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Simultaneous determination of 13-*cis*- and all-*trans*-retinoic acids and retinol in human serum by high-performance liquid chromatography

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Abstract

A simple and accurate method was developed for the simultaneous determination of 13-*cis*- and all-*trans*-retinoic acid (13cRA, tRA), and retinol (ROH) in human serum using isocratic high-performance liquid chromatography (HPLC). The serum sample (0.2 ml) was diluted with 2 ml of acetonitrile–100 m*M* ammonium acetate (1:3, v/v), pH 5.5, and extracted with 5 ml of *n*-hexane. The extract was analyzed on an ODS column with a mobile phase consisting of 70 vols. of acetonitrile–methanol (2:1) and 30 vols. of 100 m*M* ammonium acetate, pH 7.0, at 50°C. The retinoids were detected at 340 nm. Average recoveries were 88.4% for 13cRA, 82.5% for tRA at fortification levels of 5, 10 and 25 ng/ml, and 84.8% for ROH at 550 ng/ml. Within-day precision for normal human serum samples was 4.7% for 13cRA, 11.9% for tRA and 3.7% for ROH, and between-day precision was 10.4%, 14.2% and 4.7%, respectively. The limit of determination was 0.5 ng/ml in serum for the RAs. Mean concentration in 20 human sera was found to be 1.80 ng/ml for 13cRA, 1.77 ng/ml for tRA, and 487 ng/ml for ROH.

1. Introduction

All-*trans*- and 13-*cis*-retinoic acids (tRA and 13cRA) are physiological metabolites of vitamin A [retinol (ROH)] [1,2]. These compounds have been shown to be widely involved in controlling the growth and differentiation of a variety of cells through binding with specific nuclear receptors [3]. Moreover, tRA is an effective inducer for complete remission in acute promyelocytic leukemia [4]. Recently two research groups have described the presence of a pathway for the direct biogenesis of tRA from β-carotene [5,6]. These results have opened up a new field in the study on the effect of the diet on human cancer prevention [7–9]. By examining the metabolism of RAs, the relationship between the RA concentration in blood from dietary intake of ROH and β-carotene and cancer incidence, the possible role of RAs in cancer prevention should be investigated.

Although a number of HPLC methods have been published for the measurement of RAs and/or ROH in blood individually [1,5,10–14], only one group has reported an isocratic method for the simultaneous measurement of ROH,

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RAs and their oxidative metabolites. However, only the ROH concentration in plasma has been reported [15]. Epidemiological studies usually require a simple extraction and an accurate analytical method for the processing of large numbers of samples, using a small volume of serum with easy manipulation and low cost. We have evaluated the previously published methods and combined them to develop a new isocratic HPLC method. In the present paper a simple and reliable quantitative assay system for the measurement of the physiological serum concentrations of tRA, 13cRA and ROH is described.

2. Experimental

2.1. Reagent and apparatus

HPLC grade acetonitrile (MeCN), methanol (MeOH) and *n*-hexane, and reagent grade ethanol, potassium hydroxide (KOH) and ammonium acetate, were purchased from Wako Chemicals (Osaka, Japan). All-*trans*- and 13-*cis*-retinoic acids were purchased form Sigma (St. Louis, MO, USA). All-*trans*-retinol was prepared by saponification of vitamin A acetate (in oil, Wako Chemicals) and purified by HPLC to remove any trace impurities that would interfere with the RA analysis. N-Methyl-N-nitroso-*p*-toluenesulfonamide was obtained from Aldrich (Milwaukee, WI, USA). Water purified with a Milli-Q SP TOC system (Millipore, Bedford, MA, USA) was used.

Stock solutions of 13cRA and tRA were prepared by dissolving 10 mg of each in 100 ml of ethanol, and stored at −25°C. ROH stock solution was prepared as follows: the eluate from preparative HPLC was concentrated and extracted with *n*-hexane, followed by evaporation and quantitation using an E (1%/1 cm in ethanol at 324 nm) = 1800.

A serum diluting agent was prepared by mixing 1 vol. of MeCN and 3 vols. of 100 m*M* ammonium acetate, and adjusting the pH to 5.5 with acetic acid.

2.2. Chromatography

The samples were analysed on a high-performance liquid chromatographic (HPLC) system (Shimadzu, Kyoto, Japan) consisting of an LC-6AD pump, an SPD-10AV UV-Vis spectrophotometric detector, a C-R4A integrator (attenuation 1) and a CTO-6A column oven (50°C). A Chemcosorb 5-ODS-H column (150 × 4.6 mm I.D., 5-μm particle size, Chemco, Osaka, Japan) was used. The mobile phase consisted of 70 vols. of MeCN–MeOH (2:1) and 30 vols. of 100 m*M* ammonium acetate. The pH was adjusted to 7.0, and the flow-rate was 1 ml/min for sample analysis. Detection was performed at 340 nm.

Retinoid mixtures ranging from 10 to 200 ng/ml for RAs and 1 to 40 μg/ml for ROH were prepared by mixing the stock solutions and diluting with MeCN–MeOH (2:1). Linearity of the detector response was examined by injection of 20 μl of the mixture at the highest sensitivity of the instrument. The response was found to be linear up to 4 ng for RAs (equivalent to 25 ng/ml in serum) and 800 ng for ROH (equivalent to 5000 ng/ml in serum). The instrumental detection limit was 50 pg for RAs (S/N = 3), giving a practical lowest quantitation limit for analysis of a 0.2-ml serum sample of 0.5 ng/ml.

Although an unusually high column temperature (50°C) was used to obtain a better separation in a shorter period, no isomerization of the RAs and no oxidative degradation of ROH has been found during the HPLC analysis.

2.3. Extraction

A 0.2-ml human serum was mixed with 2 ml of the diluting agent for 10 s in a 10-ml ambered tube. The sample was extracted with 5 ml of *n*-hexane on a vortex-mixer for 30 s, followed by centrifugation at 1600 *g* for 5 min at 4°C. The organic phase was transferred to another tube and concentrated to less than 1 ml with a rotary evaporated (<30°C), then transferred to a 1.5-ml polyethylene tube to evaporate the solvent under nitrogen. The residue was dissolved in 25 μl of MeCN–MeOH (2:1) by mixing for 30 s. After

centrifugation at 8000 *g* for 1 min, a 20-μl aliquot was injected onto the HPLC system and peak heights of the retinoids were determined.

2.4. Quantitation

For quantitative analysis of the retinoids, a 20-μl aliquot of the standard mixture of 40 ng/ml RAs plus 4 μg/ml ROH was injected and their peak heights were determined. The peak heights of the retinoids in the serum sample were compared with those of the standards, and the amounts present were calculated.

2.5. Recovery and reproducibility

Retinoid mixtures for fortification were prepared by mixing the stock solutions and diluting with ethanol to give concentrations of 20 ng/ml and 40 ng/ml RAs, and 100 ng/ml RAs plus 2200 ng/ml ROH.

A 50-μl aliquot of the mixture was added to an ambered tube and the solvent was removed under nitrogen. The retinoids were redissolved in 0.2 ml of serum, resulting in fortification levels of 5 or 10 ng/mg serum for RAs, or 25 ng/ml for RAs and 550 ng/ml for ROH. Recovery rates were determined by comparison of the peak heights of the fortified serum sample minus the endogenous peak heights of the serum used for the fortification with the peak heights of respective amounts of retinoids. Within-day and between-days reproducibility tests were done using both normal physiological serum and the sample fortified with RAs at 5 ng/ml. ROH was not added.

2.6. Methylation

Diazomethane was generated from N-methyl-N-nitroso-*p*-toluenesulfonamide in an alkaline medium and co-distilled with ether.

The peak eluates from a serum extract, having the same retention time as the authentic 13cRA and tRA, were collected, concentrated to less than half of the initial volume, then extracted with *n*-hexane after acidification with acetic acid. After centrifugation at 1600 *g* for 3 min, the solvent was transferred to another tube and removed under nitrogen. Etherial diazomethane (50 μl) was added to the residue, then the mixture was kept at room temperature for 30 min in the dark, followed by evaporation under nitrogen in a hood. The authentic retinoids were treated in the same way. The residue was dissolved in 25 μl of MeCN and subjected to HPLC.

2.7. UV spectra

On-line UV spectra were determined with the SPD-10AV detector by stopping the flow of the mobile phase. The detector takes a spectrum by subtracting the blank absorbance (*i.e.* mobile phase) from the absorbance of a sample during scanning, resulting in a rugged spectrum when the sensitivity is very high as is the case with serum samples. Spectra of RA isomers are shown in Fig. 3 (H–J).

3. Results and discussion

3.1. HPLC separation of tRA, 13cRA and ROH

Isocratic HPLC analysis of serum RAs usually requires a clean-up step to remove ROH which is present in a concentration at least 150-fold higher than the RAs in human serum [5], otherwise ROH will hamper the RA assay due to its longer retention time [10] or its close elution with RAs [16]. We therefore tried to develop an isocratic HPLC system requiring no clean-up step for the simultaneous analysis of RAs and ROH in human serum, using a mixture of MeCN, MeOH and ammonium acetate as mobile phase.

The chromatographic behavior of the compounds was examined by changing the MeCN/MeOH ratio and the pH of the mobile phase. As shown in Fig. 1A, addition of MeCN to the mobile phase shortened the retention time of ROH, giving a possible isocratic system for the simultaneous determinations of both RAs and ROH. Fig. 1B shows the effect of pH on the

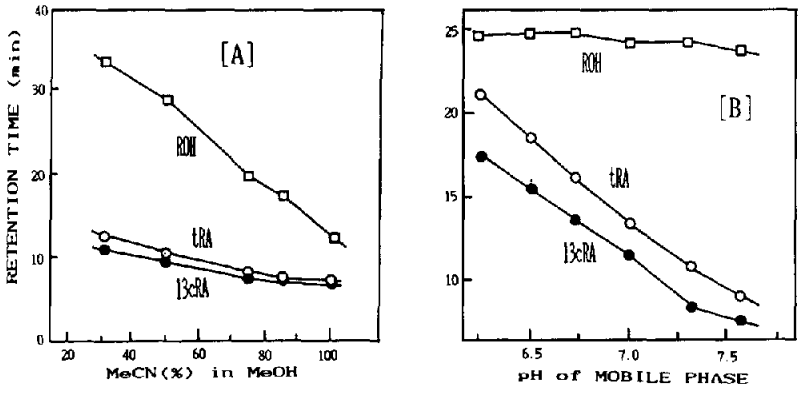

Fig. 1. Effect of MeCN–MeOH ratio (A) and pH (B) of mobile phase on HPLC retention of 13cRA, tRA, and ROH. HPLC conditions were: (A) mobile phase: 75 vols. of a mixture of MeCN and MeOH having different ratio, and 25 vols. of 10 m*M* ammonium acetate; pH was unadjusted; column temperature: 40°C; flow-rate: 1 ml/min; (B) mobile phase: 75 vols. of MeCN–MeOH (1:1) and 25 vols. of 10 m*M* ammonium acetate; pH was changed by adding acetic acid; column temperature: 45°C; flow-rate: 1.2 ml/min.

retention behavior of the retinoids, indicating that a decrease in the pH resulted in longer retention times for the RAs, but not for ROH. Although the mobile phases with a higher MeCN content gave a clear separation of 13cRA, tRA and ROH within a shorter analysis time, some serum extracts gave unsymmetrical and tailing peaks of the two RA isomers under these conditions. This insufficient separation between the isomers and endogenous compounds was resolved by lowering the organic solvent content to 70% and by adjusting the pH to 7.0. In actual analysis of serum extracts we therefore used a mobile phase consisting of 70 vols. of MeCN–MeOH (2:1) and 30 vols. of 100 m*M* ammonium acetate, pH 7.0, for the isocratic separation of the three retinoids to avoid possible overestimation of the serum RAs concentration, caused by the small endogenous peaks near the isomers. However this resulted in a somewhat longer analysis time. Under these conditions, the retention times for 13cRA, tRA and ROH were 14.9, 17.0 and 31.9 min, respectively (Fig. 2A, B).

3.2. *Extraction of retinoids from serum*

Solvent extraction methods for RAs in serum usually require an acidic medium containing an organic water-miscible solvent such as ethanol. However, treatment of serum with strong acid/base is known to release free RAs from their endogenous glucuronides, resulting in a higher apparent concentration in serum [10]. Both RAs

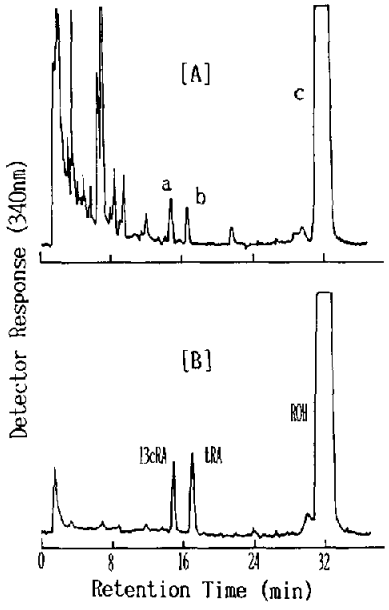

Fig. 2. HPLC separation of 13cRA, tRA, and ROH. (A) Human serum extract. Retention times of the indicated peaks were: a, 14.92 min; b, 17.04 min; c, 31.90 min. (B) Standard retinoids at a concentration of 5 ng/ml for RA isomers and 550 ng/ml for ROH. Retention times were 14.85 min for 13cRA, 16.93 min for tRA, and 31.85 min for ROH. HPLC conditions: see Experimental section.

and ROH are also susceptible to isomerization, dehydration or oxidative degradation by acid, heat, and, in particular, by light [17].

Considering the extremely low serum concentration of the RAs compared to that of ROH and the limited amount of serum sample available for analysis together with the chemical instability of the RAs and ROH described above, we improved the previously used methodology [1,10–13] by using (a) ambered tubes throughout the procedure to protect the sample from light instead of a dimly-lit room, (b) a serum diluting agent (2 ml for 0.2 ml serum sample) to avoid hydrolysis of the glucuronides instead of direct addition of ethanol and acetic acid or hydrochloric acid, and (c) a single extraction method with a rather large volume (5 ml) of *n*-hexane to enable easy and rapid manipulation instead of a conventional repetitive extraction method with a smaller volume of solvent.

3.3. Identification

Fig. 2A shows a typical chromatogram of a serum sample from a healthy volunteer. To identify chemically the species in peaks a, b, and c, these were collected by repetitive injections of serum extracts and subjected to a combination of analytical procedures including HPLC, chemical derivatization, and on-line UV spectroscopy. First, the retention times of peaks a, b, and c were compared to those of authentic 13cRA, tRA, and ROH, respectively. In all the solvent systems described above (Fig. 1A, B), having different MeCN–MeOH ratios and pHs, the retention times of these peaks changed in the same manner and extent as the peaks of the reference compounds. The collected peaks had the same retention times as those of the authentic retinoids (Fig. 3A–D) under different HPLC conditions. Second, the collected peaks were derivatized with diazomethane which specifically methylates free carboxylic groups (Fig. 3A, E–G). Upon methylation, the retention times of peaks a and b shifted from 5.88 min to 18.29 min and from 6.81 min to 20.95 min, which corresponds into the retention times of the methyl esters of 13cRA and tRA (18.26 min and 20.83 min), indicating that a and b had a free carboxylic group that was methylated. The retention time of peak c (9.17 min) remained un-

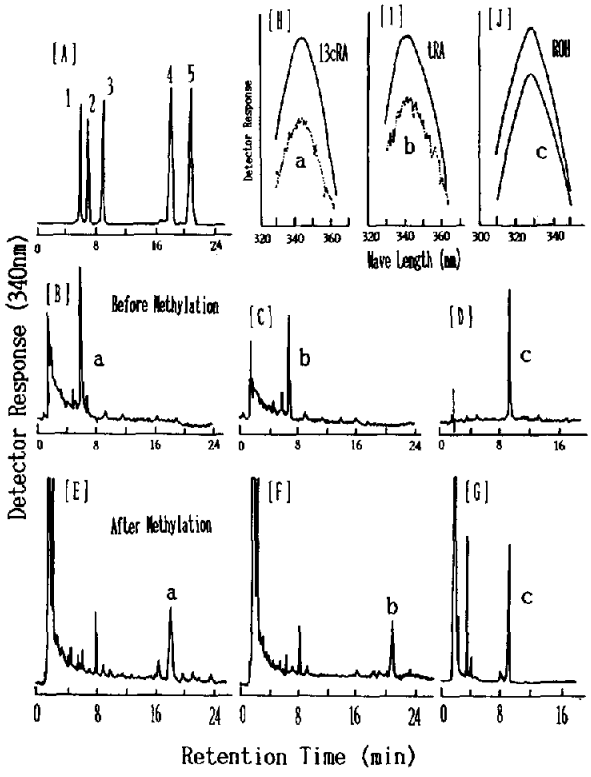

Fig. 3. Identification of peak eluates a, b, and c by comparison (A–G) of the chromatographic behavior of the peak eluates before (B, C, D) and after (E, F, G) diazomethane treatment with that of reference compounds (A, 1–5), and by on-line UV spectroscopy (H–J). (A) Reference chromatogram; peaks: 1:13cRA (5.88 min), 2 = tRA (6.81 min), 3 = ROH (9.18 min), 4 = methylated 13cRA (18.26 min), 5 = methylated tRA (20.83 min). The retention times of peak eluate a were 5.88 min (B: before treatment) and 18.29 min (E: after treatment). Those of peak eluate b were 6.81 min (C) and 20.95 min (F), respectively. Those of peak eluate c were the same (D, G: 9.17 min) both before and after treatment. HPLC conditions were: mobile phase, 80 vols. of MeCN–MeOH (1:1) and 20 vols. of 100 m*M* ammonium acetate, pH 7.0; column temperature, 50°C; flow-rate, 1 ml/min. (H) shows UV spectra of authentic 13cRA (solid line) and peak eluate a (dotted line), (I) those of authentic tRA (solid line) and peak eluate b (dotted line), and (J) authentic ROH and peak eluate c.

Table 1
Recovery of 13cRA, tRA and ROH from fortified human serum ($n = 6$)

Fortified concentration (ng/ml)	Recovery (%)	
	Mean ± S.D.	R.S.D.
13cRA		
5	89.9 ± 3.3	3.6
10	87.1 ± 6.3	7.2
25	88.2 ± 2.8	3.2
tRA		
5	85.8 ± 6.6	7.6
10	79.4 ± 3.7	3.7
25	82.2 ± 1.6	1.9
ROH		
550	84.8 ± 3.8	4.5

changed after the reaction with diazomethane. Finally, the UV spectra of peaks a, b, and c were taken and found to coincide with those of authentic 13cRA (Fig. 3H, $\lambda_{max} = 343$ nm) and tRA (Fig. 3I, $\lambda_{max} = 342$ nm) and that of ROH (Fig. 3J, $\lambda_{max} = 324$ nm), respectively. From these results, we were confident that the peaks a, b and c derived from human serum extracts were 13cRA, tRA and ROH, respectively.

3.4. Recovery and reproducibility

Recovery and reproducibility were tested by repetitive analyses of 0.2 ml of human serum with or without fortification. Data are summarized in Tables 1 and 2, respectively.

Recoveries ranged from 74.0 to 94.0% for the RA isomers at fortification levels of 5, 10 and 25 ng/ml, and that for ROH at 550 ng/ml ranged from 79.1 to 89.2%. Mean recovery rates ($n = 6$) for the three retinoids were in the range of 79.4–89.9% with standard deviations (S.D.) of 1.6–6.6% (Table 1), showing that RAs presented at extremely low concentrations in human serum were efficiently recovered together with ROH from the sufficiently diluted sample by the single hexane extraction.

Replicate analysis ($n = 5$) of normal serum samples by the HPLC method described above gave values of 1.43 ± 0.07 ng/ml for 13cRA, 1.50 ± 0.18 ng/ml for tRA and 501 ± 19 ng/ml for ROH. Thus the within-day precisions expressed as R.S.D. (relative standard deviation) were 4.7%, 11.9% and 3.7%, respectively (Table 2). The between-day precisions were 10.4%, 14.2% and 4.7%, respectively. The precision for the fortified serum samples (5 ng/ml for RAs) ranged from 1.5 to 7.7% for both the within- and between-day analysis. The results for

Table 2
Within-day and between-day reproducibility of 13cRA, tRA and ROH in normal and fortified human serum at 5 ng/ml ($n = 5$)

	Normal serum		Fortified serum	
	Mean ± S.D. (ng/ml)	R.S.D. (%)	Mean ± S.D. (ng/ml)	R.S.D. (%)
Within-day				
13cRA	1.43 ± 0.07	4.7	6.59 ± 0.17	2.6
tRA	1.50 ± 0.18	11.9	6.39 ± 0.17	2.7
ROH	501 ± 19	3.7	–[a]	–
Between-day				
13cRA	1.37 ± 0.14	10.4	6.29 ± 0.19	1.5
tRA	1.43 ± 0.20	14.2	6.09 ± 0.47	7.7
ROH	493 ± 23	4.7	–	–

[a] Not done.

0.2 ml of serum sample were thought to be acceptable for measurement of physiological concentrations of 13cRA, tRA, and ROH in human serum.

3.5. Human serum analysis

The retinoid concentrations were determined in 20 normal human sera and their mean concentrations are presented in Table 3. The serum concentration of 13cRA ranged from 1.21 to 3.46 ng/ml, that of tRA from 0.94 to 3.42 ng/ml, and that of ROH from 420 to 572 ng/ml. The mean concentrations of 13cRA, tRA and ROH were 1.80 ng/ml, 1.77 ng/ml and 487 ng/ml, respectively. The observed values were comparable with the previously published values of 1.4–1.6 ng/ml for 13cRA, and 1.3–1.8 ng/ml for tRA in normal subjects [1,10,17], but they were lower than the values published by Periquet *et al.* [11] who found a mean tRA level of 3.5 ng/ml using sodium hydroxide and hydrochloric acid for the sample extraction. Acid/base treatment has been known to give higher values of tRA due to hydrolysis of its β-glucoronide present endogenously in human serum [10].

The presented isocratic HPLC analysis coupled with a simple extraction method allows the determination of physiological concentrations of the three retinoids in a small volume of human serum (0.2 ml) in a short analysis time and can be used for large surveillance studies. The procedure requires no additional instrumentation such as an automated device for solid-phase extraction and a binary gradient elution system [1]. We successfully applied the method to the measurements of 13cRA, tRA and ROH in human serum and found the total RAs concentration to be *ca.* 0.7% of the ROH concentration.

Table 3
13cRA, tRA and ROH concentrations in human serum (ng/ml, $n = 20$)

	Concentration (ng/ml)		
	Mean ± S.D.	Min.	Max.
13cRA	1.80 ± 0.62	1.21	3.46
tRA	1.77 ± 0.75	0.94	3.42
ROH	487 ± 36	420	572

References

[1] C. Eckhoff and H. Nau, *J. Lipid Res.*, 31 (1990) 1445.
[2] M.E. Cullum and M.H. Zile, *J. Biol. Chem.*, 260 (1985) 10590.
[3] Y. Hashimoto and K. Shudo, *Cell. Biol. Rev.*, 25 (1991) 209.
[4] M. Huang, Y. Ye, S. Chen and Z. Wang, *Blood*, 72 (1988) 567.
[5] J.L. Napoli, B.C. Pramanik, J.B. Williams, M.I. Dawson and P.D. Hobbs, *J. Lipid Res.*, 26 (1985) 387.
[6] X.D. Wang, N.I. Krinsky, G. Tang and R.M. Russell, *Arch. Biochem. Biophys.*, 293 (1992) 298.
[7] R.G. Ziegler, *Am. J. Clin. Nutr.*, 53 (1991) 251S.
[8] R. Peto, R. Doll, J.D. Buckley and M.B. Sporn, *Nature*, 290 (1981) 201.
[9] T. Hirayama, *J. Epidemiol.*, 2 (1992) S-37.
[10] A.B. Barua and J.A. Olson, *Am. J. Clin. Nutr.*, 43 (1986) 481.
[11] B. Periquet, W. Lambert, J. Garcia, G. Lecomte, A.P. De Leenheer, B. Mazieres, J.P. Thouvenot and J. Arlet, *Clin. Chim. Acta*, 203 (1991) 57.
[12] R. Wyss, *J. Chromatogr.*, 531 (1991) 481.
[13] A.P. De Leenheer, W.E. Lambert and I. Claeys, *J. Lipid Res.*, 23 (1982) 1362.
[14] G. Tang and R.M. Russell, *J. Lipid Res.*, 31 (1990) 175.
[15] Y.M. Peng, M.J. Xu and D.S. Alberts, *J. Natl. Cancer Inst.*, 78 (1987) 95.
[16] R.A. Heyman, D.J. Mangelsdorf, J.A. Dyck, R.B. Stein, G. Eichele, R.M. Evans and C. Thaller, *Cell*, 68 (1992) 397.
[17] R.W. Cutley Jr. and J.W. Fowble, *Photochem. Photobiol.*, 47 (1988) 831.