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Simultaneous determination of all-*trans*-, 13-*cis*- and 9-*cis*-retinoic acid, their 4-oxo metabolites and all-*trans*-retinol in human plasma by high-performance liquid chromatography

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Abstract

All-*trans*-retinoic acid (all-*trans*-RA) and 13-*cis*-retinoic acid (13-*cis*-RA), due to their effects on cell differentiation, proliferation and angiogenesis, improved treatment results in some malignancies. Pharmacokinetic studies of all-*trans*-RA and 13-*cis*-RA along with monitoring of retinoic acid metabolites may help to optimize retinoic acid therapy and to develop new effective strategies for the use of retinoic acids in cancer treatment. Therefore, we developed a HPLC method for the simultaneous determination in human plasma of the physiologically important retinoic acid isomers, all-*trans*-, 13-*cis*- and 9-*cis*-retinoic acid, their 4-oxo metabolites, 13-*cis*-4-oxoretinoic acid (13-*cis*-4-oxo-RA) and all-*trans*-4-oxoretinoic acid (all-*trans*-4-oxo-RA), and vitamin A (all-*trans*-retinol). Analysis was performed on a silica gel column with UV detection at 350 nm using a binary multistep gradient composed of *n*-hexane, 2-propanol and glacial acetic acid. For liquid–liquid extraction a mixture of *n*-hexane, dichloromethane and 2-propanol was used. The limits of detection were 0.5 ng/ml for retinoic acids and 10 ng/ml for all-*trans*-retinol. The method showed good reproducibility for all components (within-day C.V.: 3.02–11.70%; day-to-day C.V.: 0.01–11.34%). Furthermore, 9-*cis*-4-oxoretinoic acid (9-*cis*-4-oxo-RA) is separated from all-*trans*-4-oxo-RA and 13-*cis*-4-oxo-RA. In case of clinical use of 9-*cis*-retinoic acid (9-*cis*-RA) the pharmacokinetics and metabolism of this retinoic acid isomer can also be examined.

Keywords: Retinoic acid; 4-Oxoretinoic acid; Retinol

1. Introduction

The retinoic acids, active metabolites of all-*trans*-retinol (vitamin A), play a pivotal role in morphogenesis, homeostasis and cell differentiation. All-*trans*-RA and 9-*cis*-RA influence gene transcription by interaction with nuclear receptors, the retinoic acid receptors. While all-*trans*-RA only binds to one type of receptor, the RA-receptor, 9-*cis*-RA binds to both types, the RA- and the RX-receptor. 13-*cis*-RA, another retinoic acid isomer, is not a high affinity ligand of any known nuclear receptor [1,2].

Isomerisation is one important metabolic pathway of retinoic acids, because it results in metabolites (all-*trans*-RA, 13-*cis*-RA or 9-*cis*-RA) with different effects due to different mechanisms of action. Another step in retinoic acid metabolism is the oxidation to all-*trans*-4-oxo-RA and 13-*cis*-4-oxo-

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RA by cytochrome P450, which is the main metabolic pathway after the application of pharmacological doses. At higher concentrations in vitro all-*trans*-4-oxo-RA and 13-*cis*-4-oxo-RA show the same effects on cell proliferation and differentiation as all-*trans*-RA and 13-*cis*-RA [3]. Furthermore, all-*trans*-4-oxo-RA has been identified as a potent morphogen [4].

Owing to the ability to inhibit cell proliferation and angiogenesis and to induce cell differentiation, retinoic acids gained interest in the chemoprevention and treatment of cancer [5,6]. All-*trans*-RA improved the treatment results in acute promyelocytic leukaemia [7,8]. 13-*cis*-RA, in combination with interferon α, was successfully employed in squamous cell carcinomas of skin and cervix [9,10].

Thus, there is a keen interest in developing new strategies of cancer therapy using retinoic acids. For the therapy of solid tumors, combinations of 13-*cis*-RA with 5-fluorouracil or with interferon α are discussed [11]. Furthermore, continued efforts at optimizing retinoic acid therapy are needed. In order to avoid all-*trans*-RA resistance intermittent dosing schedules are studied [12]. For the treatment of acute promyelocytic leukaemia the regular daily dose is usually 45 mg/m^2 [7,8]. A recent study showed that 25 mg/m^2 was sufficient to induce complete remission in patients with acute promyelocytic leukaemia [13]. Moreover, children are more liable to suffer severe side effects, which necessitate dose reduction or discontinuation of all-*trans*-retinoic acid therapy ([7] and personal observation).

Pharmacokinetic studies of all-*trans*-RA and 13-*cis*-RA along with monitoring of in vivo isomerisation and oxidative metabolism may help to optimize retinoic acid therapy and to develop new strategies for the use of retinoic acids in the treatment of cancer.

Therefore, a reliable and sensitive method for the simultaneous determination of all-*trans*-RA, 13-*cis*-RA, 9-*cis*-RA, their 4-oxo metabolites and all-*trans*-retinol in human plasma was needed. Several HPLC methods for the quantification of 13-*cis*-RA, all-*trans*-RA and their 4-oxo metabolites in human plasma have been reported. Apart form gradient systems the published reversed-phase methods required either column switching techniques [14] or a column oven [15]. On a silica gel column these retinoic acids were separated in an isocratic mode [16]. However, separation of 9-*cis*-RA from 13-*cis*-RA was not possible with this method [17]. In addition none of these methods was designed for the simultaneous quantification of all-*trans*-retinol.

Improving the sample preparation and the HPLC method published by Meyer et al. [18], we developed a reliable and sensitive method for the simultaneous determination of all-*trans*-RA, 13-*cis*-RA, 9-*cis*-RA, their 4-oxo metabolites and all-*trans*-retinol in human plasma.

2. Experimental

2.1. Reagents

All-*trans*-RA and 13-*cis*-RA were purchased from Sigma (Deisenhafen, Germany). All-*trans*-retinol (Ro 01-4955), 9-*cis*-RA (Ro 04-4079), all-*trans*-4-oxo-RA (Ro 12-4824), 13-*cis*-4-oxo-RA (Ro 22-6595), 9-*cis*-4-oxo-RA (Ro 47-8078) and arotinoid ethylsulfone (Ro 15-1570) were generously provided by Hoffmann–La Roche (Basel, Switzerland).

Glacial acetic acid, 2-propanol, *n*-hexane, ethanol and dichloromethane were obtained from Baker Chemicals (Groß-Gerau, Germany). Ammonium sulfate and anhydrous sodium sulfate were purchased from Merck (Darmstadt, Germany). Glacial acetic acid, 2-propanol and *n*-hexane were of HPLC grade. The remaining chemicals were of analytical grade. Water was purified with a Milli-Q system (Millipore, Eschborn, Germany).

2.2. Laboratory precautions

To avoid photoisomerisation, all handling of retinoids and blood samples was done under dim yellow light and whenever possible amberized vacutainers were used.

2.3. Preparation of stock solutions and working standards

The retinoids were individually dissolved in ethanol to produce stock solutions of 1 mg/ml for each retinoid. Working standards were prepared by serial dilution of stock solutions with ethanol to obtain concentrations of 10 000, 1000, 100 and 10 ng/ml.

Stock solutions, flushed with nitrogen, and working standards were stored at −20°C.

2.4. Instrumentation and chromatographic conditions

A Pharmacia HPLC system (Pharmacia Biosystems, Freiburg, Germany) was used, consisting of a LKB 2150 HPLC pump, a LKB low pressure mixer, a LKB LCC 2252 controller and a LKB 2151 variable-wavelength UV detector. The analytical column, a silica gel adsorption column (Nucleosil 100, 5 μm, 200×4 mm I.D.) (Macherey und Nagel, Düren, Germany) was fitted with a silica gel guard column (Nucleosil 100, 5 μm, 15×4 mm I.D.). The analysis was performed with a binary multistep gradient (Table 1) at a flow-rate of 1 ml/min, which provided a pressure of approximately 45 bar. Between each analysis (run time 45 min) the column was reequilibrated with 100% solvent A for 10 min (cycle time 55 min). UV detection was performed at a wavelength of 350 nm and a sensitivity of 0.0025 AUFS. Chromatograms were recorded with a Nelson Analytical 900 Series interface.

2.5. Sample collection

Blood samples were collected in heparinized vacutainers and kept in the dark at 4°C before centrifugation at 1500 *g* for 5 min. Plasma was removed and divided into aliquots of 600 μl and frozen at −20°C. At this temperature all-*trans*-retinol was reported to be stable over a period of 8 years [19]. At −20°C no degradation of all-*trans*-RA, 13-*cis*-RA and their 4-oxo metabolites was observed in plasma during storage for up to three months [20]. After 24 h at room temperature the degradation of 13-*cis*-RA and 13-*cis*-4-oxo-RA in plasma was shown to be about 10% [14]. Therefore, the thawed plasma samples were immediately submitted to the extraction procedure.

Table 1
Composition of mobile phase (binary multistep gradient)

Time (min)	A (%)	B (%)
0	100	0
9	100	0
9.05	92	8
14	92	8
24	45	55
24.05	0	100
34	0	100
35	100	0
55	100	0

Solvent A: *n*-hexane–2-propanol–glacial acetic acid (400:1:0.27).
Solvent B: *n*-hexane–2-propanol–glacial acetic acid (400:6:0.27).

2.6. Extraction

Extraction of retinoic acids and all-*trans*-retinol from human plasma (500 μl) involved protein precipitation with 500 μl of an ethanolic solution of the internal standard (arotinoid ethylsulfone) and 500 μl of saturated ammonium sulfate solution. After vortex mixing, 2 ml of water and 4 ml of an extraction mixture, containing *n*-hexane–dichloromethane–2-propanol (80:19:1), were added. The sample was extracted on a rotating mixer for 10 min. After centrifugation (4 min, 2700 *g*) the organic layer was removed, dried with anhydrous sodium sulfate and evaporated under a stream of nitrogen. The residue was dissolved in 100 μl *n*-hexane and a 50-μl aliquot was injected.

2.7. Calibration and system validation

Calibration graphs and analysis of linearity were performed by linear least-squares regression analysis plotting peak-area ratios of the analyte and the internal standard against the concentration of the analyte. Standards with 1, 10, 50, 100, 500 and 1000 ng/ml for retinoic acids and 50, 100, 500, 1000 and 1500 ng/ml for all-*trans*-retinol were examined for analysis of linearity. To test for reproducibility, a pool of human plasma was spiked with standards to give 10, 100 or 1000 ng/ml for retinoic acids and 100, 500 or 1000 ng/ml for all-*trans*-retinol. For within-day studies at least six samples were analysed. Day-to-day reproducibility was tested on six different days. The limit of quantification for retinoic acids and all-*trans*-retinol, defined as the lowest concentration, which could be measured with a precision and accuracy below 20% [21], was determined from spiked plasma samples in five replicates. Quantification of retinoic acids in human plasma was based on calibration curves consisting of at least three concentrations for 13-*cis*-RA, 9-*cis*-

RA, all-*trans*-RA, all-*trans*-retinol, all-*trans*-4-oxo-RA and 13-*cis*-4-oxo-RA. The calibration standards were prepared in plasma, covering the expected retinoid concentration (standard addition method). The obtained calibration curves for all-*trans*-RA, 13-*cis*-RA, all-*trans*-4-oxo-RA, 13-*cis*-4-oxo-RA and all-*trans*-retinol had a non-zero intercept with the *y*-axis due to the endogenous retinoid concentration in the plasma pool. The intercepts of the calibration curves were subtracted and unknown retinoid concentrations were calculated with the new calibration curves, having a zero intercept [18]. Recovery was determined in triplicate at concentrations of 10, 100 and 1000 ng/ml for retinoic acids and 100, 500 and 1000 ng/ml for all-*trans*-retinol by comparing the peak areas of spiked plasma samples with those of *n*-hexane standard solutions injected directly into the HPLC system. Owing to the endogenous retinoid concentrations in human plasma, the peak areas of the spiked plasma samples were corrected by subtraction of the peak areas of the corresponding retinoids in blank plasma.

3. Results and discussion

Modifying the sample preparation and the HPLC method, published by Meyer et al. [18], we developed a sensitive and reliable HPLC method for the determination of three physiologically important retinoic acid isomers, 13-*cis*-RA, 9-*cis*-RA and all-*trans*-RA, their parent compound, all-*trans*-retinol, and their 4-oxo metabolites, all-*trans*-4-oxo-RA and 13-*cis*-4-oxo-RA. To our knowledge this is the first method, which separates 9-*cis*-4-oxo-RA from all-*trans*-4-oxo-RA and 13-*cis*-4-oxo-RA.

Extraction of plasma samples was started with protein precipitation by ethanol and saturated ammonium sulfate solution. The saturated ammonium sulfate solution resulted in lower viscosity and improved the extraction of 4-oxoretinoic acids. After addition of deionized water liquid–liquid extraction was carried out with a mixture of *n*-hexane, dichloromethane and 2-propanol. The addition of more polar solvents than *n*-hexane resulted in a better extraction of the 4-oxoretinoic acids. Liquid–liquid extraction was preferred to solid phase extraction for the lower limits of detection, smaller sample volumes and additional extraction of all-*trans*-retinol [16]. Extraction of plasma in the presence of a strong acid or base induces the hydrolysis of retinoyl-β-glucuronides, endogenous compounds of human blood, and results in higher retinoic acid concentrations. We extracted the retinoids at a pH value of 5, so that hydrolysis of retinoyl-glucuronides was not expected [22]. As retinoids are susceptible to isomerisation and oxidation, extraction had to be done cautiously to avoid artificial isomerisation and oxidation. Individual analysis of each retinoid showed no degradation by the extraction procedure. The extraction procedure yielded a good recovery ranging from 80% to 95% for each retinoid. The overall percent recoveries (mean±standard deviation), calculated from individual data, were 86.60±1.40% for 13-*cis*-RA, 88.70±5.06% for 9-*cis*-RA, 88.13±5.28% for all-*trans*-RA, 86.61±8.58% for all-*trans*-retinol, 88.57±4.54% for all-*trans*-4-oxo-RA and 89.81±8.84% for 13-*cis*-4-oxo-RA, respectively.

Separation was performed on a silica gel adsorption column using a binary multistep gradient. This system allowed simultaneous determination of all-*trans*-RA, 13-*cis*-RA, 9-*cis*-RA, all-*trans*-4-oxo-RA, 13-*cis*-4-oxo-RA and all-*trans*-retinol. Furthermore, 9-*cis*-4-oxo-RA was separated from all-*trans*-4-oxo-RA and 13-*cis*-4-oxo-RA. The corresponding k' values and resolution factors (R) are given in Table 2. Fig. 1 shows plasma spiked with the retinoids in question. Gradient systems with silica gel columns have on occasion been associated with

Table 2
Retention factors (k') and resolution (R) of adjacent peaks (derived from Fig. 1)

Retinoid	k'	R
13-*cis*-RA	2.60	
		2.52
9-*cis*-RA	3.03	
		1.59
all-*trans*-RA	3.39	
all-*trans*-retinol	16.26	
		7.89
all-*trans*-4-oxo-RA	18.56	
		3.77
9-*cis*-4-oxo-RA	19.32	
		2.18
13-*cis*-4-oxo-RA	19.73	

The resolution of two adjacent peaks was calculated as follows: $R=1.177(t2-t1)/(W_{1/2}1+W_{1/2}2)$, with $W_{1/2}$ being the peak width at half maximum peak height.

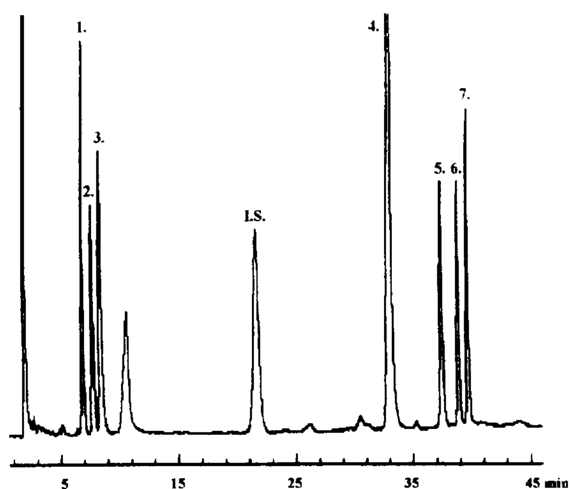

Fig. 1. Chromatogram of plasma spiked with retinoids (100 ng/ml). Peaks: 1=13-*cis*-RA, 2=9-*cis*-RA, 3=all-*trans*-RA, I.S.=internal standard, 4=all-*trans*-retinol, 5=all-*trans*-4-oxo-RA, 6=9-*cis*-4-oxo-RA, 7=13-*cis*-4-oxo-RA.

irreproducible retention times due to loss of water from the solid phase [23,24]. Reequilibration with 100% solvent A for 10 min at a flow-rate of 1 ml/min led to reproducible retention times (Table 3). Therefore, the gradient system was retained, as it required less expense in chromatographic equipment than a column switching or a pre-column switching mode [14,15]. Examination of linearity yielded a correlation ≥0.9999 for retinoic acids and ≥0.999 for all-*trans*-retinol. Within-day precision studies showed coefficients of variation between 11.70% and 3.70% for low concentrations and 5.93% and 3.02% for high concentrations (Table 4). The coefficients of variation for the day-to-day analysis were 6.78–11.34% for low concentrations and 0.01–1.51% for high concentrations (Table 5), which is sufficient for the quantification of drugs in biological matrices [21]. UV detection was performed at a wavelength of 350 nm, which is near the absorption maximum of the retinoic acids of interest. All-*trans*-retinol, however shows an absorption maximum at a wavelength of 325 nm. Therefore, the limit of quantification for all-*trans*-retinol was 50 ng/ml (C.V. 17.10%). As the physiological concentrations of all-*trans*-retinol in human plasma range between 100–1000 ng/ml [25], quantification of all-*trans*-retinol in human plasma is possible. The limit of quantification was 1 ng/ml for retinoic acids with coefficients of variation between 6.24% and 15.74% (Table 6), which allowed detection of physiological concentrations of all-*trans*-RA (1.32±0.46 ng/ml), 13-*cis*-RA (1.63±0.85 ng/ml) and 13-*cis*-4-oxo-RA (3.68±0.99 ng/ml) in human plasma (Fig. 2) [15]. The limits of detection (signal-to-noise ratio >3) were 0.5 ng/ml for retinoic acids and 10 ng/ml for all-*trans*-retinol. No 9-*cis*-RA was found in plasma from healthy human volunteers and in plasma from patients undergoing retinoic acid therapy for cancer treatment. Thus, if 9-*cis*-retinoic acid was a physiological component in human plasma, the concentrations must be below 0.5 ng/ml. Fig. 3 and Fig. 4 show chromatograms of

Table 3
Reproducibility of retention times (determined on a given day, $n=8$)

Retinoid	Retention time (mean±S.D.) (min)	C.V. (%)
13-*cis*-RA	6.42±0.17	0.23
9-*cis*-RA	6.93±0.33	0.44
all-*trans*-RA	7.55±0.17	0.22
Internal standard	23.47±0.36	1.26
all-*trans*-Retinol	34.05±0.27	0.80
all-*trans*-4-oxo-RA	38.53±0.25	0.66
13-*cis*-4-oxo-RA	40.67±0.26	0.64

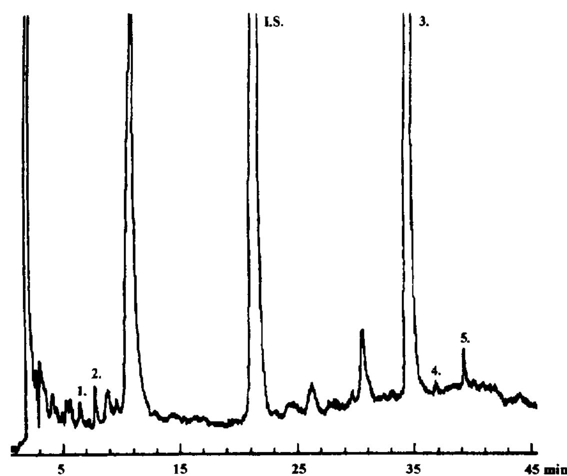

Fig. 2. Chromatogram of blank plasma (500 μl). Retinoid concentrations: 13-*cis*-RA, 1.01 ng/ml (1); all-*trans*-RA, 1.18 ng/ml (2); all-*trans*-retinol, 640.91 ng/ml (3); all-*trans*-4-oxo-RA, <1 ng/ml (4); 13-*cis*-4-oxo-RA, 1.33 ng/ml (5).

Table 4
Within-day reproducibility (determined from one standard curve on a given day)

		13-*cis*-RA	9-*cis*-RA	all-*trans*-RA	all-*trans*-retinol	all-*trans*-4-oxo-RA	13-*cis*-4-oxo-RA
Added (ng/ml)	(n=6)	1000.00	1000.00	1000.00	1000.00[a]	1000.00	1000.00
Found (ng/ml)	Mean	999.54	990.73	972.17	996.26	964.25	974.44
	S.D.	30.35	44.59	29.38	59.10	34.74	31.18
	C.V. (%)	3.04	4.50	3.02	5.93	3.60	3.20
Added (ng/ml)	(n=8)	100.00	100.00	100.00	500.00[b]	100.00	100.00
Found (ng/ml)	Mean	109.19	107.03	109.99	508.96	111.49	111.94
	S.D.	5.84	5.16	6.09	39.45	6.18	6.66
	C.V. (%)	5.35	4.82	5.54	7.75	5.54	5.95
Added (ng/ml)	(n=8)	10.00	10.00	10.00	100.00	10.00	10.00
Found (ng/ml)	Mean	11.32	11.40	10.23	113.12	10.48	9.85
	S.D.	1.31	0.74	0.77	13.24	0.39	0.82
	C.V. (%)	11.57	6.50	7.52	11.70	3.70	8.32

[a] n=8.
[b] n=6.

Table 5
Day-to-day reproducibility (determined from single standard curves on different days)

		13-*cis*-RA	9-*cis*-RA	all-*trans*-RA	all-*trans*-retinol	all-*trans*-4-oxo-RA	13-*cis*-4-oxo-RA
Added (ng/ml)	(n=6)	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00
Found (ng/ml)	Mean	999.98	999.21	999.82	994.17	999.83	1000.40
	S.D.	0.18	0.75	0.13	15.00	0.40	0.27
	C.V. (%)	0.02	0.08	0.01	1.51	0.04	0.03
Added (ng/ml)	(n=6)	100.00	100.00	100.00	500.00	100.00	100.00
Found (ng/ml)	Mean	100.06	107.92	102.05	513.12	102.45	97.68
	S.D.	1.98	7.64	1.43	33.76	2.89	2.00
	C.V. (%)	1.98	7.08	1.40	6.58	2.82	2.04
Added (ng/ml)	(n=6)	10.00	10.00	10.00	100.00	10.00	10.00
Found (ng/ml)	Mean	10.31	10.84	8.74	106.31	9.98	10.61
	S.D.	0.89	0.74	0.76	12.06	0.75	0.97
	C.V. (%)	8.61	6.78	8.67	11.34	7.51	9.17

Table 6
Precision and accuracy at the limit of quantification ($n=5$)

Retinoid	Added (ng/ml)	Found (mean±S.D.) (ng/ml)	C.V. (%)	Accuracy (%)
13-*cis*-RA	1.00	0.84±0.13	15.74	84.00
9-*cis*-RA	1.00	1.00±0.16	15.48	100.00
all-*trans*-RA	1.00	1.04±0.14	13.59	104.19
all-*trans*-retinol	50.00	43.88±7.50	17.10	87.76
all-*trans*-4-oxo-RA	1.00	1.19±0.13	10.59	119.32
13-*cis*-4-oxo-RA	1.00	1.13±0.07	6.24	113.08

plasma from patients receiving all-*trans*-RA or 13-*cis*-RA for cancer treatment. In both cases small amounts of 9-*cis*-RA were detectable.

4. Conclusion

We developed a reproducible, accurate and sensitive HPLC method for the determination of retinoic acid isomers, 4-oxoretinoic acid isomers and all-*trans*-retinol in human plasma. With limits of quantification of 1 ng/ml for retinoic acids and 50 ng/ml for all-*trans*-retinol, the determination of physiological retinoid concentrations in human plasma is possible. Intensive retinoid monitoring in patients, who receive retinoic acid for cancer treatment, will complement data about the pharmacokinetics and metabolism of retinoic acids in children and adults and provide useful information on the influence of co-administered drugs (cytotoxic or immunemodulating drugs) on the pharmacokinetics and metabolism of retinoic acids. This may give some indication of why children are more susceptible to retinoic acids, and will be necessary for optimizing retinoic acid therapy and developing new strategies in cancer treatment.

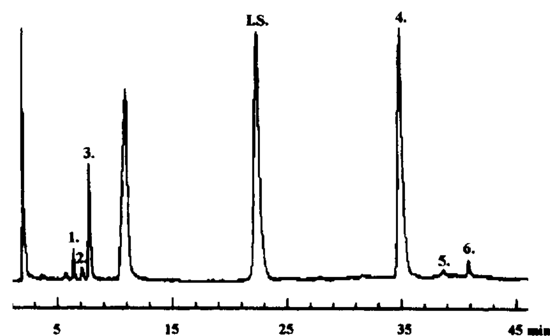

Fig. 3. Chromatogram of patient plasma (400 μl) 30 min after ingestion of all-*trans*-RA (45 mg/m^2 body surface area). Retinoid concentrations: 13-*cis*-RA, 7.43 ng/ml (1); 9-*cis*-RA, 3.14 ng/ml (2); all-*trans*-RA, 34.18 ng/ml (3); all-*trans*-retinol, 729.26 ng/ml (4); all-*trans*-4-oxo-RA, 3.90 ng/ml (5); 13-*cis*-4-oxo-RA, 7.77 ng/ml (6).

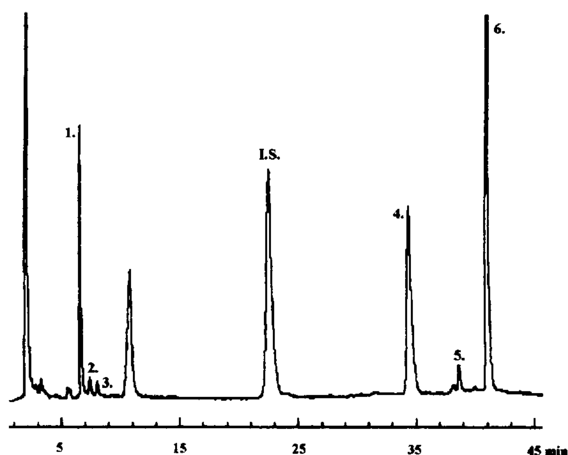

Fig. 4. Chromatogram of patient plasma (500 μl) 20 h after ingestion of 13-*cis*-RA (1 mg/kg body weight). Retinoid concentrations: 13-*cis*-RA, 62.36 ng/ml (1); 9-*cis*-RA, 5.81 ng/ml (2); all-*trans*-RA, 4.87 ng/ml (3); all-*trans*-retinol, 474.59 ng/ml (4); all-*trans*-4-oxo-RA, 9.04 ng/ml (5); 13-*cis*-4-oxo-RA, 164.31 ng/ml (6).

This method is currently used for pharmacokinetic and metabolic studies of all-*trans*-RA and 13-*cis*-RA in children and adults, who receive one of those retinoic acids for cancer treatment. The results will be reported elsewhere.

When 9-*cis*-retinoic acid is introduced for clinical use retinoid monitoring regarding pharmacokinetics and metabolism is possible with the described method.

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