A., 4-Oxo-13-Cis-R.A., 13-Cis-R.A. and All-Trans-R.A. from Serum Albumin Solution

Compound	% Recovery[a] (Mean ± S.D.)[b]
4-Oxo-All-Trans-R.A.	98 ± 8.0
4-Oxo-13-Cis-R.A.	92 ± 6.8
13-Cis-R.A.	97 ± 1.5
All-Trans-R.A.	95 ± 9.7

a) the concentrations of all added compounds were 1000 ng/ml
b) n = 5

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Calibration, Limit of Detection

Our method permits quantitative determination of retinol, all-trans-R.A., 13-cis R.A., 4-oxo-13-cis-R.A., and 4-oxo-all-trans-R.A. by measuring peak areas (external standardization). Standard curves were calculated from data obtained by processing

1) mouse plasma, amniotic fluid or embryo homogenate samples to which known amounts of these substances were added and the precipitation step with isopropanol had been carried out
2) spiked serum albumin solution, which was directly injected into the HPLC system.

There was a linear relationship between peak areas obtained and concentration for the 5 retinoids in plasma, serum albumin solution, amniotic fluid and mouse embryo homogenate, over the range from 0.5 ng to 500 ng of injected retinoid. The correlation coefficients of all 5 retinoids in the standard curves exceeded 0.98 for mouse embryo homogenate and 0.99 for plasma, serum albumin solution and amniotic fluid (Table 6). The sensitivity limit which allowed precise quantitation was about 5 ng/ml of body fluid or 5 ng/g of tissue with a sample size of 0.1 ml or 0.1 g, respectively. With this concentration the signal to noise ratio was typically 5. Lower concentrations could be detected (1 - 2 ng/ml or g), but precision was in this case insufficient.

Experimental Use

Typical chromatograms are shown of mouse plasma (Fig. 3, left) and mouse embryo homogenate (Fig. 4, left) from untreated animals. Only retinol is detectable and not retinoic acid. Figure 3 (middle) shows the plasma levels, Fig. 4 (middle) the embryo levels and Fig. 5 the amniotic fluid levels of 13-cis-R.A. and its metabolites as well as physiological retinol, after NMRI mice were orally administered 10 mg/kg 13-cis-R.A. on day 11 of gestation. Figure 3, (right) shows the plasma levels, and Fig. 4 (right) the embryo levels of all-trans-R.A. and its metabolites and retinol 2 hours after NMRI mice were administered 10 mg/kg all-trans-R.A. on day 11 of gestation.

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TABLE 6

Coefficient of Correlation for Calibration Curves with 5, 50, 500, 5000 ng/ml or g

Compound	Serum Albumin r =	Amniotic Fluid r =	Mouse Plasma r =	Embryo Homogenate r =
4-Oxo-All-Trans-R.A.	0.9995	0.9927	0.9980	0.9879
4-Oxo-13-Cis-R.A.	0.9996	0.9913	0.9981	0.9885
13-Cis-R.A.	0.9998	0.9978	0.9938	0.9841
All-Trans-R.A.	0.9998	0.9958	0.9956	0.9867
Retinol	0.9999	0.9959	0.9958	0.9861

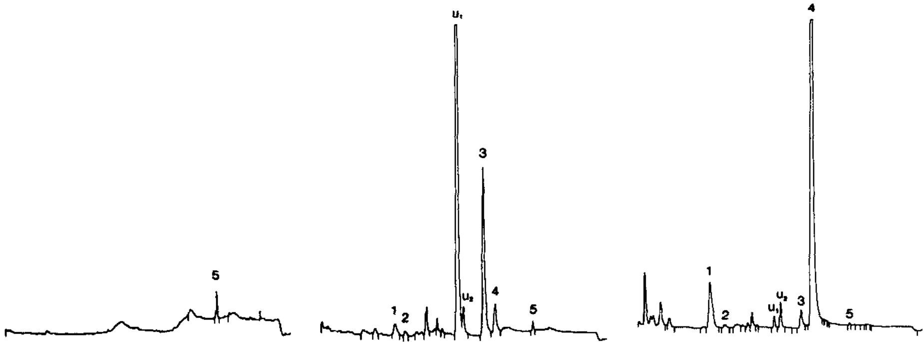

FIGURE 3, left. Typical chromatogram of HPLC analysis of mouse plasma of an untreated animal. The endogenous presence of retinol (peak 5) is seen (28 ng/ml). Sensitivity: 0.005 a.u.f.s.

FIGURE 3, middle. Chromatogram of HPLC analysis of mouse plasma 2 hours after treatment with 10 mg/kg 13-cis-R.A. Peaks: 1 = 60 ng/ml 4-oxo-all-trans-R.A.; 2 = 40 ng/ml 4-oxo-13-cis-R.A.; 3 = 460 ng/ml 13-cis-R.A.; 4 = 80 ng/ml all-trans R.A.; 5 = 18 ng/ml retinol; u1, u2 = unknown metabolites. Sensitivity: 0.01 a.u.f.s.

FIGURE 3, right. Chromatogram of HPLC analysis of mouse plasma 2 hours after treatment with 10 mg/kg all-trans-R.A. Peaks: 1 = 300 ng/ml 4-oxo-all-trans-R.A.; 2 = 40 ng/ml 4-oxo-13-cis R.A.; 3 = 100 ng/ml 13-cis R.A.; 4 = 2640 ng/ml all-trans-R.A.; 5 = 6 ng/ml retinol. Sensitivity: 0.02 a.u.f.s.

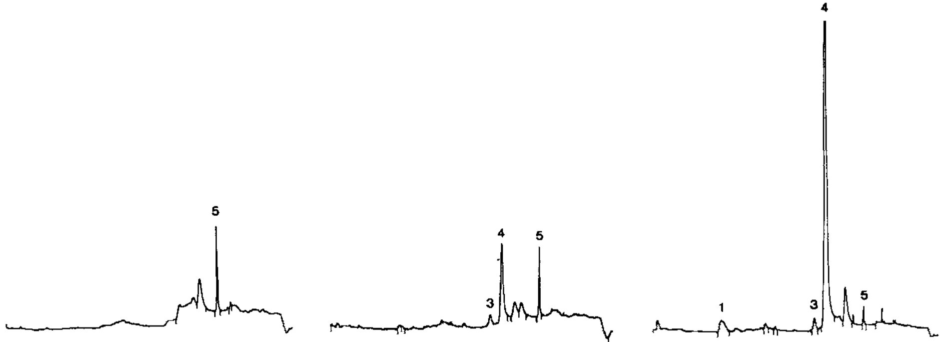

FIGURE 4, left. Typical chromatogram of HPLC analysis of mouse embryo homogenate of an untreated animal. Peak 5 = 200 ng/g retinol, sensitivity: 0.005 a.u.f.s.

FIGURE 4, middle. Chromatogram of HPLC analysis of mouse embryo homogenate 2 hours after treatment with 10 mg/kg 13-cis-R.A. Peaks: 3 = 60 ng/g 13-cis-R.A.; 4 = 200 ng/g all-trans-R.A.; 5 = 160 ng/g retinol. Sensitivity: 0.005 a.u.f.s.

FIGURE 4, right. Chromatogram of HPLC analysis of mouse embryo homogenate 2 hours after treatment with 10 mg/kg all-trans-R.A. Peaks: 1 = 108 ng/g 4-oxo-all-trans-R.A.; 3 = 64 ng/g 13-cis-R.A.; 4 = 2000 ng/g all-trans-R.A.; 5 = 52 ng/g retinol. Sensitivity: 0.01 a.u.f.s.

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DISCUSSION

Retinoids are unstable compounds which are highly susceptible to isomerization and degradation. Therefore, the time necessary for a complete analysis and particularly for sample preparation should be as short as possible and all procedures must be carried out under amber light. Our method offers a number of advantages over other assays which require lengthy work-up techniques. The sample preparation developed here which causes no isomerization of the compounds requires only the addition of isopropanol, precipitation in the cold and subsequent centrifugation prior to injection into the HPLC system. Tissues must additionally be sonified. There is no need for an internal standard because there is no sample loss as is sometimes the case during lengthy extraction procedures. For this reason,

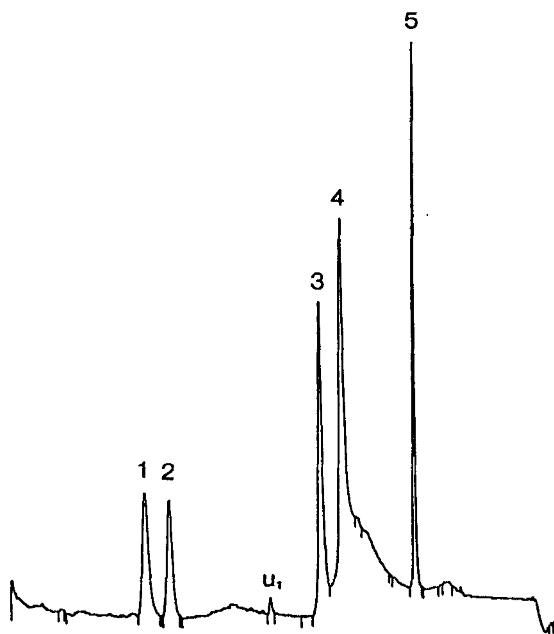

FIGURE 5. Chromatogram of HPLC analysis of mouse amniotic fluid 2 hours after treatment with 10 mg/kg of 13-cis-R.A. Peaks: 1 = 114 ng/ml 4-oxo-all-trans-R.A.; 2 = 116 ng/ml 4-oxo-13-cis-R.A.; 3 = 252 ng/ml 13-cis-R.A.; 4 = 364 ng/ml all-trans-R.A.; 5 = 360 ng/ml retinol. Sensitivity: 0.005 a.u.f.s.

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and because the recovery was found to be > 90% for all compounds, our method allows for the external standard method. Because of the poor availability of sufficient amounts of mouse blank samples, routine calibration can be done with a spiked 5% human serum albumin solution, injected directly, because recovery is comparable to the mouse standards.

The same simple procedure of sample pretreatment can not only be applied to plasma, amniotic fluid and embryo homogenates but also to serum, placental tissue and media for whole-embryo- or cell-cultures. Due to the automated operation of the HPLC equipment, a considerable number of samples can be analysed, without the risk of sample degradation, because the samples are kept at 7°C in a thermostated sample tray protected from light.

Finally, it should be noted that a small sample size (0.1 ml or 0.1 g or less) is needed for each analysis. This can be of particular importance and advantage in studies involving small laboratory animals and embryos during organogenesis or patients during treatment, but it can also be limiting in cases where abundant sample volumes are available and very low retinoid concentrations are present. We are currently testing if larger sample volumes can also be injected which would further increase the sensitivity of the assay and would allow to investigate endogenous R.A. concentrations in plasma and embryo; considerable controversy persists in this area of research at present [24-26].

The method described here is well suited for experimental pharmacokinetic studies as shown in our most recent publication [10]. Here we showed that after either the administration of 100 mg/kg all-trans-R.A. or 13-cis-R.A., only all-trans-R.A. is reaching the embryo in large amounts. This correlated well to the fact that all-trans-R.A. is highly teratogenic in mice and 13-cis-R.A. only marginally so. The chromatograms shown here show the same phenomena, but at a lower dosage of 10 mg/kg of either compound. They show that only all-trans-R.A. is reaching the embryo in large amounts. Even after the administration of 13-cis-R.A., all-trans-R.A. is found in the embryo at higher concentrations than 13-cis-R.A.. It is also shown that after the administration of 13-cis-R.A. (10 mg/kg) all five investigated retinoids appear in the amniotic fluid.

In conclusion, the excellent chromatographic resolution, the small sample size required, the rapid analysis to minimize degradation and isomerization and the high degree of automation make this method well suited for experimental or clinical pharmacokinetic studies following administration of pharmacologic or toxic doses.

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ACKNOWLEDGEMENT

This work was supported a grant of the Deutsche Forschungsgemeinschaft to the Sonderforschungsbereich 174 (C-6). We would like to thank Prof. Dr. D.M. Kochhar, Jefferson University,

Philadelphia, for his helpful discussions and encouragement, Thekla Sparenberg for technical assistance and Renate Ebbinghaus for the preparation of the manuscript.

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