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Journal of Chromatography B, 798 (2003) 309–316
JOURNAL OF CHROMATOGRAPHY B
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# Automated solid-phase extraction and liquid chromatographic method for retinoid determination in biological samples

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Received 12 June 2003; received in revised form 2 October 2003; accepted 2 October 2003

**Abstract**

In this study, a method for partly automated sample preparation and fully automated solid-phase extraction method for plasma, kidney and liver samples for various retinoids like all-*trans*-4-oxo-retinoic acid, 13-*cis*-4-oxo-retinoic acid, 13-*cis*-retinoic acid, 9-*cis*-retinoic acid, all-*trans*-retinoic acid, retinol and retinyl palmitate was established. Plasma, embryo-, kidney-and liver-homogenates were automatically mixed and extracted on multiple usage solid-phase (C2) extraction cartridges immediately before HPLC analysis. Automated cleaning, preconditioning and incorporation of the loaded cartridge to fully automated HPLC separation and quantification of the various retinoids in a single HPLC run was established. The recovery of the retinoids was generally between 80 and 90%. Intra-day repeatability was <11.7%. As little as 1.2 ng/ml could be quantified in lipid-mixture standard samples. This method allows a highly automated sample preparation and a fully automated solid-phase extraction with good selectivity for the study of endogenous retinoids and retinoids after nutritional supplementations and pharmacological applications in several biological samples.


*Keyword:* Retinoid

## 1. Introduction

Retinoids, derivatives of Vitamin A alcohol (retinol), are involved in several physiological processes such as embryonic development, growth and differentiation [1,2]. Retinoids are important micronutrients in the human diet and are mainly taken up as retinyl esters and retinol [3]. Retinol is metabolised to various polar and unpolar metabolites, namely retinoic acid isomers, 4-oxo-retinoic acid isomers and retinyl esters (for a review [4,5]). Some of these retinoids transmit important physiological functions via mediation of gene transcription as ligands of the nuclear retinoic acid receptors and/or the retinoid-X-receptors [6].

Currently methods are available for determination of polar retinoids or unpolar retinoids and a few of them for the simultaneous detennination of polar and unpolar retinoids (for a review see: [7–9]). In comparison to the highly laborious liquid-liquid extraction methods including nitrogen drying with a high risk of inadvertent oxidation and isomerisation of labile retinoids [10] automated routine analysis HPLC methods including semi-automated column switching methods [11–16] or solid-phase extractions have been established [17–19].

In the present study, we developed a partly automated sample preparation, fully automated solid-phase extraction and immediately following fully automated reversed-phase gradient HPLC method. The separation, quantification and identification of various retinoids like 4-oxo-retinoic acid isomers, retinoic acid isomers, retinol and retinyl palmitate (Fig. 1) could be performed for various tissues (plasma, embryo-, kidney- and liver-homogenates).

## 2. Experimental

### 2.1. Chemicals

All-*trans*-retinoic acid, 13-*cis*-retinoic acid, 9-*cis*-retinoic acid, retinyl palmitate and retinol were kindly provided by BASF AG (Ludwigshafen, Germany), all-*trans*-4-oxo-retinoic acid and 13-*cis*-4-oxo-retinoic acid by Roche

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doi:10.1016/j.jchromb.2003.10.001

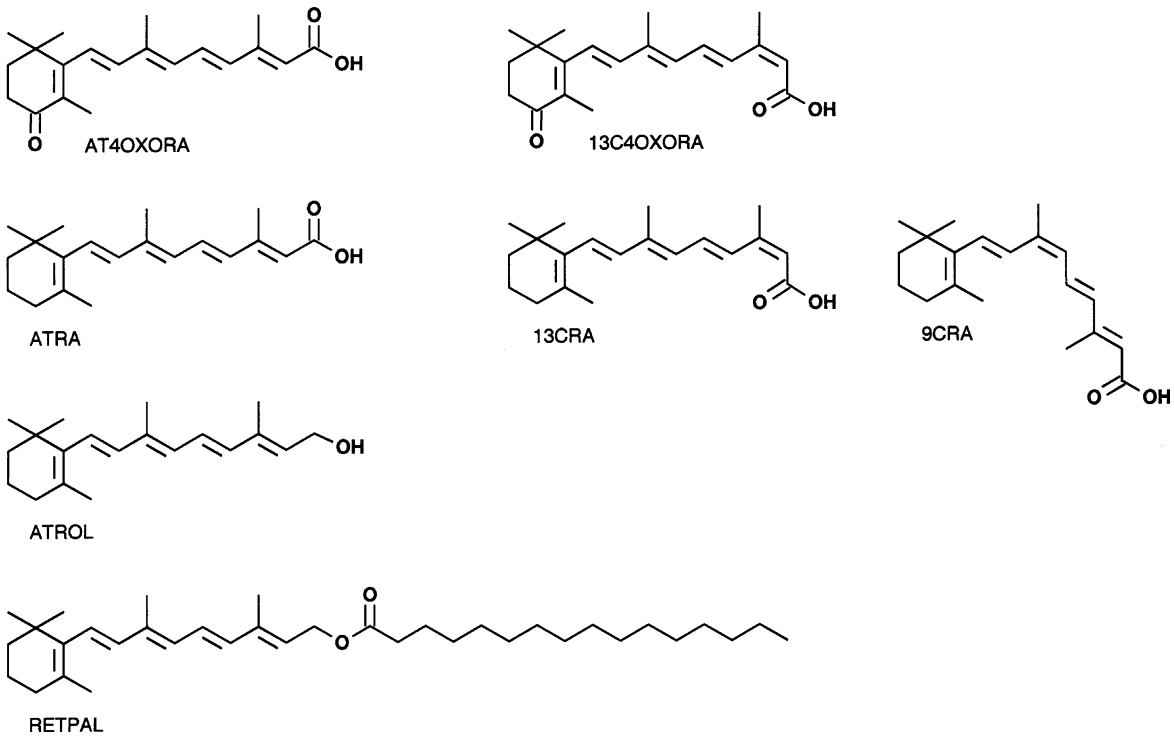



Fig. 1. Structural formulas of the retinoids (AT4OXORA: all-*trans*-4-oxo-retinoic acid; 13C4OXORA: 13-*cis*-4-oxo-retinoic acid; 13CRA: 13-*cis*-retinoic acid; 9CRA: 9-*cis*-retinoic acid; ATRA: all-*trans*-retinoic acid; ROL: retinol; RETPAL: retinyl palmitate).

Vitamins (Basle, Switzerland). Lipid-mixture 1 (a mixture of non-animal derived fatty acids) and ammonium acetate were obtained from Sigma (Deisenhofen, Germany), methanol, acetonitrile and isopropanol were purchased from Roth (Lage, Germany). Water was deionised and purified by a Reinstwassersystem TYP HP 6 UV/UF (TKA-Lab, Germany).

### 2.2. Laboratory precautions

As retinoids are sensitive to light, all experiments (sample preparation as well as the HPLC analysis) were carried out under dim yellow light. Due to standardised HPLC analysis and sample preparation all laboratory work was performed at 23 °C.

### 2.3. Sample collection

NMRI mice were treated with all-*trans*-retinoic acid (10 mg/kg body weight) by oral application in 25% aqueous Cremophor EL solution (5 ml/kg body weight) [20], and killed by decapitation after 4 h. Kidney samples were collected and immediately stored at −80 °C until analysis. Mouse embryo samples of gestational day 12.5 were obtained from pregnant NMRI mice (Charles River, Sulzfelden, Germany) and rat plasma was taken from male Wistar rats (Charles River). All experiments were approved by the respective authorities from Land Berlin or Brandenburg, Germany.

### 2.4. Sample pre-treatment

About 125 μl of lipid-mixture standard samples (as a non-Vitamin A containing standard for biological fluids) or plasma/serum were accurately diluted with a three-fold excess of isopropanol. After 3 min of shaking the precipitated protein was pelleted by centrifugation. Kidneys from one animal were pooled and diluted with 1 volume of water, embryo samples were not diluted and the organs were minced with scissors. One hundred twenty-five microlitres of this preparation was diluted with a three-fold excess of isopropanol and disrupted by ultrasonic treatment on ice. Liver was diluted with a nine-fold excess of ice-cold 0.9% aqueous NaCl solution and homogenised with a Teflon-glass potter. One hundred twenty-five microlitres of the homogenate was diluted with a three-fold excess of isopropanol and disrupted by ultrasonic treatment on ice [17,21]. Each of the biological sample dilutions (serum/embryo/kidney/liver) were centrifuged for 6 min and 410 μl of the supernatant were placed into the AS3000 (Thermoquest, Darmstadt, Germany) autosampler (Table 1).

### 2.5. Automated solid-phase extraction

Solid-phase extraction and clean up were achieved using an on-line solid-phase extraction unit, consisting of a L-7100A intelligent pump (Thermoquest, Darmstadt, Germany), a WE-IIb, electric actuator (Valco, Schenkon, Switzerland) and an autosampler AS3000 (Thermoquest,

Table 1
AS3000 program in sample preparation mode. "Dilution" configuration for automated sample preparation

| | | | |
|---|---|---|---|
| 1 | Load[a] | 500 μl | S1-buffer[a,b] |
| 2 | PickLG[a] | 100 μl | Sample[a] |
| 3 | Add[a] | 600 μl | To sample[a] |
| 4 | Mix[a] | 0.5 min | |
| 5 | Load | 400 μl | S1-buffer |
| 6 | PickLG | 100 μl | Sample |
| 7 | Add | 500 μl | To sample |
| 8 | Mix | 0.5 min | |
| 9 | Load | 300 μl | S1-buffer |
| 10 | PickLG | 100 μl | Sample |
| 11 | Add | 400 μl | To sample |
| 12 | Mix | 1 min | |

[a] These terms are variables in the dilution mode of the AS3000.
[b] S1-buffer (optional solvent delivery of the AS3000: in our method 2% aqueous ammonium acetate solution).

Darmstadt, Germany) (Fig. 2). The autosampler was connected to the sample enrichment cartridge (manu-CART "4" cartridge holder), LiChroCART cartridges (4 mm i.d., 20 mm length) controlled by the WE-IIb electric actuator and fitted inside LiChroCART sealing elements (1.51254) (Merck KGaA, Darmstadt, Germany) self-packed with C2-modified silica (isolute-C2 sorbent, 40–70 μm, ICT Handels-GmbH, Bad Homburg, Germany). In-between the autosampler and the sample enrichment cartridge a manual injection port (Rheodyne 1700, Merck Eurolab, Bruchsal, Germany) with a 20 μl sample loop was installed (Fig. 2). The sample containing cartridges were integrated by a valve switching technique into the analytical cycle. C2 cartridges were used up to 100 times or exchanged earlier due to non-appropriate recovery and separation of the different standard retinoids in standard samples which were run routinely every day.

Prior to sample injection the cartridges were preconditioned and equilibrated. The AS3000, with connection to 3 bar air pressure at the "AIR IN" outlet, a 1500 μl sample coil, a 2500 μl sample preparation syringe and a 2500 μl sample syringe containing 50% methanol/50% 60 mM aqueous ammonium acetate solution as purge solution automatically prepares before each HPLC analysis the biological extract for solid-phase extraction (Table 1). Fifteen hundred microlitres of this freshly prepared biological extract were then automatically injected into the 1500 μl sample loop of the AS3000. The biological extract was transferred by a solvent composed of 2% aqueous ammonium acetate onto the cartridge. Solvent A (methanol) was used for cleaning of the cartridge; B and C (85% (0.5% aqueous ammonium acetate solution (B)): 15% acetonitrile (C)) for loading and washing of the cartridge after sample solid-phase extraction and D (2% aqueous ammonium acetate solution) for preconditioning and transfer of the biological sample onto the cartridge. This whole procedure was controlled by a program (Table 2) of the L-7100A (the L-7100A must be connected without the internal mixing chamber), which is automatically started by the AS3000 after completion of sample preparation and filling the sample loop (Fig. 2).

Time events were defined in the time program of the L-7100A for the sample enrichment, clean up, sample cartridge incorporation, WE-IIb electronic actuator functions and start of the computer program and multilinear gradient of the L-7100A (Table 2). Before each HPLC analysis the cartridge was cleaned, preconditioned and loaded.

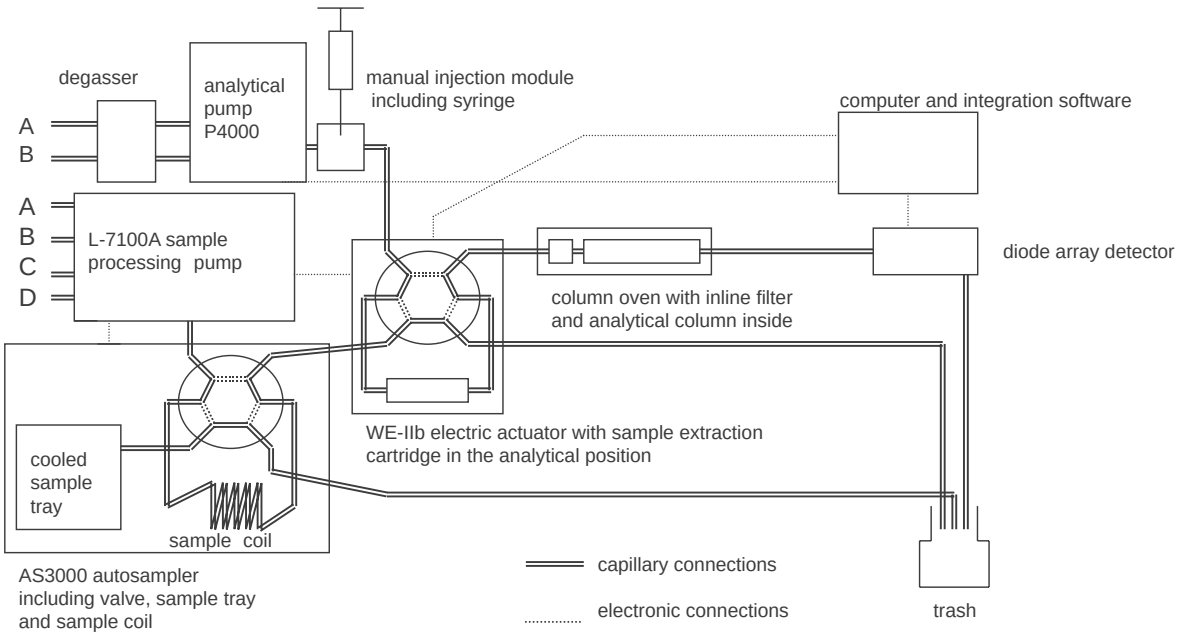



Fig. 2. Schematic representation of the HPLC pre-column switching system.

Table 2
L7100A pump program

| Time (min) | Solvent | | | | Flow (ml/min) | Event | Description |
|---|---|---|---|---|---|---|---|
| | A | B | C | D | F | | |
| 0.0 | 0 | 85 | 15 | 0 | 0 | 12 | WE-IIb valve in position B (sample preparation position) |
| 0.1 | 0 | 85 | 15 | 0 | 1.0 | | |
| 2.6 | 0 | 85 | 15 | 0 | 1.0 | | |
| 2.7 | 0 | 85 | 15 | 0 | 0 | | |
| 3.8 | | | | | | 22 | WE-IIb valve in position A (analysis position) |
| 3.9 | | | | | | 30 | Start for pump-and computer program |
| 47.0 | | | | | | 12 | WE-IIb valve in position B |
| 47.1 | 100 | 0 | 0 | 0 | 1.0 | | |
| 47.2 | 100 | 0 | 0 | 0 | 2.0 | | |
| 48.2 | 100 | 0 | 0 | 0 | 2.0 | | |
| 48.5 | 100 | 0 | 0 | 0 | 2.0 | | |
| 48.6 | 0 | 0 | 0 | 100 | 2.0 | | |
| 50.0 | 0 | 0 | 0 | 100 | 2.0 | | |
| 50.9 | 0 | 0 | 0 | 100 | 2.0 | | |
| 51.0 | 0 | 100 | 0 | 0 | 0 | | |
| 52.0 | 0 | 100 | 0 | 0 | 0 | | |

A: preparation solvent A (methanol); B: preparation solvent B (0.5% aqueous ammonium acetate solution); C: preparation solvent C (acetonitrile); D: preparation solvent D (2% aqueous ammonium acetate solution).

### 2.6. Chromatographic system

The HPLC system consisted of an HPLC pump P4000 (Thermoquest, Darmstadt, Germany) connected to a system controller SN4000 including a PC-interface and Chrom Quest-software, as a controller and integrator. A UV6000LP diode array detector was also part of the analytic equipment (all from Thermoquest, Darmstadt, Germany). The eluents were degassed using a SCM1000 (Thermoquest, Darmstadt, Germany) prior to mixing, then passed through an in-line filter (1–2 μm) (Knauer, Berlin, Germany) before reaching the analytical column (120 mm × 4 mm i.d. with Spherisorb ODS2, 3 μm) (Bischoff Chromatography, Leonberg, Germany) embedded in a column thermostat jetstream 8 plus (Knauer AG, Berlin, Germany). A multilinear gradient was formed from solvent A (methanol) and solvent B (60 mM aqueous ammonium acetate solution:methanol (1:1, v/v)). The gradient consisted of the following steps: 0.0 min 90% B, 8.0 min 45% B, 17.0 min 1% B, 41.0 min 1% B and 41.5 min 90% B. The flow rate was adjusted at 0.7 ml/min and the column was heated at 60 °C comparable to Tzimas et al. [22]. The detection wavelengths adjusted at the Chrom Quest-software for detection and integration were 326 nm for retinol and retinyl palmitate and 354 nm for retinoic acids and oxo-retinoic acids. The UV spectra of the several retinoids could additionally be qualified by determination of UV spectra from the diode array UV detector UV6000LP via the Chrom Quest-software.

### 2.7. Standard solutions

Stock solutions of the seven retinoids were prepared by dissolving 10 mg of each substance in 100 ml ethanol, to give a final concentration of 100 μg/ml. All stock solutions were stored in darkness at −20 °C.

### 2.8. Calibration

The reference retinoids used in the assay validation were all prepared by spiking a lipid-mixture I solution (as a non-Vitamin A containing standard for biological fluids), human serum, rat kidney extract and mouse liver extract with ethanolic solutions of the retinoids (all-*trans*-4-oxo-retinoic acid, 13-*cis*-4-oxo-retinoic acid, 13-*cis*-retinoic acid, 9-*cis*-retinoic acid, all-*trans*-retinoic acid, retinol and retinyl palmitate) with different concentrations of the retinoids. Ethanolic solutions were directly injected into the manual injection port when the extraction cartridge is incorporated into the analytical cycle. The multilinear calibration was carried out by measuring the lipid-mixture standards of six different concentrations (5, 10, 50, 100, 500, 1000 ng/ml) of the seven retinoids in the spiked validation mixtures.

## 3. Results

### 3.1. Recovery

Recovery was determined by comparing the peak areas obtained from the different retinoids which have been spiked to the lipid-mixture samples (LMS) ($n = 5$) with those of direct manual injection ($n = 5$) of three different concentrations (10, 100, 1000 ng/ml) and from spiked serum, kidney ($n = 3$) and liver extract ($n = 3$) at 100 ng/ml. The recoveries of retinoids spiked to samples were >72% LMS, >81%

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

| Retinoid | Recovery (%) | | |
|---|---|---|---|
| | 10 ng/ml (<±7)[a] | 100 ng/ml (<±4)[a] | 1000 ng/ml (<±5)[a] |
| | 10 ng/ml LMS ($n = 5$) | 100 ng/ml LMS ($n = 5$) | 1000 ng/ml LMS ($n = 5$) |
| AT4OXORA | 74 ± 4 | 81 ± 5 | 85 ± 4 |
| 13C4OXORA | 79 ± 8 | 97 ± 5 | 102 ± 4 |
| 13CRA | 76 ± 4 | 75 ± 2 | 81 ± 5 |
| 9CRA | 80 ± 8 | 76 ± 2 | 81 ± 5 |
| ATRA | 77 ± 5 | 74 ± 3 | 81 ± 4 |
| ATROL | 81 ± 6 | 72 ± 2 | 72 ± 5 |
| RETPAL | 95 ± 8 | 95 ± 2 | 98 ± 3 |
| | 100 ng/ml in | | |
| | Plasma ($n = 5$) | Kidney extract ($n = 3$) | Liver extract ($n = 3$) |
| AT4OXORA | 85 ± 1 | 81 ± 3 | 87 ± 2 |
| 13C4OXORA | 108 ± 1 | 100 ± 4 | 89 ± 5 |
| 13CRA | 83 ± 4 | 87 ± 4 | 93 ± 1 |
| 9CRA | 81 ± 2 | 86 ± 4 | 96 ± 1 |
| ATRA | 95 ± 2 | 83 ± 5 | 95 ± 2 |

Analysis of standard samples with 10, 100, 1000 ng/ml of the respective retinoids. Values given are based on the mean peak area values of five standard samples and five direct injections. AT4OXORA: all-*trans*-4-oxo-retinoic acid; 13C4OXORA: 13-*cis*-4-oxo-retinoic acid; 13CRA: 13-*cis*-retinoic acid; 9CRA: 9-*cis*-retinoic acid; ATRA: all-*trans*-retinoic acid; ROL: retinol; RETPAL: retinyl palmitate; LMS: lipid-mixture sample.
[a] Direct injection ($n = 6$).

serum, >81% rat kidney extract and >87% rat liver extract (Table 3) for all determined retinoids.

### 3.2. Intra-day and inter-day variation

Intra-day variation (relative standard deviation, R.S.D.) was <8.3% ($n = 5$), and inter-day variation of three consecutive days <11.7% ($n = 3$) for all determined retinoids in LMS standards (Table 4).

### 3.3. Limit of detection

As little as 1.2 ng/ml of all retinoids could be quantified in LMS standard using a sample weight of 125 μl or mg, whereas 94 μl or mg were used after liquid extraction. Retinol and retinyl palmitate were detected at 326 nm and the other retinoids at 354 nm.

### 3.4. Linearity

Examination of linearity based on LMS standards with 5/10/50/100/500/1000 ng/ml yielded a coefficient of regression >0.999 for all seven retinoids.

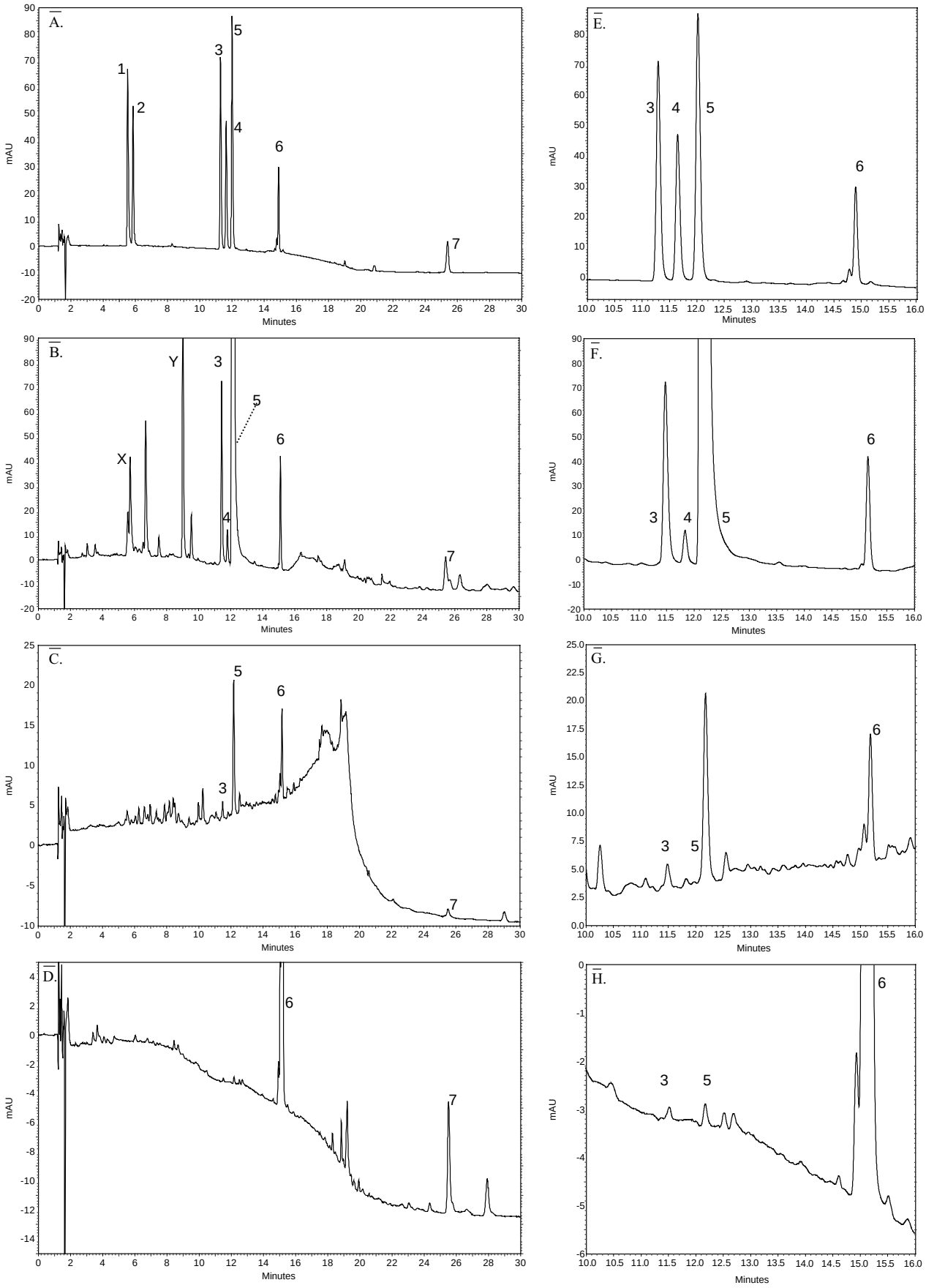
### 3.5. Application

Fig. 3A and E shows a typical chromatogram of a LMS standard solution of all determined retinoids; Fig. 3B and F shows a typical chromatogram of a mouse kidney 4 h after oral treatment of a NMRI mice with 10 mg all-*trans*-retinoic acid/kg body weight; Fig. 3C and G shows a typical chromatogram of endogenous retinoids in pooled embryos of gestational day 12.5 and Fig. 3D and H a typical chromatogram of endogenous retinoids in rat plasma.

Table 4
Intra-day and inter-day variations expressed as relative standard deviations

| Retinoid | Intra-day variation (%) ($n = 5$) | | | Inter-day variation (%) ($n = 3$) |
|---|---|---|---|---|
| | 10 ng/ml LMS-standard | 100 ng/ml LMS-standard | 1000 ng/ml LMS-standard | 100 ng/ml LMS-standard |
| AT4OXORA | 3.9 | 5.0 | 4.0 | 2.6 |
| l3C4OXORA | 8.3 | 4.9 | 4.3 | 3.0 |
| 13CRA | 4.0 | 2.3 | 4.7 | 11.3 |
| 9CRA | 8.2 | 2.2 | 4.6 | 10.5 |
| ATRA | 5.3 | 2.5 | 4.2 | 11.7 |
| ATROL | 5.9 | 2.2 | 4.8 | 8.7 |
| RETPAL | 7.5 | 2.4 | 3.1 | 3.9 |

AT4OXORA: all-*trans*-4-oxo-retinoic acid; 13C4OXORA: 13-*cis*-4-oxo-retinoic acid; 13CRA: 13-*cis*-retinoic acid; 9CRA: 9-*cis*-retinoic acid; ATRA: all-*trans*-retinoic acid; ROL: retinol; RETPAL: retinyl palmitate; LMS: lipid-mixture sample.


A.
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2
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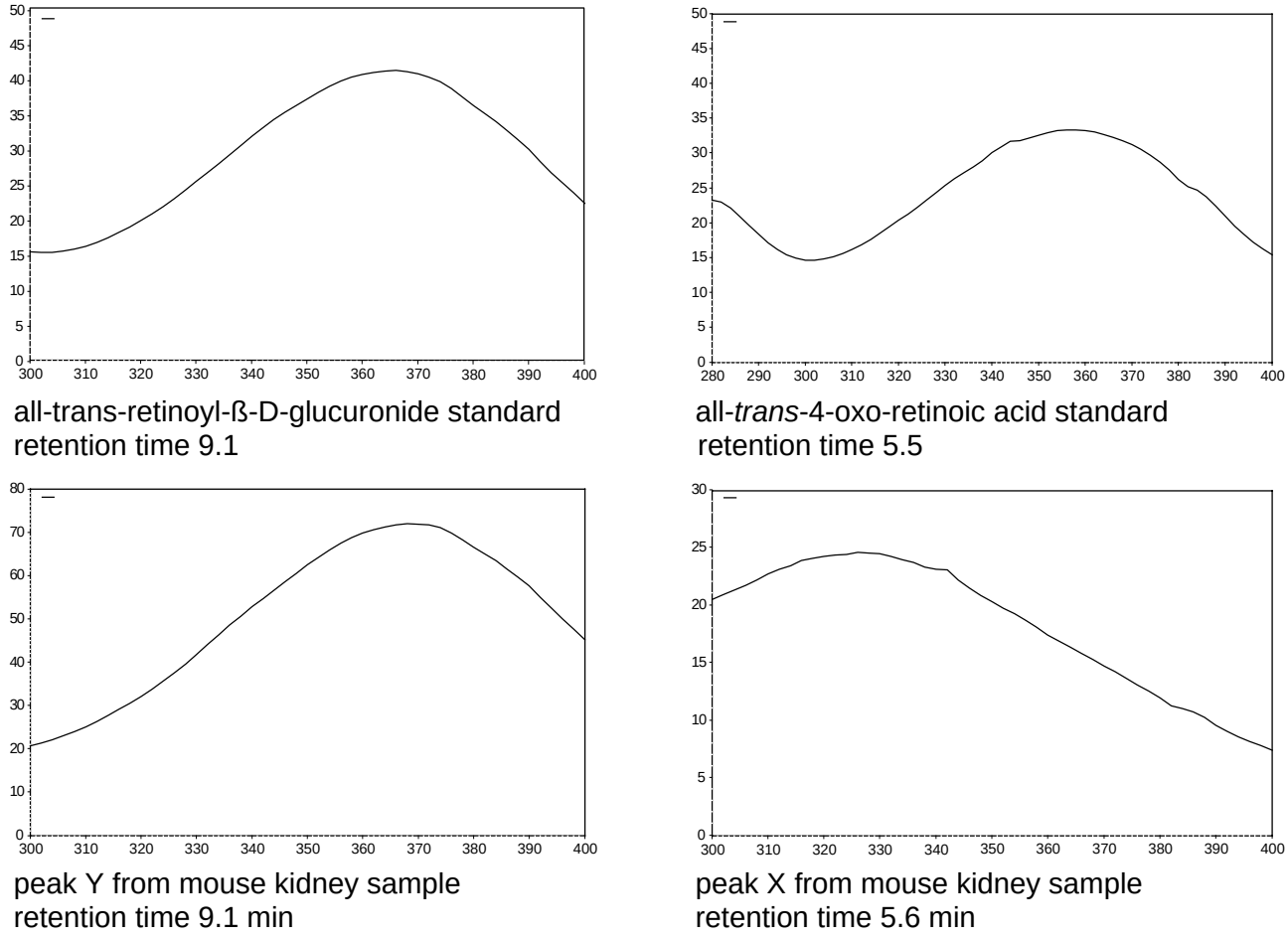


Fig. 4. Comparison of the UV spectra of peaks X and Y from mouse kidney 4 h after oral treatment with 10 mg all-*trans*-retinoic acid/kg body weight (Fig. 3B) in comparison to UV spectra of reference compounds.

Retinoid metabolites like all-*trans*-retinoyl-β-D-glucuronide (ATRAG) in mouse kidney extracts from all-*trans*-retinoic acid treated animals could be identified by comparison of retention time (9.1 min for standard ATRAG and peak Y) and UV spectra with comparable $UV_{max}$ of 366 nm for the standard and 367 nm for peak Y (Fig. 4). Additionally it could be ensured that peak X (retention time 5.6 min) nearly coeluting with the reference compound all-*trans*-4-oxo-retinoic acid (AT4OXORA; retention time 5.5 min) is not by mistake calculated and identified ($UV_{max}$ of AT4OXORA: 356 nm; peak X: 326 nm) as AT4OXORA (Fig. 4).

## 4. Discussion

This method enables quantitative determination of polar and unpolar retinoids (Fig. 1) in plasma and tissues in a single-run HPLC separation. This method is automated to a maximum extent. Especially the solid-phase extractions is totally automated including an AS3000 autosampler and L-7100A intelligent pump for highly automated sample preparation and solid-phase extraction and immediately following reversed phase HPLC analysis. Sample pre-treatment step is kept simple by diluting the sample or sample homogenate with a three-fold volume of isopropanol,

Fig. 3. Typical HPLC chromatograms of: (A and E) LMS standard mixture sample of all-*trans*-4-oxo-retinoic acid (1), 13-*cis*-4-oxo-retinoic acid (2), 13-*cis*-retinoic acid (3), 9-*cis*-retinoic acid (4), all-*trans*-retinoic acid (5), retinol (6) and retinyl palmitate (7). (B and F) Mouse kidney sample 4 h after oral treatment with 10 mg all-*trans*-retinoic acid/kg body weight [20]. (C and G) Mouse embryo sample of gestational day 12.5. (D and H) Rat plasma sample. All chromatograms were detected at 354 nm. Chromatograms at the left panel (A–D) show entire chromatogram in a range between 0 and 30 min and chromatograms at the right panel (E–H) a range between 10 and 16 min of the chromatogram for improved determination of retinoic acids and retinol with different kinds of magnifications, indicated by the area units under full scale (mAU).

centrifugation and incorporation of the sample extract to the autosampler. The automation ensures that all samples are processed in the same way and that time-consuming and highly laborious sample preparation can be reduced to a minimum. The sample preparation is comparable to [17,21], but this method is more automated and could be applied on commercial available technical equipment, whereas the used AASP (automatic sample processor, Varian) and AASP cartridges (ICT, Frankfurt, Germany) for the methods [17,21,23] are not commercially available any more for years making it necessary to use more automated extraction systems which are commercially available by various producers.

The assay quantification limit was 1.2 ng/ml (ng/g) for all retinoids using a sample weight of 125 μl or mg, whereas 94 μl or mg was used after liquid extraction. The low quantification limit is of particular importance due to low endogenous concentrations of retinoic acids in the organism [16,18,24,25]. The quantification limit is comparable or slightly better than other methods, based on calculations using 125/94 μl or mg of sample amount [10–14], whereas methods using electrochemical detection [18] or narrow bore columns were more sensitive [19,25]. The method has been determined to be linear in the range of 5–1000 ng/ml for all retinoids mentioned.

Another advantage of the described method is the use of a sensitive diode array detector (UV6000LP) in comparison to mono or dual wavelength UV detectors [9–19,22,23] or electrochemical detectors [18] which enables high selectivity for identification of reference retinoids in biological samples. In our experiments retinoids coeluting with reference compounds like all-*trans*-4-oxo-retinoic acid and all-*trans*-retinoyl-β-D-glucuronide could easily positively and/or negatively be identified by comparison of UV spectrum (Fig. 3).

A drawback of the method is that the endogenous retinoid 9,13-di-*cis*-retinoic acid [26] an in vivo metabolite of Vitamin A [22,27] and isomerisation product of 9-*cis*-retinoic acid [28] could not be separated in this gradient system from 13-*cis*-retinoic acid. Different gradient systems described by Sass and Nau [29], Wyss and Bucheli [13] and Horst et al. [26] have been shown to be appropriate for 13-*cis*- and 9,13-di-*cis*-retinoic acid separation, but without determination and quantification of retinol and/or retinyl palmitate.

High sensitivity, a maximum degree of automation, simple and quick sample preparation, high repeatability and high selectivity makes this method convenient and reliable for the use in animal and human studies for the determination, quantification and identification of endogenous retinoids as well as several retinoids after nutritional supplementation or pharmacological/toxicological treatment with retinoids.

## Acknowledgements

The authors thank Prof. M. Elmazar (Helwan University, Cairo, Egypt), Prof. H. Nau (Department of Food Toxicology, School of Veterinary Medicine Hannover, Germany) and Elke Thom (Max Rubner Labor, Deutsches Institut für Ernährungsforschung) for help with the animal experiments.

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