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Isocratic reversed-phase liquid chromatography of all-*trans*-retinoic acid and its major metabolites in new potential supplementary test systems for developmental toxicology

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Abstract

An isocratic reversed-phase high-perfomance liquid chromatographic procedure for the determination of all-*trans*-retinoic acid (all-*trans*-RA) and its metabolites, all-*trans*-4-oxo-RA, 5,6-epoxy-RA, 9-*cis*-RA and 13-*cis*-RA, in mouse plasma and embryo and in new in vitro potential test systems for developmental toxicology has been developed. These compounds, their biological precursor retinol (vitamin A) and the internal standard were resolved on a Spherisorb ODS-2 (5 μm) column (250×4.6 mm I.D.) with acetonitrile–water–methanol–*n*-butyl alcohol (56:37:4:3, v/v) containing 100 m*M* ammonium acetate and 70 m*M* acetic acid as the elution system, with a total run time of 23 min. The assay was linear over a wide range, with a lower limit of quantitation of 50 ng/ml or 10 ng/mg of protein for all-*trans*-RA, 13-*cis*-RA and 9-*cis*-RA and of 25 ng/ml or 5 ng/mg protein for the 4-oxo- and 5,6-epoxy-metabolites. At these concentrations, intra-assay coefficients of variation (C.V.) of the retinoids were 3–9%. Mean intra-assay C.V. averaged 5–7% in the tissues studied. Its use is discussed for RA measurements in some of the new test systems — *Drosophila melanogaster*, sea urchin embryos and cultured human keratinocytes — that have to be evaluated in toxicological testing, supplementary to standard assays in mammals.

Keywords: Retinoic acid; Oxoretinoic acid; Epoxyretinoic acid; Retinol; Vitamins

1. Introduction

Drug measurements are particularly important when new techniques for toxicity testing are to be validated and it becomes essential to compare these results with those of established toxicological assays in mammals. Because of binding to constituents of the medium and uptake or release mechanisms within the cells, it cannot be assumed that the concentration of a probe in the medium of a culture system always corresponds to the biologically active concentration [1]. Differences in drug metabolism in various toxicity assays must also be taken into consideration [2]. As a consequence, the probe and its active metabolite(s) should be measured in the system under study, therefore, precise and reproducible assays are essential.

All-*trans*-retinoic acid (all-*trans*-RA), a metabolite of retinol (vitamin A) and clinically used for the treatment of acute promyelocytic leukemia [3], is a teratogen in man and animals [4,5] and was employed as a probe in a study evaluating cultures of

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eukaryotic cells, gametes and embryos as potential test systems for developmental toxicity [6]. An analytical method is needed to resolve this compound from its various metabolites and other endogenous retinoids (see [7] for review). The usual procedures are generally based on reversed-phase high-performance liquid chromatography (HPLC) with ultraviolet (UV) absorbance detection in gradient and/or column-switching mode [8–11]. A few isocratic HPLC methods have been described, but they are focused exclusively on the detection of all-*trans*-RA, its photoisomer 13-*cis*-RA and their biological precursor, retinol [12], or on the use of normal-phase elution to separate the two RA photoisomers and their 4-oxo- metabolites [13]. Based on available extraction procedures of retinoids from mammalian body fluids [14,15], we developed an isocratic, reversed-phase HPLC method for the simultaneous measurement of all-*trans*-RA and its metabolites, all-*trans*-4-oxo-RA, 5,6-epoxy-RA, 13-*cis*-RA and 9-*cis*-RA (Fig. 1), in mouse plasma and embryo and in some of the new in vitro systems under study — *Drosophila melanogaster* and sea urchin embryos, and in cultured human keratinocytes.

2. Experimental

2.1. Chemicals

All-*trans*-RA and retinol were supplied by Fluka (Milan, Italy) and 13-*cis*-RA by Sigma (Milan, Italy). All-*trans*-4-oxo-RA and its 13-*cis*-isomer, 5,6-epoxy-RA and 9-*cis*-RA were kindly supplied by Hoffman-La Roche (Basel, Switzerland) and the internal standard, 4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-anthracenyl)benzoic acid (I.S.) [16] was supplied by CIRD-Galderma (Valbonne, France).

Stock solutions of all-*trans*-RA, its metabolites, retinol and the I.S. were prepared in amber-coloured volumetric flasks by dissolving the compounds in methanol, each at a concentration of 1 mg/ml. Working standard solutions were prepared from the stock solutions by dilution with methanol.

Acetonitrile (HPLC grade) was obtained from

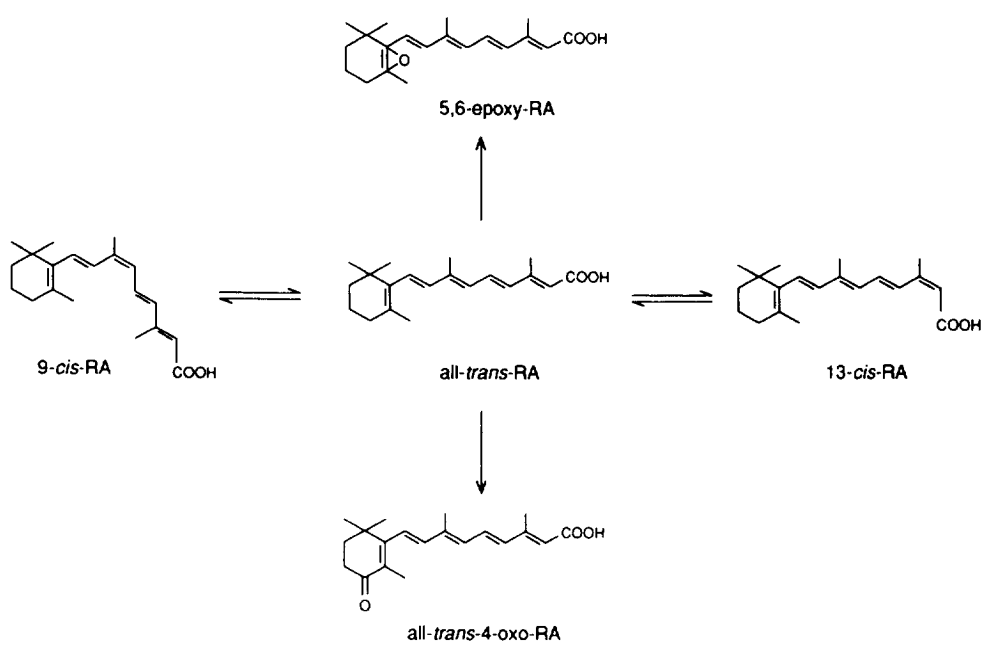

Fig. 1. Chemical structures of the retinoids studied and their metabolic relationship.

Carlo Erba (Milan, Italy). Methanol, ammonium acetate, sodium acetate and acetic acid (Merck, Darmstadt, Germany) and *n*-butyl alcohol (Carlo Erba) were of analytical-reagent grade.

2.2. Biological samples and extraction procedure

Timed-Pregnant Crl:CD1(ICR)BR mice (Charles River, Italy) were used. Procedures involving animals and their care were conducted in accordance with the institutional guidelines that are in compliance with national (D.L. n. 11, G.U., suppl. 40, 18 Febbraio 1992) and international laws and policies (EEC Council Directive 86/609, OJ L 358,1, Dec. 12, 1987; NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985).

On day 11 of gestation, mice were given 10 mg/kg of all-*trans*-RA orally, dissolved in 0.9% NaCl, 3% ethanol, 0.5% Tween 80 and 6.7 m*M* NaOH and were killed 2 h after dosing. Blood samples were collected in tubes and were wrapped in aluminium foil, centrifuged in an Eppendorf centrifuge and the plasma was stored at −80°C until assayed. Embryos were rapidly removed and stored at −80°C.

Drug-free and all-*trans*-RA-cultured (10 μM) sea urchin embryos were kindly supplied by Dip. di Biologia Cellulare e dello Sviluppo "A. Monroy", Palermo, Italy. The technique for cultivation of these embryos has been described [17]. Drug-free and all-*trans*-RA microinjected *Drosophila melanogaster* (150 ng/*Drosophila*) and a pool of their ovaries were kindly provided by the Dip. di Genetica, Biologia Generale and Molecolare, University of Naples, Italy. The experimental conditions have already been described [6]. Human keratinocytes were isolated from a skin biopsy and cultured in the Laboratorio di Differenziamento Cellulare, Centro di Biotecnologie Avanzate, Genova, Italy, as described by Pellegrini et al. [18].

Plasma aliquots (0.2 ml) were used for all-*trans*-RA and for analysis of metabolites. Tissue and cell samples were resuspended in 0.01 *M* phosphate buffered saline, pH 7.4 (0.2 ml/mg protein), homogenized by ultrasonication, and 0.2-ml aliquots were generally used for drug analysis. Sample preparation involved a one-step extraction procedure using direct protein precipitation with acetonitrile. After addition of 10 μl of a methanolic solution of the I.S. (50 μg/l) and shaking with 0.8 ml of acetonitrile for 60 s in a vortex-mixer, samples were centrifuged for 2 min at 12 000 *g* in an Eppendorf centrifuge and the supernatant was evaporated to dryness under a nitrogen stream. The residue was reconstituted with 0.2 ml of the mobile phase, samples were vortex-mixed and injected onto the HPLC system.

2.3. Chromatographic apparatus and conditions

HPLC analysis was performed on a Beckman System Gold (Beckman Instruments, San Ramon, CA, USA), equipped with a programmable solvent module 126, a detector module 166 and a NEC PC-8300 controller system and a Model C-R6A Chromatopac Shimadzu integrator (Shimadzu, Kyoto, Japan). A reversed-phase Techsphere 80/5 ODS-2 (Spherisorb ODS-2) 5 μm column (250×4.6 mm I.D.; theoretical plates 86 778 p/m) was used, fitted with a Techsphere 5 ODS pre-column (10×3 mm I.D.) (Analytical Technology, Macclesfield, UK), maintained at room temperature. The mobile phases were filtered through a 0.45-μm filter, degassed before use and were delivered isocratically at a flow-rate of 1.0 ml/min.

2.4. Assay calibration and performance

Standard calibration graphs were constructed by linear least-squares regression analysis of the plot of the peak-height ratios between each compound and the I.S. responses, against the concentrations in the standard samples. The computer-generated parameters were used to convert the relative response of the unknown samples to concentrations.

Standard curves with five to seven concentrations of all-*trans*-RA, its 13- and 9-*cis*-isomers (0.05–2.5 μg/ml or 0.01–0.5 μg/mg protein, respectively) and 4-oxo- and 5,6-epoxy-metabolites (0.025–1.25 μg/ml or 0.005–0.25 μg/mg protein) were analysed concurrently with each set of quality control (QC) and unknown samples. The lowest concentration used for these curves was equal to the lower limit of quantitation, i.e. the lowest concentration of the

compounds that can be measured with acceptable precision [19].

The quality of the analytical results was checked by analyzing biological samples containing known amounts of all-*trans*-RA and its metabolites (QC). For mouse plasma and human keratinocytes, small (0.025–0.05 μg/ml or 0.005–0.01 μg/mg protein), medium (0.25–0.5 μg/ml or 0.05–0.1 μg/mg protein) and high-level QC samples (1.25–2.5 μg/ml or 0.25–0.5 μg/mg protein) were prepared at the start of the study. The intra-assay precision was established by replicate analysis of these QC on the same day ($n=5$). The inter-assay precision and reproducibility of plasma analysis was checked by assaying plasma QC samples along with freshly prepared calibration curves on different days. For the embryo tissues, only the intra-day precision was obtained and only at the lowest level.

3. Results and discussion

The chromatographic behaviour of all-*trans*-RA and its metabolites was tested on a Spherisorb ODS-2 5 μm column [20], initially varying the composition of an aqueous acetonitrile elution system containing either 10 m*M* ammonium acetate [8] or sodium acetate. Progressively lowering the content of acetonitrile from 80% to 65% improved the separation of the analytes, but the resolution of all-*trans*-RA, 9-*cis*-RA and 13-*cis*-RA remained insufficient. The ammonium acetate was superior to the sodium acetate salt, possibly because of an ion-pair effect of the NH_4^+ to the solutes, at the relatively high acetonitrile content (Fig. 2A and B). A higher concentration of the ammonium salt (100 m*M*, pH adjusted to 7.4) increased the ionic strength of the mobile phase, enhancing the efficiency of separation (Fig. 2C). However, the 4-oxo- and 5,6-epoxy-derivatives still eluted too close to the solvent front. To further improve the resolution, the composition of the mobile phase was sequentially optimized by adding small amounts of methanol and *n*-butyl alcohol and adjusting the acidity as well. The capacity factor (k) of all compounds (Fig. 2) increased with the increasing acidity (i.e. increasing the content of acetic acid) and separation was acceptable in a relatively short run time with acetonitrile–water–methanol–*n*-butyl alcohol (56:37:4:3, v/v) containing 100 m*M* ammonium acetate/70 m*M* acetic acid, pH 6.3.

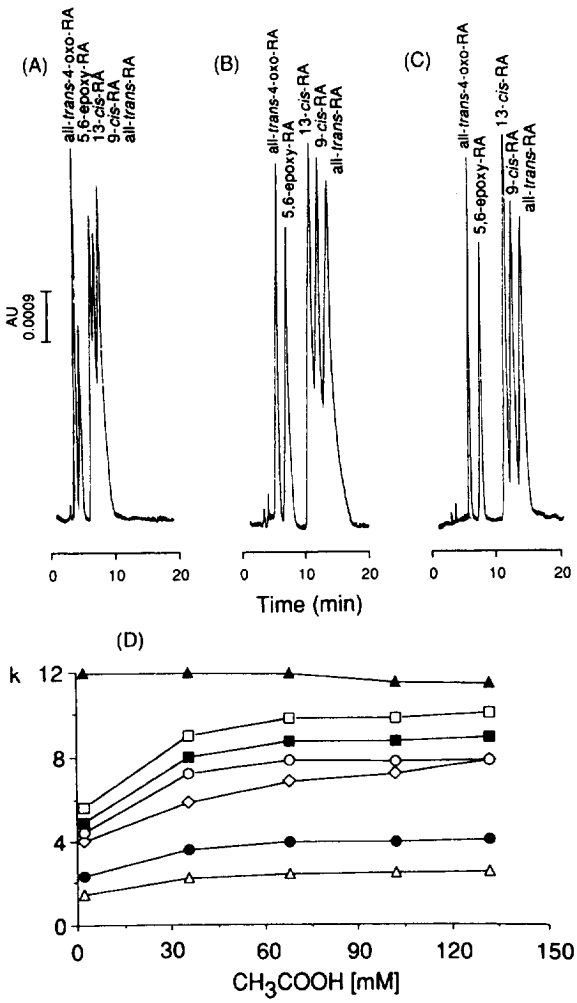

Fig. 2. Chromatograms showing the separation of the retinoids studied using a CH_3CN–H_2O mobile phase (65:35, v/v) with 10 m*M* CH_3COONa, pH 7.4 (A) and 10 m*M* (B) and 100 m*M* (C) CH_3COONH_4, pH 7.4. Also shown (D) is the influence of CH_3COOH concentrations in CH_3CN–H_2O–CH_3OH–*n*-C_4H_9OH (56:37:4:3, v/v) with a 100 m*M* CH_3COONH_4 elution system on the retention of all-*trans*-4-oxo-RA (△), 5,6-epoxy-RA (●), 13-*cis*-RA (○), 9-*cis*-RA (■), all-*trans*-RA (□), retinol (▲) and I.S (◇). The hold-up time, t_0, was determined as the time from injection to the first distortion of the baseline [24]. Each capacity factor (k') value is the mean of five analytical runs.

Under these chromatographic conditions, the retention times were 6.7 min for all-*trans*-4-oxo-RA (and its 13-*cis*-isomer), 9.9 min for 5,6-epoxy-RA,

15 min for the I.S., 17 min for 13-*cis*-RA, 19 min for 9-*cis*-RA and 21 min for all-*trans*-RA (Fig. 3). Although the 4-oxo-isomers co-eluted together, this was accepted because 13-*cis*-4-oxo-RA is generally undetectable in plasma and embryonic tissues of animals given all-*trans*-RA [21–23]. The retention time of retinol was 25 min so it did not interfere with the detection of its metabolites (Fig. 3).

Available procedures [13,14] were tested for extraction of all-*trans*-RA and its main metabolites from plasma, embryonic tissues and from human keratinocytes. Solid-phase extraction on Bakerbond SPE octadecyl columns [13] efficiently extracted the drug, its 13-*cis*-isomer and their 4-oxo-derivatives from all matrices, but recovery of 9-*cis*-RA and 5,6-epoxy-RA was too inconsistent for their simultaneous quantitation.

Considering the small amounts of biological material generally available for our analysis, we tested the direct protein precipitation with acetonitrile, developed for all-*trans*-RA and the synthetic retinoid

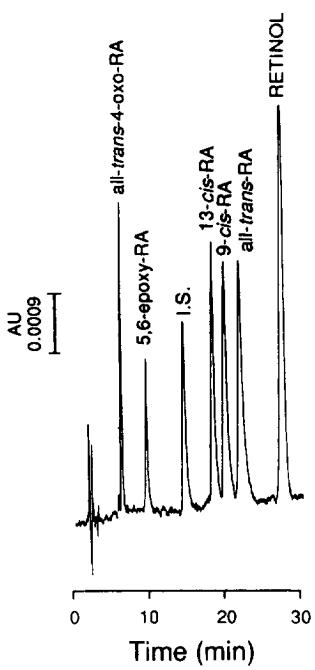

Fig. 3. Chromatographic separation of all-*trans*-4-oxo-RA, 5,6-epoxy-RA, 13-*cis*-RA, 9-*cis*-RA and all-*trans*-RA and their biological precursor, retinol, using CH_3CN–H_2O–CH_3OH–*n*-C_4H_9OH (56:37:4:3, v/v) containing 100 m*M* CH_3COONH_4/70 m*M* CH_3COOH, pH 6.3 at a flow-rate of 1.0 ml/min.

derivative, Am 580 [14,15]. This enabled us to extract the parent drug and its metabolites easily and rapidly from all the biological matrices evaluated. The overall mean recovery, determined by comparing the peak height of the retinoids with those obtained from direct injection of the compounds dissolved in mobile phase, averaged 85–99% for all-*trans*-RA, 89–95% for 13-*cis*-RA, 87–102% for 9-*cis*-RA, 87–103% for all-*trans*-4-oxo-RA and 93–94% for 5,6-epoxy-RA, with no significant dependence on concentration over the range investigated.

The relationship between the peak-height ratios of all-*trans*-RA, all-*trans*-4-oxo-RA, 5,6-epoxy-RA, 13-*cis*-RA and 9-*cis*-RA to the I.S. and the amount of the compound added to the various matrices were always linear, with correlation coefficients invariably exceeding 0.99. Typical standard curves (±S.D.; $n=5$) from homogenized cells are represented by the equation $y=0.014\ (0.001)x-0.038\ (0.003)$ for RA, $y=0.018\ (0.001)x-0.073\ (0.005)$ for 9-*cis*-RA, $y=0.015\ (0.001)x-0.111\ (0.009)$ for 13-*cis*-RA, $y=0.037\ (0.003)x-0.037\ (0.003)$ for 4-oxo-RA and $y=0.047\ (0.004)x-0.059\ (0.004)$ for 5,6-epoxy-RA. The lower limit of quantitation was about 50 ng/ml or 10 ng/mg of protein for all-*trans*-RA, 13-*cis*-RA and 9-*cis*-RA and 25 ng/ml or 5 ng/mg of protein for all-*trans*-4-oxo- and 5,6-epoxy-RA, using 0.2 ml of plasma or about 1 mg of tissue protein. At these concentrations, intra-assay coefficients of variation (C.V.) of the retinoids were 3–9%, regardless of the tissue considered. Mean inter-assay C.V. averaged 5% in mouse plasma and 7% in human keratinocytes.

Examples of chromatograms of extracts from drug-free plasma (A) and from plasma from a pregnant mouse given 10 mg/kg of all-*trans*-RA and killed 2 h after dosing (B) are shown in Fig. 4. Sample B contained 87 ng/ml of all-*trans*-RA and 30 ng/ml of all-*trans*-4-oxo-RA. The corresponding concentrations in embryos were 301 ng/g for all-*trans*-RA and 225 ng/g for the all-*trans*-4-oxo-metabolite. Concentrations of 13-*cis*-RA, 9-*cis*-RA and 5,6-epoxy-RA were below the limit of the analytical procedure in both plasma and fetus. Fig. 5A shows chromatograms of extracts from ovaries of all-*trans*-RA-treated *Drosophila* (150 ng or about 0.5 nmol) containing 122, 145, 71, 11 and 10 ng/mg protein of all-*trans*-RA, 13-*cis*-RA, 9-*cis*-RA, 5,6-epoxy-RA

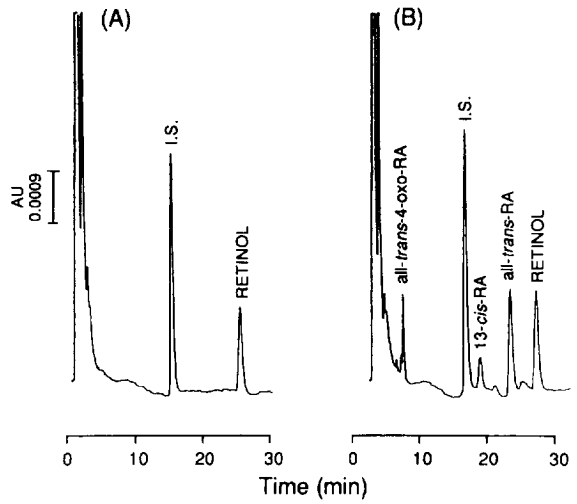

Fig. 4. Chromatograms of extracts of (A) drug-free plasma and (B) plasma from a pregnant mouse 2 h after oral dosing with 10 mg/kg all-*trans*-RA, containing 87 ng/ml of all-*trans*-RA and 30 ng/ml of all-*trans*-4-oxo-RA. Concentrations of 13-*cis*-RA, 9-*cis*-RA and 5,6-epoxy-RA were below the limit of detection of the analytical procedure.

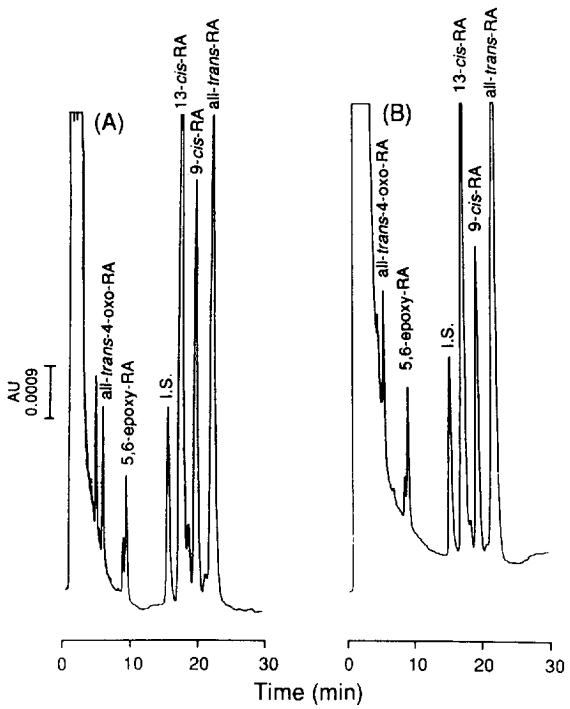

Fig. 5. Examples of chromatograms of extracts from ovaries of all-*trans*-RA-treated *Drosophila melanogaster* (A: 150 ng or about 0.5 nmol) containing 122, 145, 71, 11 and 10 ng/mg of protein of all-*trans*-RA, 13-*cis*-RA, 9-*cis*-RA, 5,6-epoxy-RA and all-*trans*-4-oxo-RA, respectively. Also shown is the chromatogram of the extract from the carcass (B) which contained similar amounts of these retinoids on a ng/mg of protein basis.

and all-*trans*-4-oxo-RA, respectively. Also shown is the chromatogram of the extract from the carcass (Fig. 5B) which contained similar amounts of these retinoids on a ng/mg of protein basis (133, 81, 41, 10 and 5 ng/mg of protein). As with mouse plasma and embryonic tissue, there were no peaks that could interfere with the analysis of RA in this test system.

Chromatograms from sea urchin embryos incubated with all-*trans*-RA at a concentration (10 μM) that could alter development [17] appear in Fig. 6A. This sample contained measurable concentrations of all-*trans*-RA (20 ng/mg of protein) and traces of its 9- and 13-*cis*-isomers (<10 ng/mg of protein), as in all cultured embryos analyzed so far (5–50 μM). Similar concentrations of all-*trans*-RA were measured in keratinocytes cultured with 10 μM all-*trans*-RA (Fig. 6B). These greatly influence skin development when keratinocyte colonies are growing and not stratified or fully organized in a multilayered tissue (G. Pellegrini, personal communication). We observed no interfering peaks in several samples of drug-free sea urchin embryos and human keratinocytes.

In conclusion, the isocratic HPLC procedure described, coupled with a simple deproteinization procedure, allows rapid and simple determination of all-*trans*-RA, its 13- and 9-*cis*-isomers and the oxidized metabolites 4-oxo- and 5,6-epoxy-RA, with some advantages in terms of analysis time and simpler instrumentation than found with the gradient elution technique. The method is suitable for analysing a variety of biological specimens and is currently used in our laboratory to measure concentrations of drugs and their metabolites in tissues which are employed in new potential in vitro test systems for developmental toxicity. Preliminary drug measurement studies in the *Drosophila* assay indicate that the pattern of all-*trans*-RA metabolism is qualitatively similar to that in mammals and that all compounds rapidly enter the ovaries, achieving concentrations similar to, or even higher than, those found in whole body. The drug is also rapidly taken up by cultured sea urchin embryos and human

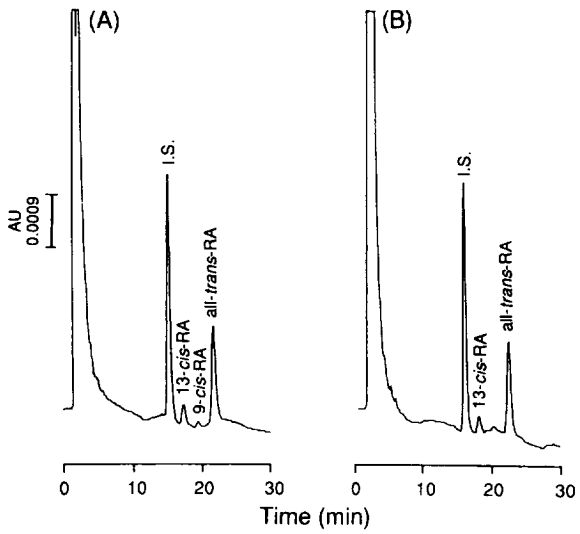

Fig. 6. Chromatograms of extracts of sea urchin embryos (A) and human keratinocytes (B) incubated with all-*trans*-RA at concentrations of 10 μM. The concentrations of unchanged drug were 20 and 18 ng/mg of protein in embryos and cells, respectively. The concentrations of all-*trans*-4-oxo-RA, 5,6-epoxy-RA, 13-*cis*- and 9-*cis*-RA were below the limits of sensitivity of the procedures used in both test systems (<5–10 ng/mg of protein).

keratinocytes, although in variable amounts and with qualitative and quantitative differences in the metabolic pattern.

Studies are now in progress to determine the effective doses or medium concentrations of all-*trans*-RA and its metabolites perturbing the reproductive and developmental processes for each test system. Comparison to in vivo results derived from mammals using all-*trans*-RA and other teratogenic drugs will allow an evaluation of the predictive potential of new assays.

Acknowledgments

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