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Quantitative determination of endogenous retinoids in mouse embryos by high-performance liquid chromatography with on-line solid-phase extraction, column switching and electrochemical detection

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Abstract

An isocratic high-performance liquid chromatographic method for the determination of 9-*cis*-retinoic acid, 13-*cis*-retinoic acid, all-*trans*-retinoic acid and all-*trans*-retinol in mouse embryos using on-line solid-phase extraction and column switching in combination with electrochemical detection has been developed. The method was validated using retinoids in albumin solutions and 13-*cis*-acitretin was used as internal standard. About 370 μl of albumin solution was injected on a 10×2.1-mm I.D. pre-column packed with Bondapak C_{18}, 37–53-μm particles. The proteins were washed to waste within 5 min using as mobile phase, a 1:3 dilution of mobile phase 2, which consisted of acetonitrile–methanol–2% ammonium acetate–glacial acetic acid (79:2:16:3, v/v). Components retained on the pre-column were back-flushed to and separated on the 250×4.6-mm I.D. Suplex pKb-100 analytical column using mobile phase 2. The retinoids were detected electrochemically at +750 mV using a coulometric electrochemical detector. The total analysis time was about 20 min. Recoveries were in the range of 86–103%. The mass limits of detection were about 10 pg and 25 pg for the retinoic acids and all-*trans*-retinol, respectively. The intra-assay precision, reported as relative standard deviation, was in general better than 4% (n=6) for the four retinoids. Inter-assay precision was in the range 3–4% (n=10). The method was applied for determination of endogenous retinoids in 9.5 day-old mouse embryos. A 340-μl solution containing 100 μl of embryo homogenate (1.64 embryos) was analyzed. The concentrations of all-*trans*-retinol and all-*trans*-retinoic acid were found to be 279 pg per embryo and 75.8 pg per embryo, respectively. The amount of 13-*cis*-retinoic acid and 9-*cis*-retinoic acid was below the detection limit.

Keywords: Column switching; Retinoids; Retinoic acids

1. Introduction

Retinoids are involved in the regulation of proliferation and differentiation of many cell types during fetal development as well as for most cell types throughout life. These effects are known to be mediated by specific nuclear transcription factors such as the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs). The activation of the RARs and RXRs are dependent upon binding of their ligand. Ligands for RARs are all-*trans*-retinoic acid (all-*trans*-RA), 9-*cis*-RA, 3,4-didehydro-RA and 4-oxo-RA as well as 4-oxo-retinol. The RXRs are, however, only activated by 9-*cis*-RA. It is not known

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whether retinoic acid response elements (RAREs) are activated in vivo due to an increase in the concentration of the ligands or a change in the concentration of receptors and/or other interacting factors.

Existing quantification methods for retinoids require large amounts of tissue making the determination of retinoid content in pre-implantation embryos a most challenging task. Endogenous levels of retinoids in mouse embryos have so far only been determined using high-performance liquid chromatography (HPLC) from day 9.0 of development [1]. Ang et al. [2] detected biological activity resembling all-*trans*-retinoic acid in mice at day 7.5 (E7.5) of development by using a tissue explant bioassay, but could not detect any retinoids at day 6.5 (E6.5). Similar results were found by Rossant et al. with a *lac*Z transgene linked to a retinoic acid response element [3]. The simultaneous appearance of several enzymes, believed to be capable of converting retinol, retinal or retinyl esters to retinoic acid have suggested that retinoic acid may not be important prior to gastrulation (day 7.5). Nevertheless, the receptors RAR and RXR are present at a much earlier stage [4], and we speculate that their ligands might be present at concentrations too low to be detected with existing methods.

To test this hypothesis we needed an extremely sensitive method capable of separating the different geometrical isomers of retinoic acid. Many procedures have been devised for determining the quantities of naturally occurring and synthetic retinoids in biological samples. The majority of these involve reversed-phase HPLC (RP–HPLC). Retinoids are a class of highly unstable compounds. To preserve the geometric orientations of the retinoids, severe precautions must be taken to protect the sample from light, heat and air exposure during sample work-up and analysis. In this regard extraction conditions as mild as possible are of utmost importance. An elegant and time-saving approach to these problems is the use of on-line solid-phase extraction and column switching [5,6]. The technique allows trace enrichment and clean-up in a single step. Minimal loss due to light-induced isomerisation, air exposure and adsorption to glassware during pretreatment is also achieved. This makes the technique especially suited for retinoids because of their photo- and thermo-sensitive nature. The detection technique traditionally used is UV detection, showing good selectivity and high sensitivity for retinoids.

In a narrow bore column-switching system with UV detection, Gundersen and Blomhoff were able to detect 60 pg (200 fmol) of all-*trans*-retinoic acid [7]. However, in order to determine retinoic acid in the earlier stages of embryonic development even lower detection limits will be needed. Several approaches have been done with HPLC–mass spectrometry. A limit of detection (LOD) down to 1 pg (3 fmol) of the pentafluorobenzyl derivatives of all-*trans* and 13-*cis*-retinoic acids and their 4-oxo metabolites in human plasma using microcolumns (0.32 mm I.D.) is described [8]. The sample throughput was however, very low and the limit of quantification was as high as 300 pg/ml. Laser-induced fluorescence (LIF) detection is feasible for retinol detection, but not for retinoic acid unless reacted with a fluorescent tag. LIF provides the lowest LOD obtained for retinol (32 amol) [9] when in complex with retinol-binding protein or cellular retinol-binding protein, but a similar procedure for retinoic acid has not been reported. Electrochemical detection of retinoids is possible and a 82-pg detection limit (269 fmol) of retinol and its metabolites has been reported [10,11] Recently, a capillary liquid chromatographic system with a specially designed amperiometric electrochemical detector was published [10]. The authors claim to have obtained detection limits of 472 amol and 267 amol using standard solutions of all-*trans*-retinoic acid and retinol, respectively. The method was not validated nor applied for routine analysis or applied for determination of retinoids in embryonic tissue.

In this paper, we present an isocratic RP–HPLC method with on-line solid-phase extraction (SPE) and column switching combined with coulometric electrochemical detection for the quantitative determination of all-*trans*-retinoic acid, 13-*cis*-retinoic acid, 9-*cis*-retinoic acid and all-*trans*-retinol (Fig. 1) in embryonic tissue. As the amount of embryonic tissue is very limited, a method allowing the whole sample to be analyzed and providing a low mass limit of detection (mLOD) was sought. The column-switching system allows large volumes to be injected and very mild sample work up with no need for evaporation of solvents. The method allows for

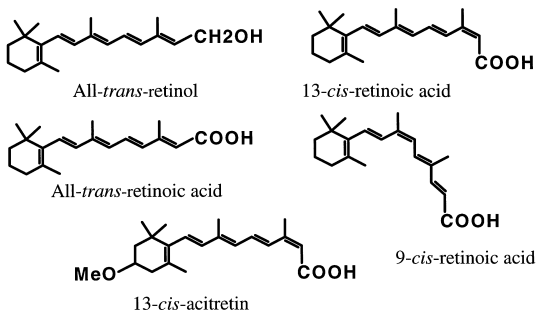

Fig. 1. Chemical structure of the retinoids and the internal standard 13-*cis*-acitretin.

routine determination of as low as 7.7 pg (25 fmol) all-*trans*-retinoic acid in tissue from mouse embryos. This reduction in LOD is crucial to limit the number of embryos required to be collected at the earliest stages of development. The method was successfully applied for the determination of endogenous amounts of retinoids in 9.5 day-old embryos. The results from retinoid analysis in earlier stages will be published elsewhere.

2. Experimental

2.1. Materials and reagents

Acetonitrile (HPLC grade) was obtained from E. Merck (Darmstadt, Germany) or Rathburn (Walkerburn, UK). Methanol (analytical-reagent grade), glacial acetic acid (analytical-reagent grade) were also obtained from E. Merck. Ammonium acetate was obtained from E. Merck or Fischer Scientific (UK). Ethanol, 96% was obtained from Arcus (Oslo, Norway). The water used was Milli-Q water with resistivity 18.2 MΩ cm. All-*trans*-retinol, 13-*cis*-retinoic acid, and all-*trans*-retinoic acid were supplied by Sigma (St. Louis, MO, USA). F. Hoffman–La Roche (Basle, Switzerland) kindly provided the 9-*cis* isomer of retinoic acid and acitretin. Albumin from bovine (fatty acid free) was supplied by Sigma. Helium and argon both 99.998%, were obtained from Hydro Gas (Oslo, Norway). Supelco (Bellefonte, PA, USA) supplied Amber vials. Upchurch Scientific supplied the polyether ether ketone (PEEK) tubing used to make all the connections. The pellet grinder was obtained from Kontes Scientific (Vineland, NJ, USA). All standards were kept under argon at −18°C, and protected from light at all times.

2.2. Preparation of standard solutions

Stock solutions of the retinoids were prepared, under red light, by dissolving 1 mg in 5 ml of 96% ethanol. The concentrations were also determined spectrophotometrically using literature ϵ values [6]. All the three retinoic acids were combined and further diluted to give a stock solution of 0.2 ng of each retinoic acid per μl of 96% ethanol. All-*trans*-retinol was separately diluted with 96% ethanol to 0.4 ng/μl. These solutions were combined and further diluted with 5.2 m*M* ammonium acetate in acetonitrile–water (pH 4) (45:55) yielding concentrations 1.50, 6.52, 9.00, 11.6, 18.0 pg of each retinoic acid and 1.47, 6.37, 11.5, 16.7, 23.9 pg of all-*trans*-retinol per μl solution. These standard solutions were used to determine the linearity of the detector using the direct injection chromatographic system. For validation of the method the stock solutions of retinoic acids (0.2 ng of each retinoic acid/μl) and all-*trans*-retinol (0.4 ng/μl) were combined and diluted further with 0.1% albumin solution. Replicates of five different concentrations [0.40 (n=6), 1.32 (n=1), 1.80 (n=6), 2.32 (n=1), 3.30 (n=6) pg/μl)] for the three retinoic acids and [0.398 (n=6), 1.59 (n=1), 2.39 (n=6), 3.58 (n=1), 4.38 (n=6) pg/μl)] for the all-*trans*-retinol were prepared and analyzed in the manner described below. Final ethanol concentration was below 1%. These solutions were also used for quantification of retinoids in embryo analysis. All solutions were flushed with argon and frozen in amber glass containers at −18°C.

A stock solution of acitretin was prepared in 96% ethanol and was irradiated with UV light at 350 nm for 1 min. The solution was injected on a direct injection HPLC system connected to a UV detector. The fraction containing 13-*cis*-acitretin was collected in amber vials and flushed with argon. The concentration was determined spectrophotometrically and was 0.234 ng/μl.

2.3. Embryo

Timed mated F1 hybrid mice (C57 female×CBA male) were killed by cervical dislocation at day 9.5 of gestation. The embryos were dissected into ice-cold 0.9% NaCl. A Kodak filter No. 25 was used to avoid exposure of UV light during microscopy. They were staged according to Theiler [12]. All embryonic tissues were stored at −70°C protected against light. The mice were 43 days old and were fed with ordinary pellet diet (Arex, Mollesentralen, Oslo, Norway).

2.4. Chromatographic system

The on-line SPE and column-switching system was used with the configuration shown in Fig. 2.

A Waters pump M-590 (Milford, MA, USA) was used to deliver mobile phase 1 to the pre-column at the flow-rate of 2.4 ml/min. A second HPLC pump, Rheos 4000 (Flux Instruments, Sweden) was used to deliver mobile phase 2 to the analytical column at a flow-rate of 1.2 ml/min. The detection was done by a coulometric detector, Coulochem 2 (ESA, Chelmsford, MA, USA) equipped with an analytical cell Model 5010 and a guard cell model 5020. The guard cell was placed between the pump 2 and the switch valve, while the analytical cell was placed after the analytical column. The guard cell was set at a potential of +750 mV. The analytical cell consisted of two cells in series, the screening cell which was set at a potential of +450 mV and the detection cell which was set at the potential of +750 mV. A 2-μm graphite interchangeable filter housed in a PEEK assembly was located in front of both the guard cell and the analytical cell. An Orlita pulse dampener (Giessen, Germany) was placed before the guard cell filter. The set of valves needed for the column-switching system were the following: one PEEK Rheodyne injector 9125 equipped with either a 20-μl PEEK loop (for direct injection) or a 2-ml titanium loop (for on-line SPE and column switching) and one steel Rheodyne 7000 flow selection valve. A Waters LC spectrophotometer M-481 was used for UV detection during the optimization of the clean-up step. All sample-contacting parts, including tubing, were made of PEEK or titanium.

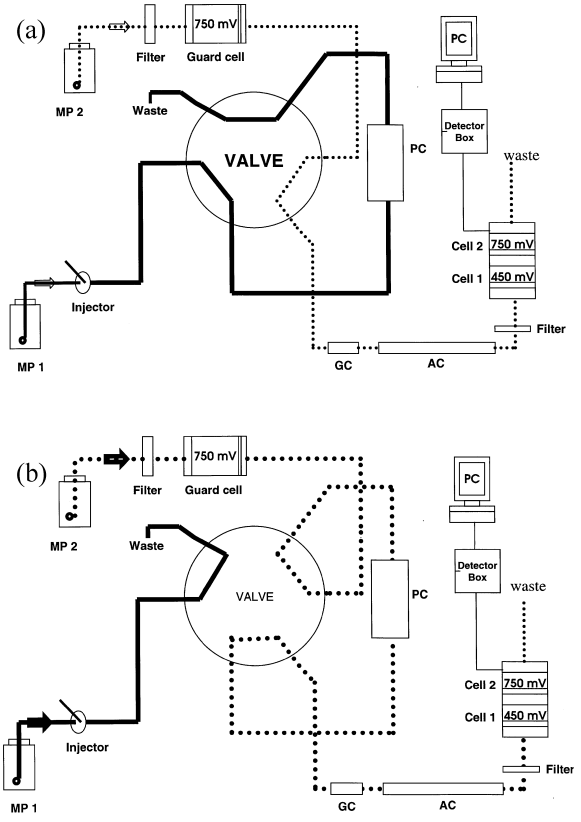

Fig. 2. Schematic representation of the final column-switching system. (A) Forward-flush position. (B) Back-flush position. See Section 2.4 for detailed description.

A HP ChemStation, HPLC software, did the collection and manipulation of the data (Hewlett–Packard).

2.5. Columns and mobile phases

The analytical column (AC) was a 250×4.6 mm I.D. Suplex pKb-100 (5 μm) from Supelco, with a 20×4.6-mm Pelliguard C_8 guard column (GC) (Supelco) in an integrated guard column housing. The pre-column (PC), a 10×2.1-mm I.D. PEEK cartridge with a steel body and 2-μm PAT frits (Jour Research, Onsala, Sweden), was dry packed with Bondapak dimethyl-octadecylsilyl bulk packing material, 37–53 μm, 300 Å (Waters, Millipore Corp.). Mobile phase 1 (M1) consisted of mobile phase 2 (M2)–water (1:3, v/v) and mobile phase 2 of acetonitrile–methanol–2% ammonium acetate–glacial acetic acid, (79:2:16:3, v/v). The 2% ammonium

acetate solution was made by dissolving 20 g in 1000 ml Milli-Q water and filtering it through a 0.22-μm Duropore filter (Millipore).

2.6. Voltammogram

A 5-μl direct injection chromatographic system was used for generating the voltammograms. The standard solution containing 55 pg/μl of all the three retinoic acids was injected at different potentials ranging from 550 mV to 950 mV and the response was recorded from the detector. At each potential, the background current was recorded as well.

2.7. Analytical procedure

2.7.1. Preparation of standard solutions

A 200-μl aliquot of the retinoids in albumin solution was transferred to a 4-ml amber vial and 10.0 μl of the internal standard solution and 20 μl of ammonium acetate buffer (0.25 *M*, pH 4.0) were added. After mixing, 330 μl of acetonitrile was added and the sample was vortex mixed. The mixture was centrifuged for 5 min at 3000×*g* and 4°C. A volume of 280 μl of the clear supernatant was transferred to an amber vial and 90 μl of water was added and mixed. The resulting mixture (370 μl, corresponding to 100 μl of albumin solution) was analyzed.

2.7.2. Preparation of the sample

The whole embryos in ammonium acetate buffer (0.25 *M*, pH 4.0) were homogenized with a motorized pellet grinder. A 200-μl aliquot of embryo homogenate was transferred to a 4-ml amber vial and 10.0 μl of the internal standard solution was added and mixed. After mixing, 300 μl of acetonitrile was added and the sample was vortex mixed. The mixture was centrifuged for 5 min at 3000×*g* and 4°C. A volume of 255 μl of the clear supernatant was transferred to an amber vial and 85 μl of water was added and mixed. The resulting mixture (340 μl, corresponding to 100 μl of embryo homogenate) was analyzed.

2.7.3. HPLC analysis

A 370-μl albumin solution (or 340-μl embryo homogenate solution) was injected on to the pre-column with a flow-rate of 2.4 ml/min. After 5 min, the valve was switched from forward-flush position to back-flush position. At this point the mobile phase 2, at a flow-rate of 1.2 ml/min, purged the pre-column and the components retained on the pre-column were transferred to the analytical column for separation. After 1 min, the valve was again switched to the forward-flush position and mobile phase 1 re-equilibrated the pre-column for the next injection.

Detection of the retinoids was done by the coulometric detector at a potential of +750 mV.

2.8. Recovery

Recoveries of the retinoids were examined by spiking 0.1% albumin solution with known amounts of the different retinoid isomers and comparing the resulting peak area with the peak areas obtained by injecting the same amount of retinoids, dissolved in 5.2 m*M* ammonium acetate in acetonitrile–water (45:55) using the direct injection system.

2.9. Quantitative determinations

For the quantification of retinoids in the embryo, the validation standard solutions in 0.1% albumin were used to establish the calibration curve. The ratios of the peak area of the retinoids and the internal standard were plotted against the concentration of the retinoids. The concentration of the analytes were determined by interpolation obtained by linear least-squares regression.

3. Results and discussion

3.1. Electrochemical detection of retinoids

Electrochemical detection generally provides lower detection limits for electroactive compounds as compared to UV detection. Hence the combination of column switching and electrochemical detection was expected to give detection limits below that reported earlier [4,5,7]. A direct injection of biological samples on this type of detector will give a very high background making the determination of early eluting compounds impossible.

In preliminary experiments, different commercially available electrochemical detectors were examined for detection of the retinoids. The coulometric detector used in this work was found to provide the lowest detection limits while also providing a stable baseline. Voltammograms of the retinoids using this detector is presented in Fig. 3. The lowest detection limits were obtained using +750 mV relative to the reference electrode for detection, while keeping the screening cell at +450 mV. A standard analytical column and analytical cell was used in these experiments. The combination of microbore column and microbore cell did not give lower mass limit of detection limits. Therefore, the standard analytical system, which was more robust, was used in further experiments.

3.2. Electrochemical detection and column switching

When replacing the UV detector in the system described earlier [6] with the electrochemical detector, several problems arose. Firstly, when back-flushing, a large front appeared in the chromatogram and this precluded the detection of the retinoids. Secondly, the baseline drift was severe. The problems were probably caused by small differences in pH, ion strength and dielectric constant of the mobile phases 1 and 2. The problems were eliminated by using as mobile phase 1, a dilution (with water) of mobile phase 2. It was important that mobile phase 1 was prepared of the same batch of mobile phase 2 as the one used at the time. A chromatogram showing the separation and electrochemical detection of retinoids using the on-line SPE column-switching system is shown in Fig. 4. The pKb-100 column provides isocratic separation of retinoic acids and all-*trans*-retinol [6]. When using UV detection, a mobile phase consisting of acetonitrile-1-butanol–methanol-2% ammonium acetate–glacial acetic acid (69:2:10:16:3, v/v) was used. However, when electrochemical detection was performed, it was found beneficial to replace the alcohols with acetonitrile to achieve a low background current. Some methanol was necessary to achieve sufficient selectivity. The optimal composition of the mobile phase (M2) was acetonitrile–methanol–2% ammonium acetate–glacial acetic acid (79:2:16:3, v/v). Using this mobile phase, baseline separation of *cis* and *trans*-isomers of retinoic acid and all-*trans*-retinol could be obtained in less than 15 min, resulting in a total analysis time of 20 min using the column-switching system.

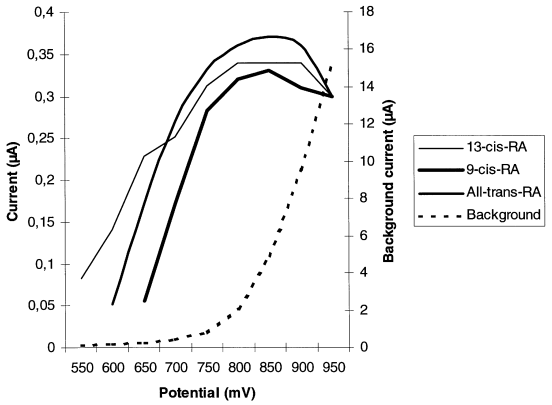

Fig. 3. Background current and voltammograms for 13-*cis*-RA, 9-*cis*-RA and all-*trans*-RA. See Section 2.6 for detailed description.

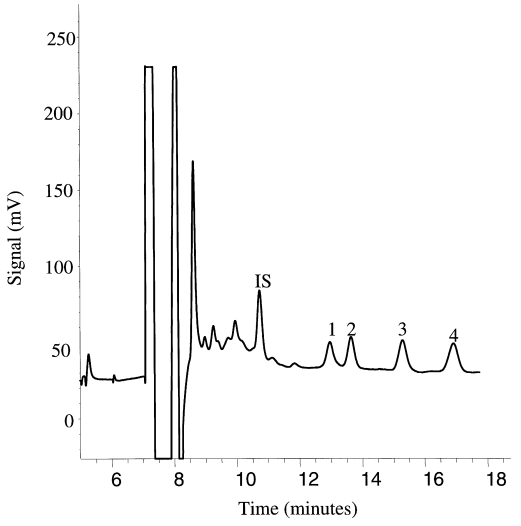

Fig. 4. Chromatogram showing separation, on pKb-100 using mobile phase M2, of the four retinoids: 13-*cis*-RA (1) 330 pg; all-*trans*-ROH (2) 438 pg; 9-*cis*-RA (3) 330 pg; all-*trans*-RA (4) 329 pg; and the internal standard, 13-*cis*-acitretin (I.S.) in a 100-μl albumin solution (370-μl injection volume) injected on the pre-column and back-flushed on to the analytical column. The column temperature was ambient and the detector potential 750 mV.

3.3. Clean-up and pre-concentration

The ideal way of using the on-line SPE column-switching technique is the direct injection of untreated samples onto the pre-column. When performing blood serum analysis, a simple dilution only is necessary before injection. However, tissue homogenates contain particulate. Therefore, after addition of acetonitrile for protein precipitation, the homogenate was centrifuged. The supernatant was diluted with water to an acetonitrile content of 45% before being injected onto the SPE column. It was found that at least 1000 μl of the solution containing 45% acetonitrile could be injected onto the pre-column and washed for at least 20 min at 1.2 ml/min without breakthrough (results not shown). An optimal clean-up time was found by monitoring elution of the residual proteins from the pre-column using an UV detector set at 260 nm (Fig. 5). Most of the proteins were eluted at 5 min, which was chosen as the duration of the clean-up step.

3.4. Choice of internal standard

The internal standard must have electrochemical and chromatographic properties quite similar to the retinoids, while still being separated from the retinoids. The most frequently used internal standard in retinoid determination is acitretin (4-methoxy-2,3,6-trimethylphenyl (TMMP)–retinoic acid) [5]. In this work the possible use of acitretin and 13-*cis*-acitretin as internal standard was explored. Both could be detected electrochemically at the same potential and at about the same sensitivity as the retinoic acids. However, acitretin co-eluted with 13-*cis*-retinoic acid in the chromatographic system used. The retention time of 13-*cis*-acitretin was less than that of the retinoic acids (Fig. 4), but was sufficiently well separated from the front components. Thus, 13-*cis*-acitretin was used as internal standard in this work.

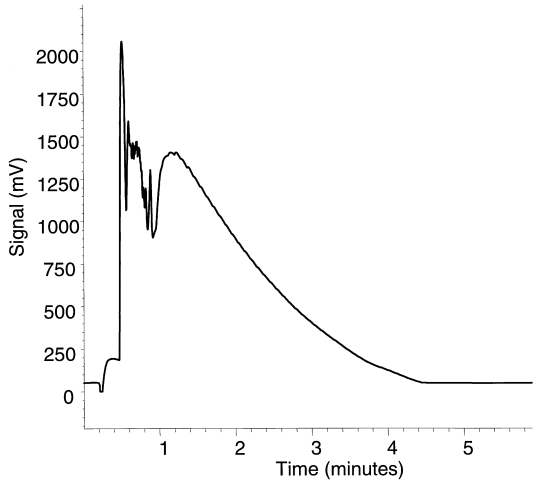

Fig. 5. Chromatogram showing the elution from the pre-column during the clean-up monitored with UV detection at 260 nm.

3.5. Validation

Due to the low expected concentration of the retinoids in the embryonic tissue samples, it was most important that the method could quantify low concentrations. Higher concentrations can more easily be determined using UV detection. Hence the method was validated for low concentrations only. To minimize the loss due to adsorption to the vial, it is preferable that the retinoids are protein bound. This will give a more accurate estimation of recovery. Albumin is known to bind retinoic acid, therefore we chose to perform the validation studies using 0.1% albumin solutions.

3.5.1. Linearity of the detector

The detector response was examined in the range of 30–360 pg for each of the retinoic acids and 30–480 pg for all-*trans*-retinol. The response was found to be linear in the investigated range with correlation factors better than 0.999 for the retinoic acids and better than 0.99 for all-*trans*-retinol.

3.5.2. Linearity of the method

The validation solutions were also used for estimation of the linearity of the method. Peak area ratios (peak area of analytes/peak area of the internal standard) were plotted against concentration. Their correlation was found to be linear in the investigated range at least. The coefficient of correlation (r) was better than 0.990 for all four retinoids (Table 1).

3.5.3. Limit of detection

The limits of detection were found using albumin solutions and the column-switching system and

Table 1
The mass limit of detection, equations for the linear regression lines and the coefficients of correlation for each retinoid where y represents the peak area ratio and x the analyte concentration in pg

Retinoid	Equation	Coefficient of correlation (R)	mLOD (pg) (signal-to-noise=3)
13-*cis*-RA	$y=0.0018x-0.0132$	0.98962	12
All-*trans*-retinol	$y=0.0015x-0.0276$	0.99542	25
9-*cis*-RA	$y=0.0024x-0.0030$	0.99465	10
All-*trans*-RA	$y=0.0029x+0.0021$	0.99557	7.7

calculated using a signal-to-noise ratio of 3. The mass limits of detection are given in Table 1.

3.5.4. Intra-assay and inter-assay precision

The intra-assay precision (within-day repeatability) is shown in Table 2. All experiments were performed by the same analyst and on the same day. The inter-assay precision was estimated by performing the same determination on the same samples every day for 10 days. The results are shown in Table 2.

3.5.5. Recovery

Recovery studies using embryonic samples are not feasible due to the lack of blank sample and the poor availability and high expense. Recovery studies therefore, were performed using 0.1% albumin solutions. The results are shown in Table 2. The recovery was found to be in the range 86–103% for the four retinoids.

3.5.6. Stability and robustness

Stability of standard solutions have been reported elsewhere [13]. The pre-column was changed every one hundred injections or when the baseline drift increased and/or the solvent front became enlarged. The guard column was changed every 300 injections. The PEEK in-line filters were changed when the back-pressure increased by 10 bar or more. Reproducible retention of the retinoids was highly dependent of the pH of the mobile phase. Also, whenever M1 was changed, M2 was changed and vise versa. This was essential to minimize equilibrium changes on the analytical column leading to

Table 2
Validation results

Isomer	Level		Recovery (%)	Precision				
	Added (pg)	Found (pg)		Intra-assay		Inter-assay		
				S.D. (pg) (n=6)	R.S.D. (%)	Found (pg)	S.D. (pg) (n=6)	R.S.D. (%)
1	40.0	40.4	101	0.669	1.7	–	–	–
	180	173	96.1	5.24	3.0	177	6.50	3.7
	330	340	103	10.6	3.1	–	–	–
2	39.8	36.0	90.5	1.26	3.5	–	–	–
	239	205	85.8	5.48	2.7	198	6.32	3.2
	438	415	94.7	4.47	1.1	–	–	–
3	40.0	39.5	98.8	0.654	1.7	–	–	–
	180	162	90.0	10.9	6.7	167	7.25	4.3
	330	326	98.8	6.56	2.0	–	–	–
4	39.8	38.8	97.5	1.57	4.1	–	–	–
	179	176	98.3	7.18	4.1	179	5.17	2.9
	329	328	99.7	8.17	2.5	–	–	–

13-*cis*-Retinoic acid (1), all-*trans*-retinol (2), 9-*cis*-retinoic acid (3), all-*trans*-retinoic acid (4).

artificial peaks. Different batches of chemicals, especially buffer components, gave extensive variations in background current. Chemicals specially designed for electrochemical detection did not show better performance as compared to analytical-reagent grade qualities. It was crucial to use freshly made Milli-Q water or of better quality.

3.6. Application

The method was used for quantification of retinoids in 9.5 day-old mice embryos (Fig. 6). Only two of the retinoids, all-*trans*-retinol and all-*trans*-retinoic acid were present at concentration levels above the detection limit. The amount of all-*trans*-retinol (2) and all-*trans*-retinoic acid (4) was 279 pg per embryo [R.S.D.=1.6% (n=3)] and 75.8 pg per embryo [R.S.D.=3.0% (n=3)], respectively. By using the column-switching system in combination with the electrochemical detector only about 10 mg of embryonic sample was needed per analysis, while about 350 mg was required when UV detection was utilized [1]. Our findings are in agreement with the results obtained with the UV method. This method has also been used for quantification of retinoids in 7.5- and 8.5 day-old mouse embryos. The results of these analyses will be published elsewhere.

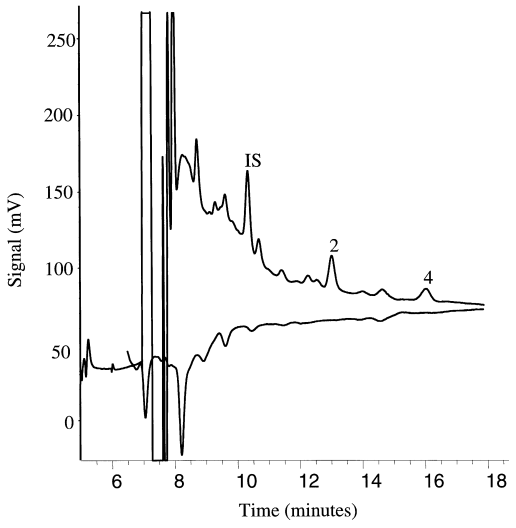

Fig. 6. Chromatograms of a blank albumin sample (lower inverted trace), and embryo homogenate sample (1.64 embryos) showing endogenous retinoids and the internal standard (upper trace). The amount of all-*trans*-retinol (2) and all-*trans*-retinoic acid (4) was 279 pg per embryo and 75.8 pg per embryo, respectively. Separation was accomplished on the 250×4.6-mm pKb-100 column using the column-switching system and ambient column temperature. Detector potential was 750 mV.

4. Conclusion

At optimal conditions, the method which combines electrochemical detection and on-line solid-phase extraction with column switching, was found to be sensitive and reproducible. Extraction, separation and quantification of pg amounts of retinoids in embryonic tissue were achieved in a single step. The method has been successfully applied for the determination of retinoids in 9.5 day-old mice embryos. The measured amount of all-*trans*-retinoic acid and all-*trans*-retinol in 9.5 day-old mouse embryos was 279 pg per embryo and 75.8 pg per embryo, respectively. Samples of 100 μl homogenate (10 mg tissue, 1.64 embryos) were used. The method is however, capable of handling a sample volume three times larger (30 mg tissue). To be able to quantify the retinoid content at much earlier stages, the number of embryos will most likely have to be increased substantially. Since the mass of the embryos at earlier stages is smaller, the maximum applicable volume will not be a limiting factor. Therefore, we believe that this method enables us to determine retinoids at much earlier stages of embryonic development. In further research, we will apply this method for the determination of retinoids in embryos kept in culture to the blastocyst stage. To isolate embryos from the placenta of mice at stages earlier than this is very difficult, and therefore further investigations must be done on embryos kept in culture. This is possible from fertilization to the blastocyst stage.

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