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Simultaneous analysis of retinol, all-*trans*- and 13-*cis*-retinoic acid and 13-*cis*-4-oxoretinoic acid in plasma by liquid chromatography using on-column concentration after single-phase fluid extraction

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Abstract

A reversed-phase high-performance liquid chromatographic method for the simultaneous analysis of retinol, all-*trans*-retinoic acid, 13-*cis*-retinoic acid and 13-*cis*-4-oxoretinoic acid in human plasma and cell culture medium is described. Sample preparation involves precipitation of proteins and extraction of retinoids with 60% acetonitrile. After centrifugation, the acetonitrile content of the supernatant is reduced to 45%, allowing on-column concentration of analytes. Injection volumes up to 2.0 ml (equivalent to 0.525 ml of sample) can be used without compromising chromatographic resolution of all-*trans*-retinoic acid and 13-*cis*-retinoic acid. Retinoids were stable in this extract and showed no isomerization when stored in the dark in a cooled autosampler, allowing automated analysis of large series of samples. Recoveries from spiked plasma samples were between 95 and 103%. Although no internal standard was used, the inter-assay precision for all retinoids was better than 6% and 4% at concentrations of 30 nM and 100 nM, respectively. The method is a valuable tool for the study of cellular metabolism of all-*trans*-retinoic acid, as polar metabolites of this compound can be detected with high sensitivity in cell culture media.

Keywords: Vitamins; Retinol; Retinoic acid isomers

1. Introduction

Retinoids are a class of naturally occurring and synthetic compounds that are structurally related to retinol (vitamin A) and play a role in a variety of physiological processes, i.e. vision, morphogenesis, growth and differentiation of tissues, reproduction and immune modulation. Nuclear retinoic acid receptors (RARs) and retinoid X receptors (RXRs) play a pivotal role in retinoid action. These ligand

dependent receptors, which form homo- or heterodimers upon retinoid binding, function as transcription factors for a number of genes by binding to retinoic acid responsive elements (RARE). Retinoids thus exert their effects on the level of regulation of gene expression. Both natural and synthetic retinoids are used in the treatment of malignancies and skin disorders. Metabolic and clinical aspects and importance of the retinoids have been reviewed extensively [1–4].

The analysis of retinoids in biological samples is a challenging problem, due to sensitivity of these

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compounds to light and heat, low endogenous concentrations and strong protein binding. Separation of retinoids is usually accomplished by HPLC in reversed-phase mode [5–15], although separation in normal-phase mode is also quite possible [16,17]. Reviews on chromatographic procedures have appeared in recent years [18,19]. Favourable spectral characteristics of most retinoids allow sensitive and selective UV detection. Mass spectrometric detection is also possible [20,21].

Many procedures for sample pretreatment have been developed [19]. Most of these procedures are based on protein precipitation using an organic solvent, followed by liquid–liquid extraction. Usually the final extract is dried under nitrogen and reconstituted in a small volume of mobile phase. To compensate for losses during the various fluid handling steps the use of an internal standard is a prerequisite. The total procedure is laborious and carries the risk of inadvertent oxidation and isomerization of labile retinoids. Another approach is the use of on-line solid-phase extraction in combination with column-switching [5–8,11]. This technique minimizes manual sample handling and exposure of samples to light and excellent results have been reported. However, the required equipment is rather complicated.

For the analysis of large numbers of samples generated by clinical studies we needed a simple and robust system for the simultaneous determination of retinoids, including retinol, all-*trans*-retinoic acid (all-*trans*-RA), 13-*cis*-retinoic acid (13-*cis*-RA) and the polar metabolite 13-*cis*-4-oxoretinoic acid (13-*cis*-4-oxoRA). For the separation of these compounds we used reversed-phase chromatography with gradient elution and UV detection. For sample clean-up we evaluated a single-phase fluid extraction procedure. Acetonitrile was added to plasma samples to effect precipitation of proteins and extraction of retinoids. To avoid a drying step, we performed direct injection of the acetonitrile extract. Peak broadening, caused by the high elution strength of the extract, made large volume injections unfeasible. However, if prior to injection, the acetonitrile content was lowered by addition of water, large volumes could be injected without compromising chromatographic resolution and in this way adequate overall sensitivity was attained. We have systematically

studied the effect of acetonitrile concentration on extraction efficiency, solubility and stability of retinoids in the final extract and also the relation between injection volume and chromatographic resolution. This led to a final procedure that combines simple and rapid sample preparation with a sensitive and reproducible detection of retinoids, allowing the analysis of large series of samples.

2. Experimental

2.1. Materials and reagents

Retinol, 13-*cis*-retinoic acid (isotretinoin) and all-*trans*-retinoic acid (tretinoin) were obtained from Sigma (St. Louis, MO, USA). 13-*cis*-4-Oxoretinoic acid and all-*trans*-4-oxoretinoic acid were kindly provided by Hoffmann-La Roche (Basel, Switzerland). Ammonium acetate and 1.0 M sodium hydroxide solution were supplied by Merck (Amsterdam, Netherlands). HPLC grade acetonitrile was obtained from Biosolve (Barneveld, Netherlands) and acetic acid, methanol (HPLC grade) and absolute ethanol from Baker (Deventer, Netherlands). HPLC-grade water was prepared from demineralized water using a Milli-Q UF Plus water purification system (Millipore, Milford, MA, USA). To prepare the 1.0 M sodium acetate buffer used for sample extraction, 5.7 ml of acetic acid was diluted to approximately 50 ml with water. After adjustment of the pH to 4.0 with 1.0 M sodium hydroxide, the final volume was brought to 100 ml.

2.2. Preparation of standards

Stock solutions of retinoids were prepared in absolute ethanol and stored at -20°C . Concentrations of the stock solutions were determined spectrophotometrically using the following molar extinction coefficients: 52 770 (325 nm) for all-*trans*-retinol; 39 750 (354 nm) for 13-*cis*-retinoic acid; 45 300 (350 nm) for all-*trans*-retinoic acid; 39 000 (361 nm) for 13-*cis*-4-oxoretinoic acid [22].

For system validation standards with concentrations of 5000, 1500, 750, 300, 150, 75, 30, 15, 7.5, 3, 1.5, 0.75 and 0.3 nM were prepared by diluting a mixture of the stock solutions with 45% acetonitrile

containing 37.5 mM sodium acetate buffer (pH 4.0). Calibration curves were constructed by linear least-squares regression analysis of peak area (y) versus concentration (x) using a weighting factor of $1/x$.

For routine quantification of retinoids in plasma samples, calibration was performed by triplicate analysis of a single plasma-based standard, containing 13-*cis*-4-oxoRA (299 nM), 13-*cis*-RA (290 nM), all-*trans*-RA (301 nM) and retinol (2050 nM). This standard was prepared by spiking a plasma pool with a mixture of individual retinoid stock solutions. Final ethanol concentration was below 1%. After thorough mixing in the dark, aliquots were snap-frozen in solid carbon dioxide–acetone and stored at -20°C .

2.3. Chromatographic system

The HPLC system consisted of a Model 616 pump and a Model 486 UV detector from Waters (Milford, MA, USA). Mobile phase was passed through a Degasys model DG2410 inline solvent degasser from Uniflows (Tokyo, Japan). A Model 717 plus automatic sample injector with cooling option from Waters was used. To allow the injection of large sample volumes, the auxiliary sample loop was installed and a syringe motor rate of $5.0\ \mu\text{l s}^{-1}$ was used. The small window in the door of the sample compartment was covered to shield the samples from light. Column temperature was controlled with a column oven and TCM temperature control module from Waters. MILLENNIUM 2010 software (version 2.10) from Waters was used for instrument control and data acquisition and processing. Analyses were performed on a 10 cm $\times$ 4.6 mm I.D. Spherisorb ODS2 C_{18} cartridge column containing 3- μm particles (Phase Separations, Deeside, UK). The analytical column was protected by an integral 1 cm $\times$ 2.0 mm I.D. reversed-phase guard column from the same supplier.

2.4. Collection and processing of samples

Blood was obtained by venapuncture and collected in EDTA containing tubes. Tubes were wrapped in aluminium foil to protect the samples from light. Within 1 h of venapuncture plasma was obtained by

centrifugation (10 min at 3000 g and 4°C) and stored in cryovials that were flushed with nitrogen before storage at -70°C .

2.5. Cell lines

Head and neck squamous cell carcinoma (HNSCC) cell lines were obtained from T.E. Carey (University of Michigan, Ann Arbor, MI, USA) and are described elsewhere [23]. The UM-SCC-35 cell line used in this study originated from a hypopharyngeal tumour. Cells were routinely cultured in DMEM (Dulbecco's modified Eagle's Medium, Flow Laboratories, Irvine, UK) with 10% FCS (fetal calf serum, Flow Laboratories) in 75-cm² flasks (Nunc, Roskilde, Denmark). Cellular doubling time was 52 h.

2.6. Sample preparation

All operations were performed in a room lit by subdued yellow light. Frozen plasma and cell culture medium samples were thawed at room temperature in the dark.

For routine experiments with 0.4-ml injection volumes, the following extraction procedure was used. To 0.35 ml of plasma, culture medium or plasma based standard, 0.05 ml of 1.0 M sodium acetate buffer (pH 4.0) was added. After mixing, 0.6 ml of acetonitrile was added and samples were immediately vortex-mixed. After centrifugation (5 min at 3000 g and 4°C), 450 μl of the clear supernatant was transferred to a conical plastic autosampler vial (Waters part No. 22476). After addition of 150 μl of water, the vials were capped, mixed by inversion and immediately put in the sample compartment of the autoinjector. The autoinjector was cooled at 7°C .

In experiments where larger injection volumes were used, 1.05 ml of sample was mixed with 0.15 ml of sodium acetate buffer and extracted with 1.8 ml of acetonitrile as described above. A 2.1-ml volume of the supernatant obtained after centrifugation was mixed with 0.7 ml of water and the extracts were stored in 4.0-ml glass vials (Waters part No. 72710).

2.7. HPLC conditions

Chromatographic conditions were as described by Eckhoff and Nau [8] with some modifications. A 40-mM ammonium acetate buffer, adjusted to pH 5.75 with acetic acid was prepared. Mobile phase A consisted of buffer–methanol (50:50, v/v) and mobile phase B was pure methanol. The gradient conditions are shown in Table 1. To avoid column damage by sudden pressure changes during injection of large sample volumes (0.4 to 2.0 ml), flow-rate was kept at 0.1 ml min⁻¹ during injection. After injection, flow-rate was increased to 0.8 ml min⁻¹ and maintained at this rate during the analysis. Data acquisition was performed at a rate of 2 Hz for 30 min. After 35 min a next injection was started. Total time between injections was approximately 40 min, depending on the sample volume injected. Column temperature was maintained at 30°C and detection was performed at 340 nm. Retinoids were quantitated on the basis of peak area using external standardization.

2.8. Assay performance

For quality control purposes three plasma pools were used. One of these pools was used without fortification (endogenous control). The other pools were spiked with a mixture of retinoids to obtain a low control and a high control plasma, respectively. Aliquots of these control samples were stored at -20°C. For determination of within-day precision, control samples (*n*=10 at each level) were determined within a single chromatographic series. Be-

tween-day precision was assessed by analyzing the control samples on 8 occasions within a two-month period.

3. Results and discussion

3.1. Chromatographic conditions

Separation was achieved by reversed-phase chromatography using a binary methanol gradient. We used the separation conditions described by Eckhoff and Nau [8] as starting point and modified buffer strength and gradient shape to obtain adequate resolution between 13-*cis*-RA and all-*trans*-RA and allow elution of the more hydrophobic retinol. Gradient conditions are shown in Table 1. As we wanted to perform large volume injections, the autoinjector was equipped with a large sample loop. Large sample loops may cause sudden pressure surges in the system during switching of the injection valve, that can reduce column performance. To avoid this potential problem we used flow programming. Flow was kept at 0.1 ml min⁻¹ during sample injection, subsequently increased to 0.8 ml min⁻¹ and kept at this rate for the remainder of the analysis.

Column temperature was kept at 30°C. Others have used higher temperatures, e.g. Takeda and Yamamoto heated the column to 50°C [15] and Eckhoff and Nau even used a temperature of 60°C [8]. Although at elevated temperatures we observed a somewhat better column performance and lower column backpressure, there was an apparent on-column degradation of retinol, resulting in diminished peak height and the appearance of an additional small unresolved peak eluting before the main retinol peak.

Fig. 1 shows the chromatogram of a standard solution. The small peak eluting between 13-*cis*-RA and all-*trans*-RA is possibly 9-*cis*-RA present as a contamination due to isomerization.

3.2. Extraction of retinoids

As we wanted a simple and rapid sample clean-up procedure, we chose to evaluate single-phase fluid extraction with a water miscible organic solvent. We used acetonitrile, which has previously been shown

Table 1
Gradient conditions

Time (min)	Flow (ml min ⁻¹)	Percent A	Percent B
0	0.1	85	15
1	0.8	85	15
20	0.8	0	100
22	0.8	0	100
23	0.8	85	15
34	0.8	85	15
35	0.1	85	15

Mobile phase A: 40 mM ammonium acetate (pH 5.75)–methanol (50:50, v/v); mobile phase B: methanol.

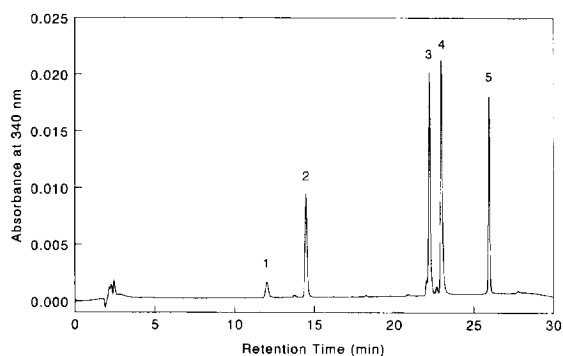


Fig. 1. Chromatogram of a retinoid standard solution. A 5- μ l volume of a combined standard in ethanol was injected (total injected amount of each retinoid 15–75 pmol). Peak 1, all-*trans*-4-oxoRA; Peak 2, 13-*cis*-4-oxoRA; Peak 3, 13-*cis*-RA; Peak 4, all-*trans*-RA; Peak 5, retinol. Chromatographic conditions as described in Section 2.7.

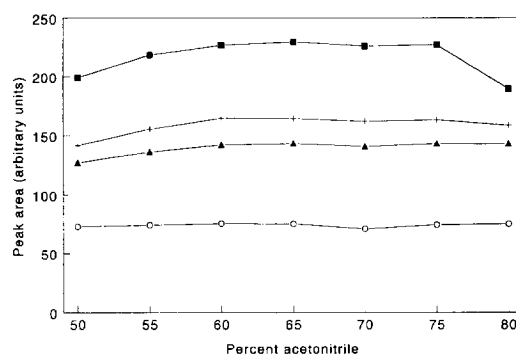


Fig. 2. Dependence of extraction efficiency of retinoids on acetonitrile concentration. Retinoids were extracted from a plasma pool, spiked with a mixture of retinoids. To 0.15 ml of spiked plasma 0.05 ml of 1.0 *M* sodium acetate buffer (pH 4.0) was added and subsequently retinoids were extracted by addition of water and acetonitrile in various proportions. Final volume was 1.0 ml. After mixing and centrifugation to remove precipitated protein, an aliquot of the supernatant was subjected to chromatography. Chromatographic conditions as described in Section 2.7. Peak area of 13-*cis*-4-oxoRA ($\circ$), 13-*cis*-RA ($\blacktriangle$), all-*trans*-RA (+) and retinol ($\blacksquare$) was determined and plotted versus acetonitrile concentration.

to accomplish effective protein precipitation and extraction of retinoic acid [10,13,14]. First we determined the minimal concentration of acetonitrile necessary for complete protein precipitation and maximal recovery of retinoids. In preliminary experiments we had observed that lowering the pH of plasma to a value between 4 and 5 before adding acetonitrile, resulted in a more effective protein precipitation. To 0.15 ml of a spiked plasma pool we added 0.05 ml 1.0 *M* sodium acetate buffer (pH 4.0) and then added water and acetonitrile in various proportions to a final volume of 1.0 ml. The final acetonitrile content varied between 30 and 80%. Only extracts with an acetonitrile content of 50% and higher gave clear supernatants and were evaluated by chromatography. The relation between peak area and acetonitrile concentration is depicted in Fig. 2. For both 13-*cis*-RA and all-*trans*-RA peak area slightly increased between 50 and 60% acetonitrile and then remained constant up to 80% acetonitrile. For retinol the same pattern was observed, with the exception that peak area dropped at 80% acetonitrile. This may be explained by the observation that at acetonitrile concentrations between 50 and 75% a finely divided protein precipitate formed, whereas at 80% acetonitrile a lumpy precipitate formed that settled very rapidly even without centrifugation. Inclusion of protein bound retinol in this precipitate may cause reduced recovery at 80% acetonitrile. Peak area of the polar metabolite 13-*cis*-4-oxoRA showed no

dependence on acetonitrile concentration. From this experiment we concluded that 60% acetonitrile is the minimal amount required to effect complete protein precipitation and maximal extraction of retinoic acid isomers and retinol.

3.3. Solubility of extracted retinoids

The elution strength of the extracts containing 60% acetonitrile was too high to allow injection of large volumes without peak broadening. We therefore studied the effect of lowering the acetonitrile content of the extracts by addition of water. As a general rule, capacity factors can be expected to increase 2–3 fold on lowering of the acetonitrile content by 10%. Therefore, loss of sensitivity by dilution of the sample is compensated by the fact that much larger volumes can be injected without loss of resolution, resulting in an overall increased sensitivity. To test solubility of the retinoids in solution as a function of acetonitrile content, a standard mixture of 13-*cis*-4-oxoRA, 13-*cis*-RA, all-*trans*-RA and retinol dissolved in 60% acetonitrile was divided over several tubes. To each tube water and acetonitrile were added in various proportions in such a way that the

total volume was kept constant. The final acetonitrile concentrations ranged from 20 to 60%. Peak area was expressed as a percentage of the area observed in the extract containing 60% acetonitrile. As can be seen from Fig. 3 the relative peak area for all components dropped as the content of acetonitrile was lowered. Curves for retinol, 13-*cis*-RA and all-*trans*-RA were nearly identical. The area of 13-*cis*-4-oxoRA was much less dependent on acetonitrile concentration, in accordance with its higher polarity. When samples were reanalyzed after storage for 6 h in the dark at 7°C in the autosampler, essentially the same peak areas were found, indicating that loss of retinoids is not a time dependent process. It may be that at low acetonitrile content of the extract, retinoids are adsorbed to components of the autosampler during injection. At 45% acetonitrile, relative peak areas of all components were higher than 93%. We therefore decided to use a final percentage of 45% acetonitrile for further experiments. This resulted in the sample preparation procedure as described in Section 2.6.

3.4. Large volume injection

We subsequently investigated injection of large volumes of extract, in order to achieve adequate

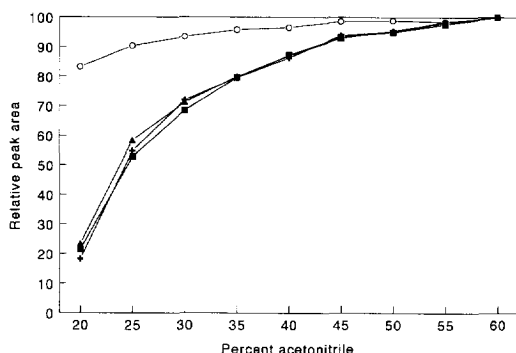


Fig. 3. Effect of acetonitrile content on solubility of retinoids. A mixture of retinoids was prepared in 60% acetonitrile containing 50 mM sodium acetate buffer (pH 4.0). To 1.0-ml aliquots of this mixture, water and acetonitrile were added in various proportions. Final volume was 3.0 ml. All samples were subjected to chromatography using conditions as described in Section 2.7. Peak area of 13-*cis*-4-oxoRA (○), 13-*cis*-RA (▲), all-*trans*-RA (+) and retinol (■) was determined and relative peak areas were calculated by dividing peak area by the area in the sample containing 60% acetonitrile.

overall sensitivity. A mixture of retinoids was prepared in 45% acetonitrile and volumes from 0.05 up to 2.0 ml were injected. For retinol, 13-*cis*-RA and all-*trans*-RA peak width was completely independent of the injection volume, up to volumes of 2.0 ml. As expected, peak widths for the early eluting compounds all-*trans*-4-oxoRA and 13-*cis*-4-oxoRA strongly increased when the injection volume exceeded 0.4 ml. From this experiment we concluded that 0.4 ml injection volumes could be used for the simultaneous analysis of retinol, 13-*cis*-RA, all-*trans*-RA and the polar metabolites all-*trans*-4-oxoRA and 13-*cis*-4-oxoRA. If analysis of the polar metabolites is not required, the injection volume can be increased to 2.0 ml without affecting peak width. Fig. 4A and B show chromatograms of a plasma based standard solution and plasma from a healthy volunteer, respectively, using an injection volume of 0.4 ml. Using this injection volume, endogenous 13-*cis*-4-oxoRA, 13-*cis*-RA, all-*trans*-RA and retinol can be measured. All-*trans*-4-oxoRA could not be detected in human plasma, in accordance with published observations [8,24].

Fig. 5 shows the effect of increasing the injection volume from 0.4 ml to 2.0 ml on the chromatogram of an unspiked plasma sample. Increasing the injection volume to 2.0 ml did not lead to peak broadening for 13-*cis*-RA and all-*trans*-RA. However, it is clear that quantification is more accurate with the 2.0 ml injection volume. In addition, using the 2.0 ml injection, the chromatogram revealed the presence of additional compounds not visible after a 0.4-ml injection.

3.5. Isomerization and degradation of extracted retinoids

Although the sample compartment of the auto-injector was shielded from light and kept at a temperature of 7°C, oxidative degradation or isomerization of the retinoids could possibly occur. It has been reported that isomerization of the synthetic retinoid acitretin is enhanced by acetonitrile [25]. To test stability, solutions of individual retinoids in 45% acetonitrile were stored in the autosampler in aliquots and injections were performed over a time span of 60 h. To check the effect of plasma components on stability and isomerization we repeated the experiment using extracts from pooled

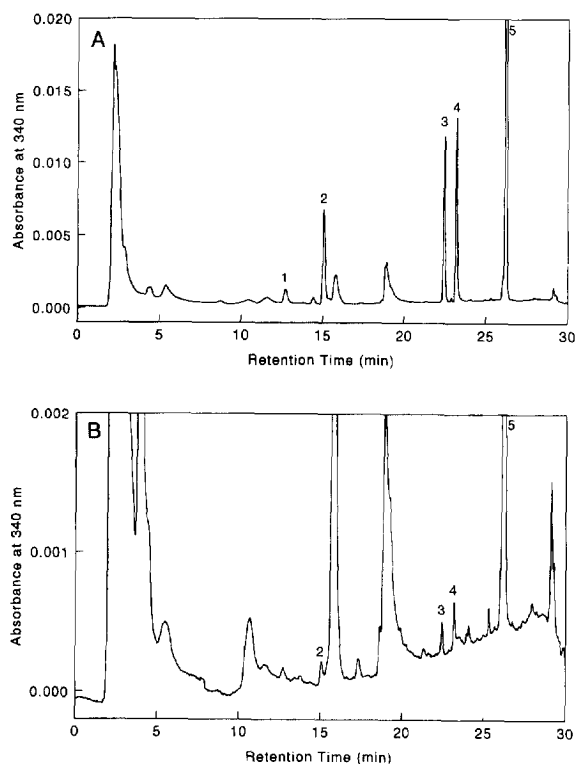


Fig. 4. Chromatograms of a plasma based retinoid standard solution (panel A), containing 13-*cis*-4-oxoRA (299 nM), 13-*cis*-RA (290 nM), all-*trans*-RA (301 nM) and retinol (2050 nM) and non-spiked plasma from a healthy volunteer (panel B), containing 13-*cis*-4-oxoRA (6.2 nM), 13-*cis*-RA (5.9 nM), all-*trans*-RA (7.6 nM) and retinol (2109 nM). To 0.35 ml of standard or plasma 0.05 ml of 1.0 M sodium acetate buffer (pH 4.0) was added, followed by 0.6 ml of acetonitrile. After vortex mixing samples were centrifuged for 5 min at 3000 g and 4°C. The supernatant (0.45 ml) was mixed with 0.15 ml of water and stored in the dark at 7°C in the sample compartment of the autosampler. An injection volume of 0.4 ml was used. Chromatographic conditions were as described in Section 2.7. Peak 1, all-*trans*-4-oxoRA; Peak 2, 13-*cis*-4-oxoRA; Peak 3, 13-*cis*-RA; Peak 4, all-*trans*-RA; Peak 5, retinol.

plasma, spiked with individual retinoids. The results are summarized in Table 2. Peak area R.S.D. values for individual retinoids were below 1% using standard solutions and below 2% using spiked plasma. These results indicate that the extracts are very stable and that no time dependent losses due to adsorption, degradation or isomerization of retinoids occur.

In a separate experiment we determined the extent of isomerization occurring during sample clean-up. This was done by spiking a plasma sample with concentrated stock solutions of either 13-*cis*-RA or

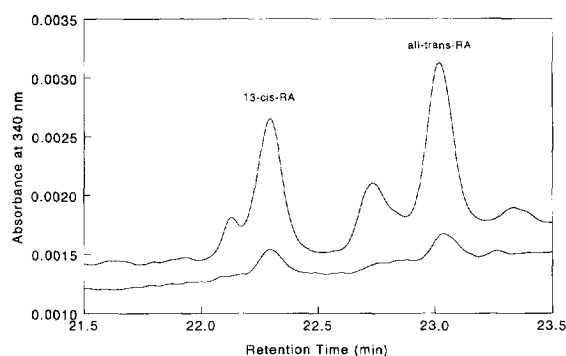


Fig. 5. Effect of injection volume on chromatography of endogenous retinoic acid isomers extracted from human plasma. To 1.05 ml of plasma 0.15 ml of 1.0 M sodium acetate buffer (pH 4.0) was added, followed by 1.8 ml of acetonitrile. After vortex mixing samples were centrifuged for 5 min at 3000 g and 4°C. The supernatant (2.1 ml) was mixed with water (0.7 ml) and stored in the dark at 7°C in the sample compartment of the autosampler. Chromatographic conditions were as described in Section 2.7 using injection volumes of 0.4 ml (lower trace) and 2.0 ml (upper trace). The plasma concentrations of 13-*cis*-RA and all-*trans*-RA were 6.2 and 6.9 nM, respectively.

all-*trans*-RA. After sample clean-up, levels of 13-*cis*-RA and all-*trans*-RA were determined to establish the extent of isomerization. Values were corrected for the amount of opposite isomer already present in the standard solutions. When plasma was spiked with 13-*cis*-RA, 0.6% was recovered as all-*trans*-RA. Conversely, 0.7% of all-*trans*-RA was recovered as 13-*cis*-RA after extraction of plasma spiked with all-*trans*-RA. These results were obtained when sample clean-up was performed under subdued yellow light. Without this precaution iso-

Table 2
Stability of retinoid extract during storage in the autosampler

Retinoid	Peak area R.S.D. (%)	
	Standard (n=14)	Spiked plasma (n=6)
All- <i>trans</i> -4-oxoRA	0.75	1.13
13- <i>cis</i> -4-oxoRA	0.28	0.22
13- <i>cis</i> -RA	0.56	1.02
All- <i>trans</i> -RA	0.54	1.68
Retinol	0.67	1.08

Individual retinoid standard solutions were prepared in 45% acetonitrile containing 37.5 mM sodium acetate buffer (pH 4.0). Pooled plasma was spiked with individual retinoids and extracted as described in Section 2.6. Standard solutions and plasma extracts were stored in aliquots in closed vials in the dark at 7°C in the autosampler. Injections were performed over a time span of 60 h.

merization values of 5% or higher were observed. These results confirm published observations [18,19] that extreme care should be taken to shield samples from light during extraction. Once extracted, isomerization of retinoids is negligible, provided the extracts are stored in the dark at 7°C.

3.6. Assay performance

Using an injection volume of 0.4 ml, calibration curves showed a linear relation between peak area and concentration up to 1.5 μM for 13-*cis*-4-oxoRA, 13-*cis*-RA and all-*trans*-RA ($r > 0.999$ for all components). As endogenous retinol concentrations are much higher than concentrations of retinoic acid isomers, linearity for retinol was tested over a wider concentration range. The calibration curve for retinol was found to be linear up to 5 μM ($r = 0.9995$). The quantification limit at a signal-to-noise (S/N) ratio of 10 was approximately 0.3 pmol on column for 13-*cis*-RA, all-*trans*-RA and retinol and 0.6 pmol on column for 13-*cis*-4-oxoRA. Using a 0.4-ml injection volume, equivalent to 0.105 ml of sample, this corresponds to a quantification limit of 3 nM for 13-*cis*-RA, all-*trans*-RA and retinol and 6 nM for 13-*cis*-4-oxoRA. For 13-*cis*-RA, all-*trans*-RA and retinol the quantification limit can be further reduced by increasing the injection volume.

Recovery was determined by spiking five plasma samples with a mixture of retinoids at three concentrations. Because endogenous concentrations of retinol are much higher than the concentrations of retinoic acid isomers, recovery of retinol was only tested at two concentrations. After a 1-h equilibration period, samples were extracted. Recovery of each retinoid was calculated by comparison of peak area in the spiked plasma minus the peak area of the unspiked plasma with the peak area obtained after direct injection of the respective amounts of retinoid. The results presented in Table 3, show that the mean recoveries ranged from 95–103% and were independent of the concentration used for spiking.

For quality control purposes, three plasma pools were used, one unfortified (endogenous pool) and two spiked with 30 nM (low control plasma) and 100 nM (high control plasma) of each retinoid, respectively. Intra-assay precision was determined by analyzing the quality control samples ($n = 10$ each) in

Table 3

Recovery of retinoids from spiked plasma samples

Compound	Spike level (nM)	Recovery (mean $\pm$ S.D.) (%)
13- <i>cis</i> -4-oxoRA	18	102.1 $\pm$ 3.8
	89	101.4 $\pm$ 3.3
	445	103.0 $\pm$ 2.2
13- <i>cis</i> -RA	52	96.7 $\pm$ 2.3
	259	101.2 $\pm$ 0.8
	1295	99.1 $\pm$ 2.0
All- <i>trans</i> -RA	54	96.4 $\pm$ 2.6
	269	102.8 $\pm$ 2.3
	1346	96.3 $\pm$ 2.4
Retinol	206	102.4 $\pm$ 7.1
	1032	95.5 $\pm$ 2.2

Plasma samples ($n = 5$) were spiked with a mixture of retinoids at three concentrations and with retinol at two concentrations.

a single chromatographic analysis series. The results are shown in Table 4. Inter-assay precision and accuracy were determined by analyzing the control samples on 8 different days over a period of two months. The same plasma based standard solution was used during this period for calibration. The results shown in Table 5 indicate that although no internal standard was used in our method, between-run precision was very acceptable and comparable to other published methods [5,6,8–11,13–17]. In order to allow quantitation of the polar metabolite 13-*cis*-

Table 4

Within-day precision for assay of retinoids in plasma ($n = 10$)

	Mean $\pm$ S.D. (nM)	C.V. (%)
<i>Endogenous control</i>		
13- <i>cis</i> -4-oxoRA	8.30 $\pm$ 0.64	7.8
13- <i>cis</i> -RA	7.08 $\pm$ 0.28	4.0
All- <i>trans</i> -RA	8.38 $\pm$ 0.31	3.7
Retinol	1920 $\pm$ 7.3	0.4
<i>Low control</i>		
13- <i>cis</i> -4-oxoRA	30.3 $\pm$ 1.3	4.3
13- <i>cis</i> -RA	34.7 $\pm$ 0.3	1.0
All- <i>trans</i> -RA	32.2 $\pm$ 0.4	1.3
Retinol	1925 $\pm$ 15.1	0.8
<i>High control</i>		
13- <i>cis</i> -4-oxoRA	103.2 $\pm$ 1.1	1.0
13- <i>cis</i> -RA	108.1 $\pm$ 1.3	1.2
All- <i>trans</i> -RA	96.1 $\pm$ 2.5	2.6
Retinol	2022 $\pm$ 25.3	1.3

Table 5
Between-day precision and accuracy for assay of retinoids in plasma ($n=8$)

	Concentration (nM)		C.V. (%)	Accuracy (%)
	Nominal	Found		
<i>Endogenous control</i>				
13- <i>cis</i> -4-oxoRA		5.60±0.97	17.3	
13- <i>cis</i> -RA		6.18±0.39	6.2	
All- <i>trans</i> -RA		7.00±0.77	11.0	
Retinol		2193±53	2.4	
<i>Low control</i>				
13- <i>cis</i> -4-oxoRA	35.6	35.1±2.0	5.8	98.6±5.6
13- <i>cis</i> -RA	36.2	36.2±0.9	2.5	100.0±2.5
All- <i>trans</i> -RA	37.0	34.3±1.0	2.8	92.7±2.7
Retinol	2223	2175±59	2.7	98.1±2.7
<i>High control</i>				
13- <i>cis</i> -4-oxoRA	105.6	112.4±3.9	3.5	106.4±3.7
13- <i>cis</i> -RA	106.2	112.7±3.4	3.0	106.1±3.2
All- <i>trans</i> -RA	107.0	105.2±2.7	2.6	98.3±2.5
Retinol	2293	2227±80	3.6	97.1±3.5

4-oxoRA, we used a 0.4-ml injection volume. Under these circumstances endogenous levels of the retinoids are rather close to the lower quantification limit of the method, explaining the relatively large inter-assay coefficient of variation in the unspiked control sample. As already shown in Fig. 5, 13-*cis*-RA and all-*trans*-RA can be analyzed with higher sensitivity using a 2.0-ml injection volume. Although we did not determine precision using 2.0-ml injection volumes, the coefficient of variation for inter-assay precision of endogenous 13-*cis*-RA and all-*trans*-RA can be expected to drop to values of ~3% using this injection volume, as observed for the low control quality control sample using an injection volume of 0.4 ml.

3.7. Analysis of retinoids in cell culture medium

The method was developed for the analysis of retinoids in plasma samples. We are currently investigating the cellular metabolism of retinoic acid by squamous cell carcinoma cell lines and we routinely use the plasma procedure for the analysis of retinoids in cell culture medium. A typical chromatogram is shown in Fig. 6, demonstrating the possibility of analyzing polar metabolites of all-*trans*-RA that appear in the medium after incubation for 24 h with the cell line UM-SCC-35. As can be seen from Fig.

6A a large number of compounds that are eluted early in the chromatogram (between 4 and 18 min), are not seen in control incubations without cells (Fig. 6B). Although none of the early eluting components are identified as yet, this experiment clearly demonstrates the potential of the current chromatographic method to study cellular metabolism of retinoids.

4. Conclusions

A very simple and rapid procedure for the simultaneous extraction of retinoic acid isomers and retinol from plasma and cell culture medium was developed. By reducing the acetonitrile concentration of the extract obtained, very large injection volumes could be used, without loss of chromatographic resolution, allowing quantification of endogenous levels of retinoids in plasma. In this way concentration of the extracts by time-consuming solvent evaporation, with the inherent risk of oxidation and isomerization, is avoided. The extracts can be stored in the auto-injector in the dark at 7°C for up to 60 h without any change in peak area or isomerization. This allows the analysis of large series of samples. We routinely analyze series of 96 samples. Because sample preparation is very straightforward, excellent precision is obtained without the use of an internal standard.

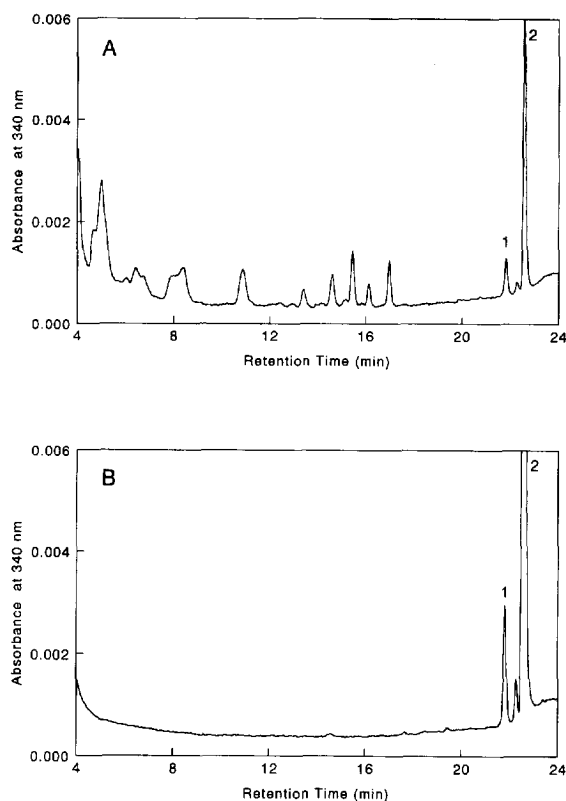


Fig. 6. Metabolism of all-*trans*-RA by the UM-SCC-35 cell line. Cells were grown in culture medium supplemented with 1 μ M all-*trans*-RA. After 24 h the medium was analyzed for retinoic acid metabolites (panel A). In a parallel experiment culture medium supplemented with 1 μ M all-*trans*-RA without cells was also analyzed after 24 h (panel B). To 0.35 ml of cell culture supernatant or control medium 0.05 ml of 1.0 M sodium acetate buffer (pH 4.0) was added, followed by 0.6 ml of acetonitrile. After vortex mixing samples were centrifuged for 5 min at 3000 g and 4°C. A 0.45-ml volume of the supernatant was mixed with 0.15 ml of water and stored in the dark at 7°C in the sample compartment of the autosampler. An injection volume of 0.4 ml was used. Chromatographic conditions were as described in Section 2.7. Only a part of the chromatogram is shown to illustrate the appearance of polar metabolites of all-*trans*-RA after incubation with cells. Peak 1, 13-*cis*-RA; Peak 2, all-*trans*-RA.

Another advantage of the current procedure is that in a single run both all-*trans*-RA and 13-*cis*-RA are analyzed, together with the more apolar retinol, as well as more polar metabolites like 13-*cis*-4-oxoRA. The combination of high sensitivity and the ability to quantitate polar metabolites make the method a

valuable tool for the study of cellular metabolism of retinoids.

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