DEVELOPMENT OF A SIMPLE METHOD FOR THE DETERMINATION OF SERUM VITAMIN A LEVEL AS REFLECTOR OF NUTRITIONAL STATUS

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SYNOPSIS

Biochemical analysis of serum vitamin A (retinol) is the recommended method for evaluating vitamin A status in nutritional and disease states. A simple and specific high performance liquid chromatographic analysis of serum retinol and its analogs is described. Two different Radial-pak columns namely μ Bondapak C18 and Nova-pak C18 were used. We have developed two elution solvent systems comprising of acetonitrile: 1% ammonium acetate (85: 15 v/v) or acetonitrile: water: acetic acid (86: 13.5: 0.5 v/v). With 200μL of serum, the limit of detection for retinol is 50μg/L. Analytical recovery is in the range of 97.0% to 99.2%. Both within-run and between-run coefficients of variation are 3%. Retinol in the serum was found to be stable for at least 30 weeks. Normal reference values of serum retinol in Singapore are 840 ± μg/L and 680 ± 90μg/L for male and female respectively, but patients with glomerulonephritis showed higher levels.

INTRODUCTION

Vitamin A is an essential nutrient for growth, vision, reproduction and regulation of epithelial cell differentiation. In dietary epidemiological studies, the nutritional status of vitamin A for an individual can be assessed by dietary survey or biochemical analysis. For the former, Hankin et al (1) stressed that dietary history is the method of choice. The procedure relies upon dietary information obtained by history and then translated into nutrient intakes by reference to food composition tables (2). However, there are difficulties arising from such a translation because of differences in the various conversion factors used to determine vitamin A potency from carotenoids, since food composition may vary by geography, season, or food preparation.

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Dietary intakes may not necessarily reflect an individual's vitamin A status because diseases like malabsorption syndrome or liver disease can lower blood vitamin A levels even though the dietary intakes are adequate. On the other hand, it is difficult to obtain correct dietary information because of the inaccuracy arising from the subject's diet recall. In his study, Jensen (3) suggested that the recall of past diet is strongly influenced by present dietary habits. In view of the validity and accuracy of dietary history, the results of dietary epidemiological studies are therefore difficult to intepret.

Biochemical analysis of serum vitamin A (retinol) or carotenoids (β-carotene) is an alternative method for assessing nutritional status. Serum β-carotene levels are dependent on recent dietary intake of carotenoids only and not on vitamin A intake of the past, whereas serum retinol concentration is an indicator of the dietary intakes of both vitamin A and carotenoids. One may argue that within the normal range of vitamin A stores, the serum vitamin A concentration is homeostatically controlled (4). Nevertheless, the situation that one has to consider may not be a state of very low serum vitamin A levels when liver storage is depleted, but a state of lower than optimal levels of vitamini A. We feel that the measurement of serum vitamin A remains the most practical available biochemical means of assessing the status of nutrition; and if cancer epidemiologic studies are required, then prospective serum vitamin A studies become the most appropriate one.

The precision of the biochemical analysis depends on the analytical procedures used to determine serum vitamin A. Basically, there are four methods of detection: colorimetry (5,6), fluorometry (7), ultra-violet inactivation (8), and high-performance liquid chromatography (HPLC) (9-13). The HPLC is recently the method of choice due to its specificity, sensitivity, accuracy, precision, and versatility. We were able to use the HPLC to separate vitamin A compounds having a wide range of polarities by a simple isocratic run. We applied this method to analyse serum samples from normal volunteers and from patients with glomerulonephritis.

MATERIALS AND METHODS

Liquid Chromatography Equipment

The liquid-chromatographic equipment (Waters Associates) consisted of a Model 510 HPLC Pump, a Model 710 WISP Autoinjector, a Model 440 Dual Wavelength Detector set at 313 nm and 465 nm, a Model U-6K Injector, a Z-module Radial Compression Separation System with either 10μ μBondapak C18 Radial-Pak Cartridge (8mm I.D. × 100mm), or 5μ Nova-pak C18 Radial-Pak Cartridge (8mm I.D. × 100mm), a Guard-pak Precolumn insert and a dual pen recorder (BBC Goerz Matrawatt SE120).

Solvents used were pre-filtered through Millipore membrane filters type FH, pore size 0.50 μm (Millipore, Bedford, Mass.) and degassed before use. The mobile phases used were acetonitrile: 1% ammonium acetate (85: 15 v/v) (Solvent A), and acetonitrile: water: acetic acid (86: 13.5: 0.5 v/v) (Solvent B) set at a flow rate of 2 ml/min. The analysis was effected at ambient temperature with a pressure limit set at 2000 psi. The sensitivity of the detector was set at 0.005 AUFS and the recorder chart speed used was 1 cm/min.

Other equipment included a SC-3 Sample Concentrator (Jencons Scientiic Ltd, Bedfordshire, England), a Model UV-260 Shimadzu UV-Visible Recording Spectrophotometer, a Model 5413 Eppendorf Centrifuge, a Model 5432 Eppendorf Mixer, and 2.2 mL Eppendorf Microtubes (Eppendorf Geratebau, Hamburg 65, W. Germany).

Chemicals

Acetonitrile, methanol and chloroform were analytical grade reagents from either Merck (Darmstadt, Germany) or J.T. Baker (Phillipsburg, N.J.), and were used without further purification. All-trans-retinol, all-trans-retinoic acid, all-trans-retinal, and all-trans-retinol acetate were purchased from Sigma Chemical Co. (St. Louis, MO. 63178). The 13-cis-retinoic acid in 10 mg capsule was purchased from Hoffman-la Roche Co. (Basle, Switzerland). Oxygen-free nitrogen and compressed air were purchased from Soxal Co. (Singapore 2261). The purity of each of the standards was further checked by measuring the $_{max}$ and extinction coefficient $\left(E^{1\%}_{1cm}\right)$. The values corresponded closely to the values reported in the Merck Index (14).

Sample extraction

Each serum sample was either assayed immediately after collection or aliquotted (200 μL) into several Eppendorf microtubes, sealed under oxygen-free nitrogen and stored at −70°C for further analysis.

To each 200 μL sample of the heparinised serum in a 2.2 mL Eppendorf microtube, 200 μL of methanol solvent was added and vortexed for 5 min. Chloroform (400 μL) was then added together with 3 ng/L of the internal standard (retinol acetate dissolved in methanol). The solutions were vortexed again before adding 200 μL) of deionized water. After vortexing for another 2 min, the tube was centrifuged at 8500 × g for 10 min. The supernatant together with the protein precipitate (in the form of a compact white disc) were removed and re-extracted twice with fresh amounts of chloroform. The chloroform extracts were pooled and evaporated to dryness over a constant flow of oxygen-free nitrogen. The dry residue was redissolved in 100 μL of methanol and 10 μL was injected directly onto the HPLC column. It is important to carry out all the extraction steps under subdued light in order to prevent any photoisomerization reactions which may isomerize or destroy the vitamin A present in the samples.

Stock solution was prepared by dissolving 10 mg of retinol in 10 mL of methanol. The stock solution was stored over oxygen-free nitrogen in the −70°C freezer and was stable for at least four months. Working standard solutions were prepared freshly before use by making the appropriate dilutions of the stock solution.

Quantitation

Standard calibration curve (peak height retinol/peak height retinol acetate as internal standard) versus mass method was obtained. This calibration curve ($y = 0.009833 + 0.000387\ x$) was then used to determine serum retinol concentrations.

Peak identification

Figure 1a illustrates the elution profile of standard vitamin A compounds using the μ Bondapak C18 column and the elution Solvent A. Their capacity ratios are presented in Table 1. The normal plasma extract showed only one peak and was identified as retinol (Fig 1b). Further confirmation of its identification was carried out by absorption spectra, by co-chromatography with standard retinol, and by using a second column-Nova-pak C18 with the elution Solvent B (Fig 2a and 2b).

Fig. 1(a)

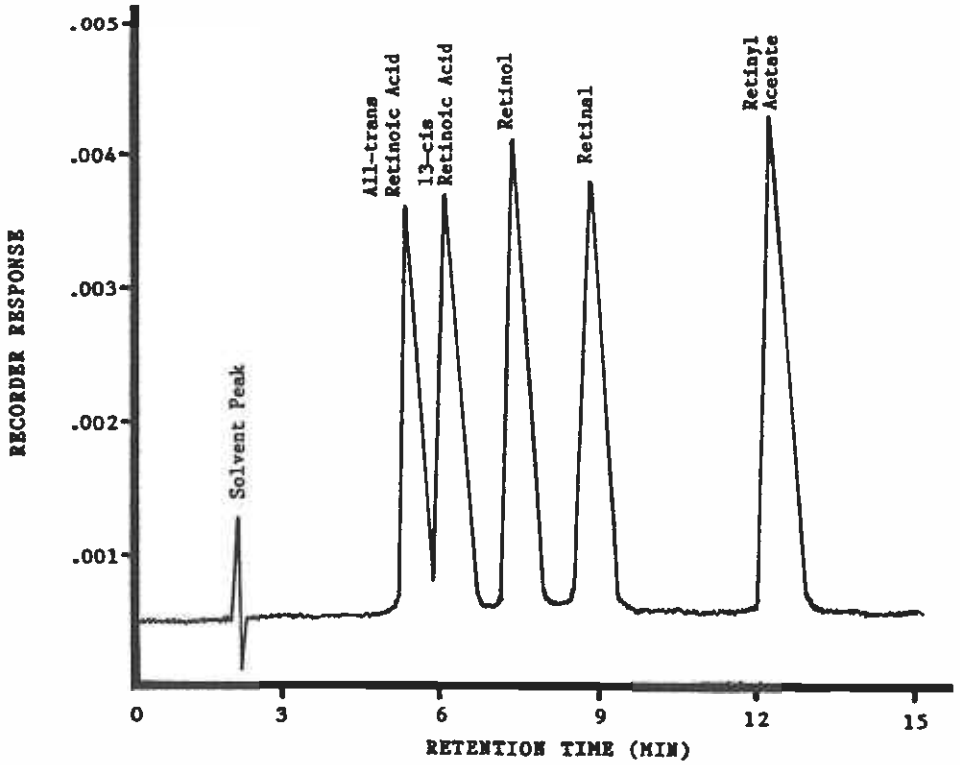

Fig. 1(b)

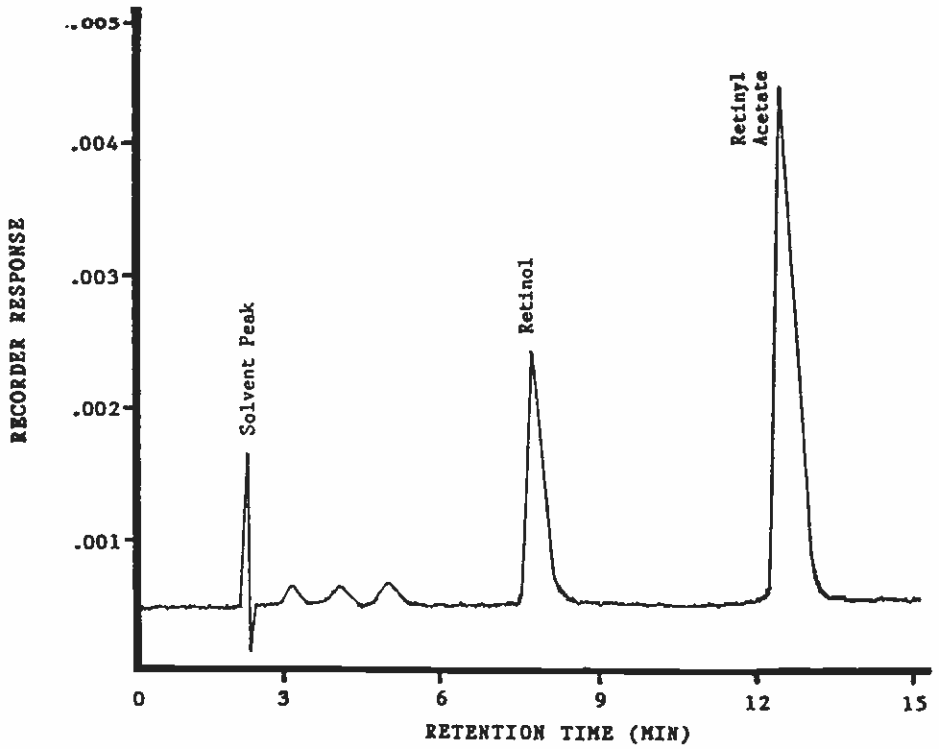

Figure 1: (a) Elution profile of standard vitamin A compounds and (b) chromatography of a normal extract plus retinyl acetate as internal standard, using 10 μBondapak C18-Radial-Pak Cartridge with mobile phase acetonitrile: 1% ammonium acetate (85: 15 v/v). The chromatographic conditions are given in the experimental section.

Fig. 2(a)

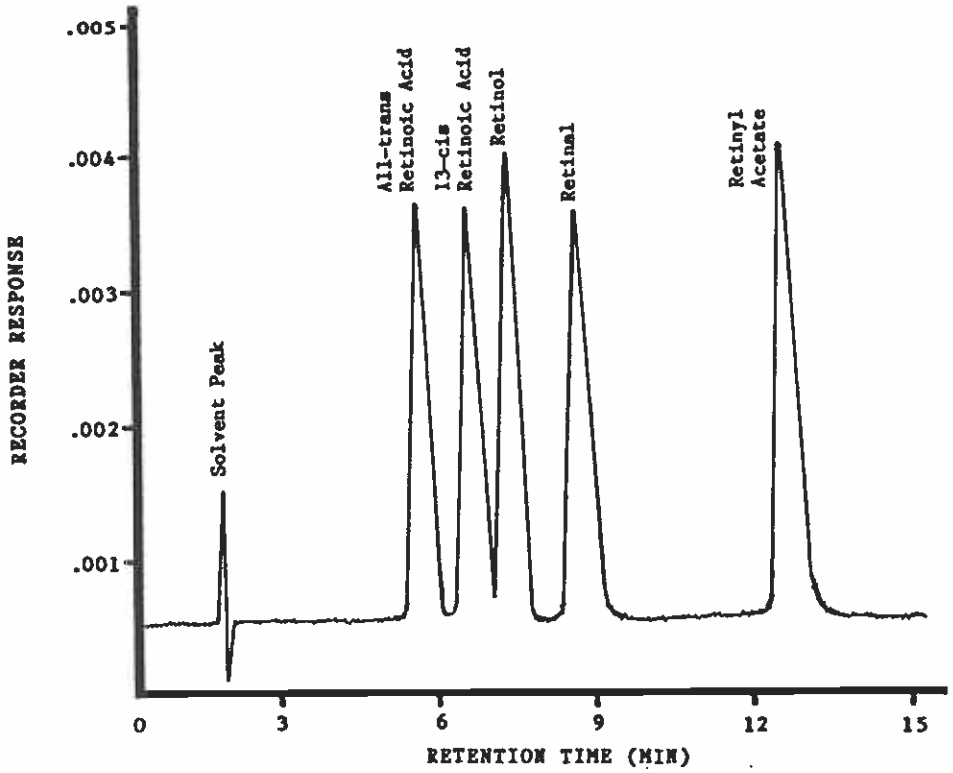

Fig. 2(b)

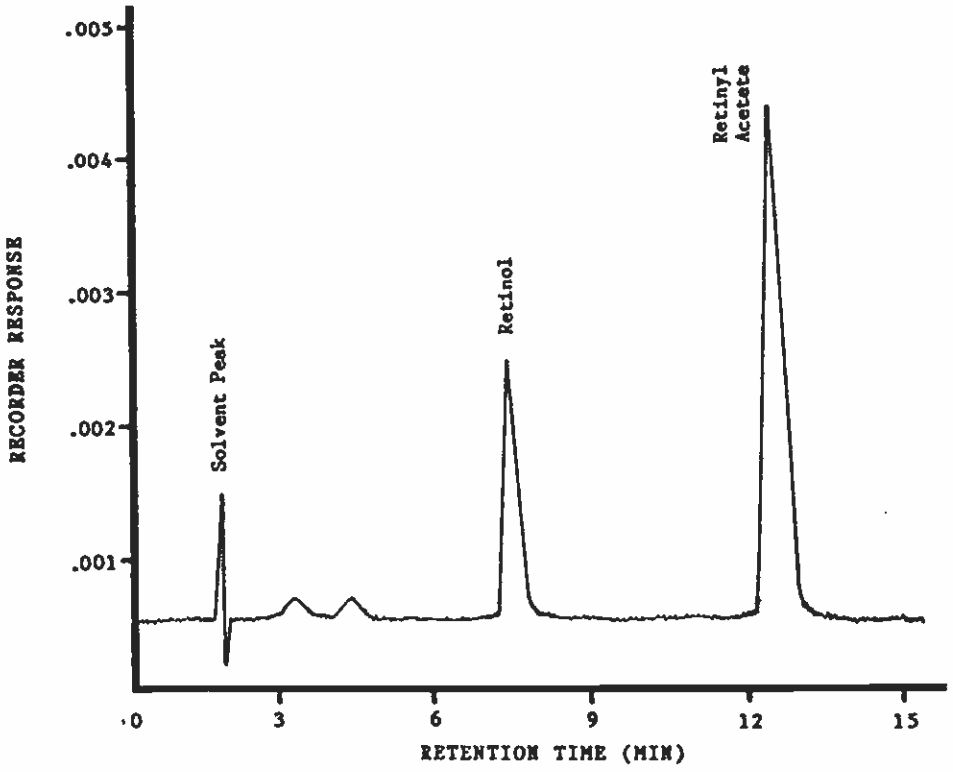

Figure 2: (a) Elution profile of standard vitamin A compounds and (b) chromatogram of a normal extract plus retinyl acetate as internal standard using 5μ Nova-pak C18-Radial-Pak Cartridge with mobile phase acetonitrile: water: acetic acid (86: 13.5: 0.5 v/v). The chromatographic conditions are given in the experimental section.

TABLE 1: CAPACITY RATIO (K')* OF STANDARD VITAMIN A COMPOUNDS USING (1) μBONDAPAK C18 COLUMN WITH MOBILE PHASE ACETONITRILE: 1% AMMONIUM ACETATE (85: 15 V/V) and (2) NOVA-PAK C18 COLUMN WITH MOBILE PHASE ACETONITRILE: WATER: ACETIC ACID (86: 13.5: 0.5 V/V)

Standard Compounds	Capacity Ratio (K') (1)	(2)
Trans-Retinoic acid	1.81	2.28
13-Cis-Retinoic acid	2.19	2.88
Retinol	2.89	3.25
Retinal	3.65	4.13
Retinol acetate	5.49	6.31

*K' value gives a measure of the ratio of the time spent by the solute (sample) in the stationery phase to the time spent in the mobile phase.

Results

Analytical recovery study

Serum pooled samples were first irradiated with long- and short- wavelengths U.V. light with constant stirring for 8 hours in order to remove all traces of endogenous retinol present. Different concentrations of the standard retinol ranging from 500—2500 μg/L were then added to the irradiated serum samples. The extraction procedures were then carried out as described above. Table 2 shows a recovery percentage for retinol ranging between 97.0% to 99.2%. Therefore, our method of extraction of retinol from serum is good.

TABLE 2: ANALYTICAL RECOVERY OF STANDARD RETINOL FROM SERUM

Initial Retinol Concentration (μg/L)	Retinol Added (μg/L)	Retinol Measured (μg/L)	Recovery %
0	500	490	98.0
0	1000	990	99.0
0	1500	1480	98.7
0	2000	1940	97.0
0	2500	2480	99.2

Precision study

Within-run (in one day) and between-run (over 15 days) precision tests were determined by assaying retinol levels from serum pooled samples. Each determination was the result of separate extractions and separate injections onto the HPLC column. The data in Table 3 show that the coefficients of variation for both types of runs were < 3%, indicating excellent reproducibility of the assay.

TABLE 3: ANALYTICAL PRECISION FOR RETINOL

	n	Retinol X	(g/L) SD	CV%
Within-run	8	435	10.0	2.3
	8	919	9.2	1.0
	8	1324	14.6	1.1
Between-run	15	500	14.1	2.8
	15	694	9.0	1.3
	15	1092	16.6	1.5

Storage effect

Vitamin A is known to be partially degraded when exposed to light and air. Since proper handling and storage of serum samples are crucial if valid data on serum vitamin A levels are to be obtained, the stability of this compound in serum stored under the conditions described earlier was examined over a period of 30 weeks. In Table 4, the X ± SD retinol values from two different serum samples were 704 ± 16.0 μg/L and 827 ± 23.4 μg/L respectively. Thus this indicates that there are no significant deterioration of serum retinol levels over a 30 weeks period.

TABLE 4: EFFECT OF STORAGE ON RETINOL CONCENTRATION IN SERUM

No. of Weeks	Serum A (μg/L)	Serum B (μg/L)
0 (Fresh sample)	710	860
1	720	835
2	710	860
4	710	820
5	710	820
6	700	825
9	700	780
12	700	810
15	690	800
18	675	815
21	710	865
24	740	815
27	695	845
30	680	835
$\bar{X}$	704	827
SD	16.0	23.4
CV%	2.3	2.8

Retinol levels in normal and renal patients

Serum samples from normal volunteers (22 males and 18 females), and from renal patients (diagnosed to have glomerulonephritis) were assayed in order to evaluate their vitamin A levels. In Table 5, the X ± SD for retinol in men and women of normal volunteers were 840 ± 90 μg/L and 680 ± 90 μg/L respectively. In renal patients, the values were 1710 ± 690 μg/L for male and 1610 ± 180 μg/L for female.

TABLE 5: SERUM RETINOL CONCENTRATIONS IN NORMAL ADULTS† AND RENAL PATIENTS* IN SINGAPORE

No. Analysed	Sex	Age Range	Retinol (μg/L) X ± SD
22†	M	18—40	840 ± 90
18†	F	19—29	680 ± 90
5*	M	25—47	1710 ± 690
3*	F	37—63	1610 ± 180

M = male
F = female

DISCUSSION

The HPLC assay described in this paper is a simple and sensitive method for the determination of vitamin A and its analogs. The limit of detection, using 10 μL injection for our method and at maximum sensitivity setting (AUFS 0.005) of the instrument, is 50 μg/L for retinol. Bakalyar & Henry (15) reported that if solvent composition can be maintained precisely, peak height measurement will yield good quantitative results. We have found that the peak height ratio allows good quantitation.

The normal reference values of serum retinol are presented in Table 5 and for comparison we have also presented the values published by other authors using their HPLC method for analysis (Table 6). It is evident that there is a range of normal reference values, and that the differences may most likely be attributed to race, diet, age and sex of the subjects used. Therefore, if cancer epidemiological study is required, then normal reference range for controls has to be developed by individual laboratory. Our data as well as some earlier reports in Table 6 indicated a higher normal reference value for retinol in male than in female.

TABLE 6: SERUM RETINOL VALUES OF HEALTHY ADULTS IN PUBLISHED PAPER

Author & Country	Sex	Serum Retinol $\bar{X} \pm SD$ (μg/L)	Age Range
McClean *et al* (10) U.S.A.	M	980 ± 18	not available
	F	820 ± 19	
Vuilleumier *et al* (11) Switzerland	M	663 ± 123	not available
	F	534 ± 128	
Biesalski *et al* (12) Germany	M	536 ± 145	19—35
	F	433 ± 104	19—35
De Ruyter & De Leenheeer (13) Belgium	U	600	not available
Wald *et al* (23) England	M	690 ± 140	35—64

M = male
F = female

Estimation of serum retinol from renal patients shows much higher values than those of normal volunteers (Table 5). The same trend was reported by De Bevere et al (16) and Yatzidis et al (17). It was suggested that this elevation in the serum retinol levels was due to decreased catabolism of retinol (18). Only one patient at the time of blood collection was put on renal dialysis and his serum retinol level was found to be 2030 μg/L. The other patients had not undergone dialysis treatment at the time of their blood collection. Steward (19) also found the constancy of hyperretinolaemia during maintenance haemodialysis, and recommended that multivitamin supplements containing vitmain A should be avoided in dialysed patients.

Our HPLC method can also be used to quantitate other retinoids besides retinol in serum, especially in evaluating the metabolism and pharmacology of retinoids. In recent years, these compounds particularly synthetic ones have been used therapeutically for cancer patients (20-22). By simply manipulating the solvent system, the resolution of desired retinoids can be optimized. As can be seen in Fig 1a and 2a, the stereoisomers namely 13-cis-retinoic acid and trans-retinoic acid could be easily separated by our HPLC system.

In conclusion, the HPLC method allows rapid, sensitive and specific analysis of retinoids. The use of the auto injector allows excellent precision. Good extraction procedures and proper storage of samples ensure accuracy of the results. Our method is therefore useful for studies on the nutritional and disease states, therapeutic values of retinoids or on their metabolism.

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