13-*cis*-Retinoic acid is an endogenous compound in human serum

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Abstract The occurence of 13-*cis*-retinoic acid as an endogenous component in human serum has been confirmed by cochromatography with standards in both normal-phase and reverse-phase high-performance liquid chromatographic (HPLC) system, by the λ_{max} of its UV spectrum recorded simultaneously with the HPLC run, and by chromatography of its methyl derivative. The method using solid-phase extraction followed by a gradient reverse-phase HPLC procedure with an internal standard and sensitive UV detector, provides an efficient and sensitive technique for the separation and quantification of serum 13-*cis*- and all-*trans*-retinoic acid. Serum levels of 13-*cis*- and all-*trans*-retinoic acid in 26 fasting volunteers ranged from 1.0 to 2.2 ng/ml (mean ± SEM = 1.4 ± 0.3 ng/ml) and from 1.1 to 1.9 ng/ml (mean ± SEM = 1.4 ± 0.2 ng/ml), respectively. The levels determined by a liquid–liquid double-phase extraction method were 90% higher in both 13-*cis*- and all-*trans*-retinoic acid than those from a solid-phase extraction. Human small intestine can isomerize all-*trans*-retinoic acid. 13-*cis*-Retinoic acid is the predominant *cis* isomer after incubation of intestinal mucosa homogenates with all-*trans*-retinoic acid. Moreover, the concentration of retinoic acid in serum is related to diet in that the level of total retinoic acid was 36% higher (n = 10) 2 h after a nonstandard breakfast than in fasting subjects. —**Tang, G., and R. M. Russell.** 13-*cis*-Retinoic acid is an endogenous compound in human serum. *J. Lipid Res.* 1990. **31:** 175–182.

Supplementary key words retinoic acid isomers • retinoids

13-*cis*-Retinoic acid (RA), an isomer of RA, is equivalent to all-*trans*-RA in supporting growth (1). As a less toxic derivative among retinoids (2), 13-*cis*-RA has been used to treat a wide variety of skin disorders including acne (3). Since it is able to inhibit the growth of tumors (4), this vitamin A analog is undergoing intensive clinical evaluation for the prevention or treatment of various cancers.

Recently, it was found that both all-*trans*- and 13-*cis*-RA are metabolized to 13-*cis*-4-oxo-RA in vitamin A-sufficient hamsters (5). Studies have demonstrated that both all-*trans*- and 13-*cis*-RA in a ratio of 2:1 are present in the intestinal mucosa of bile duct-cannulated rats shortly after the administration of all-*trans*-RA (6, 7). Further, their conjugated derivatives, 13-*cis*-retinoyl glucuronide and all-*trans*-retinoyl glucuronide, were detected in similar proportions. In addition, it has been reported that 13-*cis*-RA is not transported across mouse placenta in as large an amount as all-*trans*-RA (8, 9) and that 13-*cis*-RA is a much less potent teratogen in the mouse (10). These studies indicate that the isomerization of all-*trans*-RA to its 13-*cis* isomer is a physiologic phenomenon and suggest that 13-*cis*-RA may be an important metabolite in the metabolism of vitamin A.

In human blood, all-*trans*-RA and its glucuronide conjugate have been detected and demonstrated to be normal endogenous constitutents (11–14). While the occurrence of unidentified isomers of RA in human blood has been reported (12, 14), endogenous 13-*cis*-RA in human serum has never been confirmed. In the present study, we demonstrate the presence of 13-*cis*-RA as an endogenously circulating retinoid in human serum, and describe a quantitative assay for endogenous all-*trans*-RA and 13-*cis*-RA in human serum.

MATERIALS AND METHODS

Chemicals

HPLC grade methanol, n-hexane, water, and dioxane (Photrex™) were purchased from J. T. Baker Chemical Co., Philipsburg, NJ. Solvents were passed through a 0.45-μm membrane filter and degassed prior to use. All-*trans*-RA was purchased from Sigma Chemical Co., St. Louis, MO. 13-*cis*-, 11-*cis*-, and 9-*cis*-RA and TMMP were generous gifts from Hoffmann-La Roche Inc., Nutley, NJ. Retinoyl β-glucuronide was a gift from Drs. A. Barua and J. Olson. All retinoids were stored at −70°C and handled under red light. MNNG and MNNG-diazomethane kits were purchased from Aldrich Chemical

Abbreviations: RA, retinoic acid; HPLC, high-performance liquid chromatography; TMMP, all-*trans*-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoic acid; MNNG, 1-methyl-3-nitro-1-nitrosoguanidine; BCA, bicinchoninic acid; AUFS, absorbance unit full scale; ODS, octadecylsilane.

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Company Inc., Milwaukee, WI. Reagents for BCA Protein Assay were purchased from Pierce Co., Rockford, IL.

Serum extraction

The subjects (25–58 years old) consisted of 6 men and 20 women in good health, none of whom were being treated with Accutane™ or taking supplements containing vitamin A or β-carotene. Human blood was obtained by venipuncture in the morning after a 12-h overnight fast. Blood was also collected from nonfasting volunteers who had eaten a nonstandardized breakfast 2 h earlier. Blood was centrifuged and the serum was kept at −70°C in the dark before analysis. Pooled aliquots were used as control samples. All procedures for blood processing and analysis were done under red light.

A Bond Elut™ aminopropyl column (500 mg/2.8 ml) and a Vac Elut™ vacuum elution apparatus were obtained from Analytichem International, Harbor City, CA.

Extraction method A: aminopropyl bonded phase column extraction. The internal standard TMMP in methanol (100 μl, 10^{-7} M) was added to serum (1.5 ml). After the addition of 2 ml 0.9% sodium chloride and 5 ml chloroform–methanol 2:1 (v/v), the mixture was centrifuged. The chloroform layer was evaporated under N_2 to 0.2 ml. This extract (0.2 ml) was applied to an aminopropyl column that was placed in the Vac Elut apparatus and washed with 2 ml of hexane. The chloroform was pulled through the column by vacuum, and the serum constituents that were retained on the column were then eluted with 2 × 2 ml of chloroform–2-propanol 2:1 (v/v). This eluate consisted of neutral lipid in serum and was discarded. The column was again eluted with 2 × 2 ml of diethyl ether (with 3% acetic acid). This eluate contained RA and was evaporated to dryness under N_2. The residue was then redissolved in 150 μl (or 100 μl) of methanol, and a 40 μl (or 50 μl) aliquot was injected onto the HPLC column.

Extraction method B: liquid–liquid double-phase extraction (9). The internal standard TMMP in methanol (100 μl, 10^{-7} M) and ethanol (3 ml) were added to serum (1.5 ml) and the tube was centrifuged. Sodium hydroxide (2 N, 1 ml) was added to the supernatant, and after 10 min, the neutral and basic lipophilic constitutents were extracted with n-hexane (5 ml). The aqueous phase was acidified with hydrochloric acid (2 N, 2 ml) and extracted with n-hexane (5 ml). The n-hexane layer was evaporated under N_2, the residue was redissolved in 150 μl of methanol, and a 40-μl aliquot was injected onto the HPLC column.

HPLC analysis

The HPLC system consisted of a Series 410 LC pump, an LC-95 UV/visible spectrophotometer detector fixed at 340 nm at a maximum sensitivity (0.001 AUFS), and an LCI-100 laboratory computing integrator (Perkin-Elmer Corp., Norwalk, CT) For sample injection, either a Rheodyne Model 7125 manual injector (Rheodyne Inc., Cotati, CA) or a Perkin-Elmer ISS-100 autosampler was used.

For the identification of 13-*cis*-RA as well as its methylation product, a reverse-phase HPLC system equipped with two Pecosphere™ 3 × 3CR ODS cartridge columns (Perkin-Elmer Corp.) was used. The mobile phase consisted of methanol and water (with 1% ammonium acetate). The gradient program with a flow rate of 1.5 ml/min was as follows: methanol–water 75:25 (v/v) was eluted for 7 min, followed by an 8-min linear gradient to 100% methanol, holding 5 min at 100% methanol, followed by a 5-min linear gradient return to methanol–water 75:25 (v/v). Normal-phase HPLC with an HS-3 silica cartridge column (0.46 × 8.3 cm, Perkin-Elmer Corp.) was used for further identification of 13-*cis*-RA. The gradient procedure in the normal-phase HPLC system was done at a flow rate of 1 ml/min as follows: 5% dioxane in hexane was eluted for 1 min followed by a 15-min linear gradient to 10% dioxane in hexane with a 5-min hold and then a 3-min linear gradient return to 5% dioxane in hexane.

All quantitative analyses of serum RA by HPLC were obtained using Pecosphere™ 3 × 3CR ODS column. The mobile phase consisted two solvents: solvent A was methanol-water (65:35, v/v) and solvent B was 100% methanol. The gradient program with a flow rate of 1 ml/min was as follows: solvent A was eluted for 1 min, followed by a 14-min linear gradient to 30% solvent B, a further 10-min linear gradient to 100% solvent B, and a 5-min gradient back to 100% solvent A.

Incubation

Samples of human small intestine (jejunum) were removed during the normal course of gastrointestinal surgery and would otherwise have been discarded. The specimens were washed with 0.9% sodium chloride, then stored at −70°C until use. Homogenates (10%, w/v) of mucosal scrapings were made in 0.1 M sodium phosphate buffer (pH 7.0–7.3). This was then centrifuged at 800 *g* for 10 min. The supernatant (2 ml, triplicate) was incubated with all-*trans*-RA (26 ng in 5 μl of methanol) under N_2 in a shaking water bath at 37°C for 1, 3, 7, and 23 h. Vials (triplicate) either with boiled homogenate (kept in a boiling water bath for 10 min) or without homogenate were run as controls. In addition, vials consisting of identical components were incubated at 4°C for 3 h as an additional control. Pooled human serum (1 ml) was also incubated with all-*trans*-RA. The reactions were terminated by the addition of an internal standard (TMMP in methanol, 100 μl) and 3 ml chloroform–methanol 2:1 (v/v). The chloroform layer was evaporated to dryness, the residue was redissolved in methanol (200 μl), and an aliquot (40 μl) was injected onto the HPLC column. All the procedures with RA were carried out under red light.

Protein concentrations of specimens were determined by BCA Protein Assay (15) and ranged from 6.2 to 9.5 mg/ml for intestinal mucosa homogenates, and 59 mg/ml for pooled serum. Different homogenate dilutions were used to study the dependence of 13-*cis*-RA formation on protein concentration.

Methylation

The serum components corresponding to peaks b and c (in Fig. 1B) were collected as they eluted from the HPLC column, and the solvent was allowed to evaporate to dryness under N_2. The residue was dissolved in diethyl ether (3 ml), then reacted with diazomethane that was freshly generated from MNNG (150 mg) (16). The reaction was allowed to proceed in an ice bath for 40 min. After evaporating the reaction mixture under N_2, the residue that contained methylated derivatives of peaks b and c was redissolved in methanol and injected onto the HPLC system. All work with diazomethane was done in a well-ventilated hood and under red light.

Quantification

To quantitate RA in serum, aliquots of standard all-*trans*-RA ($E^{1\%}_{1cm}$ = 1480) and 13-*cis*-RA ($E^{1\%}_{1cm}$ = 1240) in methanol with a constant volume but increasing concentrations were injected onto the HPLC system and their response factors were determined from the standard curve thus generated. Both 13-*cis*-RA and all-*trans*-RA gave linear calibration curves over the range of 0 to 25 ng. The quantity in serum was calculated by the peak area in the HPLC chromatogram and the response factor of the corresponding standard. Levels were corrected for extraction and handling losses by monitoring the recovery of the internal standard, TMMP.

RESULTS

Peak identification

We have confirmed published reports (8, 9) that all-*trans*-RA is an endogenous retinoid in human serum based on consistent results (repeated 3 times) from co-chromatography in both reverse-phase and normal-phase HPLC systems, its UV spectrum, and chromatography after the formation of its methyl retinoate derivative. In our analysis, an unknown peak that eluted before all-*trans*-RA in a reverse-phase HPLC chromatogram, in amounts similar to all-*trans*-RA, appeared in all human sera analyzed. Most probably the unknown peak represented an endogenous compound because *a*) all the analytical work was conducted under red light, thus excluding the possibility of light-induced isomerization, and *b*) only a small amount, i.e., less than 3.1% of the *cis* isomer, was obtained from the standard all-*trans*-RA in phosphate buffer (pH 7.0) after the extraction method A (**Table 1**). Thus, the unknown peak appeared to be an endogenous component of human serum which remained to be identified.

TABLE 1. 13-*cis*-Retinoic acid (RA) formation from all-*trans*-RA in phosphate buffer[a]

All-*trans*-RA Added	n	13-*cis*-RA Formed (Mean ± SD)	Range (% Isomerized)
ng		*ng*	
16.8	8	none detectable	
84.0	6	1.98 ± 0.68	1.0 - 3.1

[a](Two ml, pH 7.0) using aminopropyl column extraction (extraction method A).

To get the UV absorption spectra for those components that were present at very low levels in serum, the extract (extraction method A) from 5 ml of pooled serum was injected onto the HPLC system and its UV spectra were recorded by diode array detector. The UV spectrum of the unknown peak in the HPLC chromatogram has λ_{max} at 340 nm and was almost identical to the spectrum of all-*trans*-RA (spectra not shown). Therefore, it appeared possible that this was a *cis* isomer of all-*trans*-RA.

The identification of this unknown peak was first carried out using a reverse-phase HPLC system using extraction method A from 2 ml of pooled serum (**Fig. 1B**). When standard 13-*cis*-RA (99% pure) was used for co-migration studies (peak b in Fig. 1A), this reverse-phase HPLC showed that the retention time of the unknown peak b (in Fig. 1B) of the extract from human serum matched that of the standard 13-*cis*-RA (peak b in Fig. 1A). Instead of the usual Pecosphere™ 3 × 3CR ODS cartridge column, an Ultrasphere™ ODS column (0.46 × 15 cm), or a 3 × 3CR ODS cartridge column with a different gradient program (methanol–water from 65:35 to 100:0 in 20 min) was used for human serum analysis, and the retention time of the unknown peak was always identical with that of authentic 13-*cis*-RA. The co-chromatographs were further analyzed by normal-phase HPLC which is capable of separating 9-*cis*-, 11-*cis*-, 13-*cis*-, and all-*trans*-RA (**Fig. 2B**). Both all-*trans*-RA (peak c) and the unknown (peak b) were found in the normal-phase chromatogram (Fig. 2A) of the extract from 4 ml of human serum using extraction method A, and their retention times were identical to those of the standards (Fig. 2B). Furthermore, after methylation of the fractions corresponding to peaks b and c in the chromatogram obtained from the serum extract, two new peaks with retention times at 19.02 min and 19.54 min (peaks d and e in **Fig. 3A**) were found in the chromatogram. The identification of these compounds was established by methylation of standard 13-*cis*- and all-*trans*-RA (peaks d and e in Fig. 3B). This experiment demonstrated that the unknown peak is a carboxylic acid.

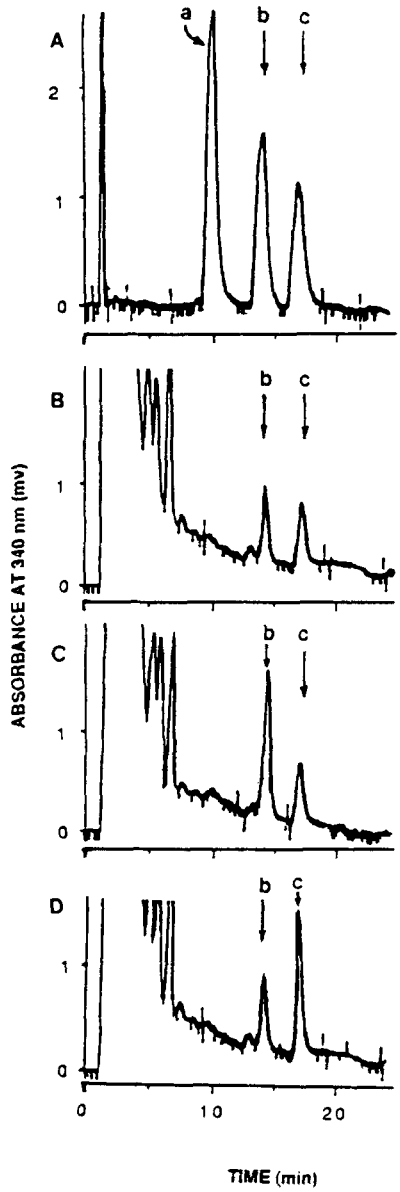

Fig. 1. Chromatograms obtained with reverse-phase HPLC. A: A standard mixture of TMMP (peak a), 13-*cis*-RA (peak b), and all-*trans*-RA (peak c); B: extract of pooled serum (2 ml); C: extract of spiked (by 13-*cis*-RA) pooled serum; D: extract of spiked (by all-*trans*-RA) pooled serum. The separation was achieved on two 3 × 3CR ODS cartridge columns (Perkin-Elmer Corp.) connected in series and eluted with methanol–water 75:25 (v/v, 1% ammonium acetate) at a flow rate of 1.0 ml/min. Detection wavelength was 340 nm, the sensitivity was 0.001 AUFS, and the slope setting was 100.

When standard 13-*cis*-RA (3.73 ng) was added to the serum and extraction method A and reverse-phase HPLC procedures were followed, peak b was increased to 5.4 ng (Fig. 1C). Similar results were obtained using normal-phase HPLC procedures. When standard all-*trans*-RA (2.10 ng) was added to serum and extraction method A and reverse-phase HPLC were followed, peak c was increased to 3.6 ng (Fig. 1D). Similar results were obtained using normal-phase HPLC procedures. Moreover, peak b (2.3 ng) in Fig. 1D and peak c (2.1 ng) in Fig. 1C were quantitatively identical to peaks b (2.4 ng) and c (2.0 ng), respectively, in Fig. 1B. Thus, the addition of standard all-*trans*-RA did not result in increased formation of 13-*cis*-RA in serum. Similarily, the addition of standard 13-*cis*-RA did not result in increased formation of all-*trans*-RA. All the chromatographic characteristics of peak b suggest that this compound is 13-*cis*-RA.

In vitro isomerization of RA

Kinetic study of the incubation of human intestinal mucosa with all-*trans*-RA (purity of 99.99%) showed that

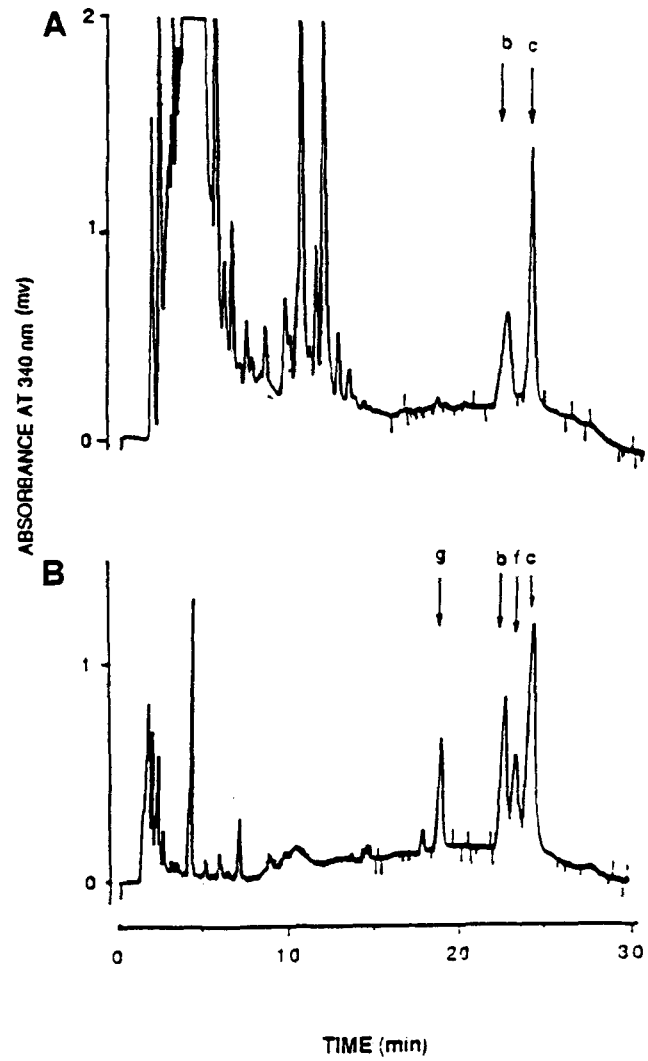

Fig. 2. Chromatograms obtained with normal-phase HPLC. A: Extract of human serum (4 ml, using extraction method A); B: a standard mixture of all-*trans*-RA (peak c), 13-*cis*-RA (peak b), 11-*cis*-RA (peak g), and 9-*cis*-RA (peak f). The separation was achieved on HS-3 silica cartridge columns (0.46 × 8.3 cm, Perkin-Elmer Corp.) with the gradient between solvents of hexane and dioxane as described in the text. The detection conditions were identical to the reverse-phase HPLC shown in Fig. 1.

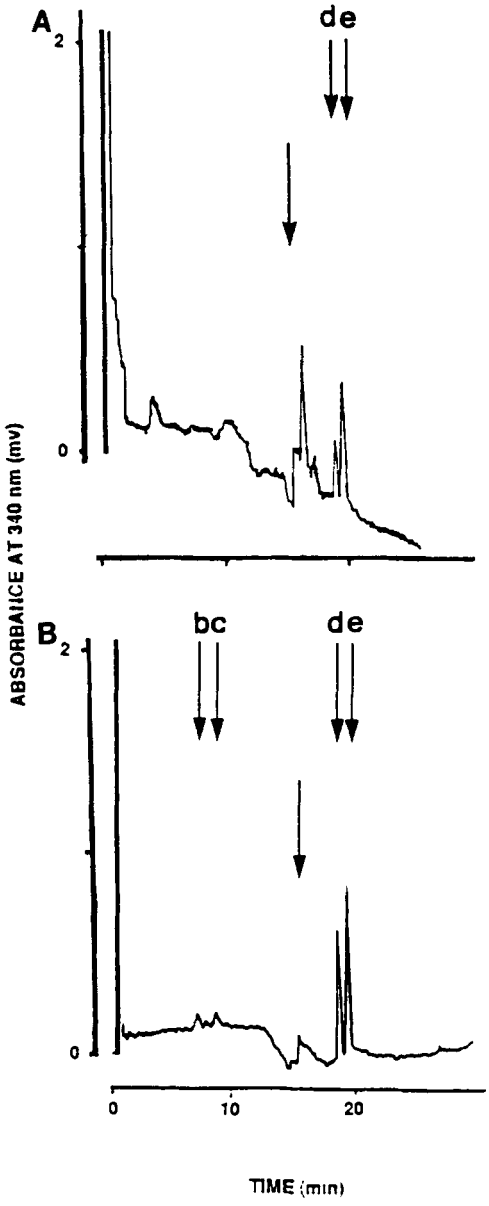

Fig. 3. Chromatograms obtained from diazomethane derivatizations. A: Extract of diazomethane methylated fraction of peaks b and c; B: methylated standard 13-*cis*-RA (peak d) and all-*trans*-RA (peak e). The detection conditions were similar to those in Fig. 1. The HPLC gradient program is described in the text. The straight line blip in the chromatogram, indicated by an arrow, is produced by the auto zero function of the detector.

the formation of 13-*cis*-RA approached maximum at 3 h, and at this time, the recovery of total RA was 94% (**Fig. 4**). The recovery of RA decreased markedly with longer incubation times. Therefore, a 3-h incubation was chosen as optimum time for this isomerization study. The 3-h incubations of boiled human intestinal mucosa homogenates and of blanks (without homogenates) with all-*trans*-RA (26 ng) showed the production of 0.9 and 1.0 ng of 13-*cis*-RA, respectively, while incubation at 4°C formed only 0.2 ng of 13-*cis*-RA. Thus, after the subtraction of 13-*cis*-RA formed from the controls (1.0 ng), the incubation of intestinal mucosa homogenates with all-*trans*-RA (26 ng) resulted in the formation of 2.8 to 8.3 ng of 13-*cis*-RA which corresponded to formation of 0.1 to 0.2 ng 13-*cis*-RA/h per mg protein. 13-*cis*-RA was the predominant *cis* isomer in the mixture (**Fig. 5**). The incubation of human serum with all-*trans*-RA for 3 h did not result in any detectable increment of the 13-*cis* isomer.

The appearance of 13-*cis*-RA was linearly dependent on the protein concentration of intestinal mucosa from the same subject (in a typical experiment, the equation of the regression line was $Y = 0.012 + 1.226X$, $r \pm 0.99996$) but not between individual subjects who had different protein concentrations. The results are shown in **Fig. 6**.

Serum RA determination

To analyze 13-*cis*-RA and all-*trans*-RA, which are labile and present in very low levels in human serum, a sensitive, quantitative, and automated method has been developed. The solid-phase extraction procedure was adapted to concentrate and isolate RA from retinol, the concentration of which is 150 times greater in serum compared to RA. The liquid–liquid double-phase extraction method was used for comparison, and the levels of RA obtained using this extraction procedure are shown in **Table 2**. The HPLC chromatograms using liquid–liquid double-phase extraction and the solid-phase extraction procedures, showed identical patterns, i.e., both 13-*cis*- and all-*trans*-RA were detected. However, the levels of 13-*cis*-RA and all-*trans*-RA obtained were higher when the liquid–liquid double-phase extraction was used than those obtained from solid-phase extraction (Table 2). Retinoyl β-glucuronide was extracted using liquid–liquid double-phase extraction. The result showed that less than 6% of it could be recovered and that more than 94% had been converted to all-*trans*-RA.

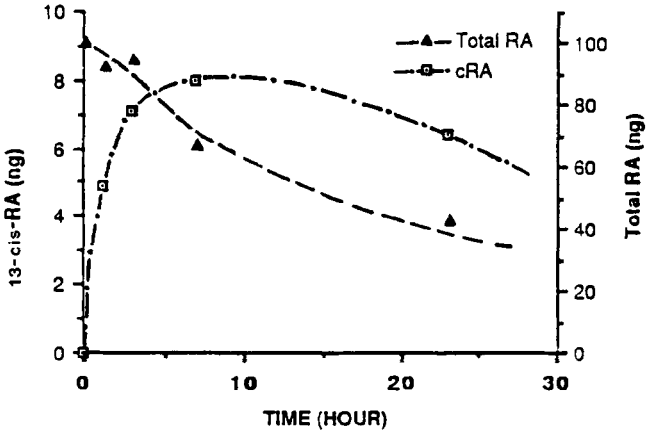

Fig. 4. Effect of incubation time on total RA and 13-*cis*-RA formation from all-*trans*-RA by human intestinal mucosa homogenate. Data represent means of triplicates with 12.4 mg protein.

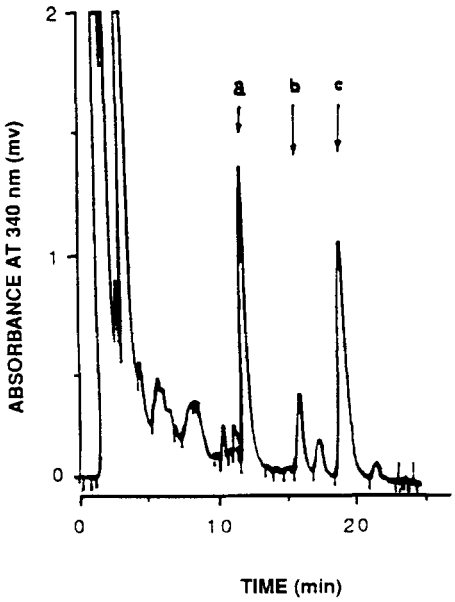

Fig. 5. Chromatogram obtained from isomerization of all-*trans*-RA to 13-*cis*-RA by human intestinal mucosa homogenates. HPLC conditions were similar to those in Fig. 1.

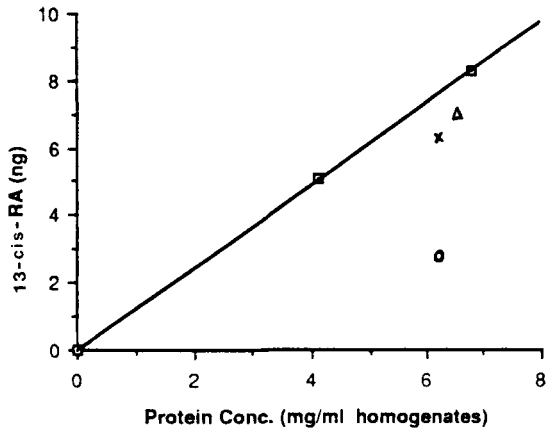

Fig. 6. Effects of the amount of human intestinal mucosa protein on the formation of 13-*cis*-RA from all-*trans*-RA. The symbols of square, triangle, cross, and circle represent different subjects.

13-*cis*-RA and all-*trans*-RA are polar hydrophobic compounds. Therefore, the solvent component of the HPLC system with the reverse-phase ODS column showed an effect on the resolution and peak shapes of 13-*cis*-RA and all-*trans*-RA. The solvent with less polarity gave shorter retention times, sharper peaks, and shorter interval times between peaks. The program which had a gradient time of 24 min from methanol–water 65:35 (v/v, 1% ammonium acetate) to 100% methanol was used for quantitative analysis since it gave the best resolution between these two isomers of RA. Because retinol is less polar and 150-fold more concentrated than RA in human serum, the isocratic system designed to separate 13-*cis*- and all-*trans*-RA was not suitable for continuous analysis of these compounds in serum. To avoid interference from the previous run, our gradient program was designed to elute all the polar, less polar, and nonpolar retinoids, complete the analysis in 30 min, and re-start the program immediately without needing to flush the column. A sensitive UV detector (0.001 AUFS) ensured a measurement as low as 0.1 ng. Both 13-*cis*-RA and all-*trans*-RA were detected in the sera of all of the 26 healthy volunteers. Our results by HPLC analysis after solid-phase extraction and using

TABLE 2. All-*trans*-retinoic acid (RA) and 13-*cis*-RA in human sera

Compound	Mean (n)	Min	Max	SD	Percentage of Total RA
		ng/ml			
Liquid–liquid double-phase extraction —— fasting sera					
All-*trans*-RA	2.7 (26)	1.5	3.8	0.6	27–60
13-*cis*-RA	2.7 (26)	1.7	4.4	0.7	40–73
Total RA	5.3 (26)	3.0	7.6	1.2	——
Solid phase extraction —— fasting sera					
All-*trans*-RA	1.4 (26)	1.1	1.9	0.2	41–64
13-*cis*-RA	1.4 (26)	1.0	2.2	0.3	36–59
Total RA	2.8 (26)	2.2	4.0	0.5	——
Solid phase extraction —— nonfasting sera[a]					
All-*trans*-RA	1.9 (10)	1.0	2.9	0.7	34–71
13-*cis*-RA	1.9 (10)	1.1	2.8	0.6	29–66
Total RA	3.8 (10)	2.9	5.5	0.8	——

[a]Blood was drawn 2 h after a nonstandardized breakfast.

TABLE 3. Analytical recovery of exogenous 13-*cis*-retinoic acid (RA) (8.4 ng) and all-*trans*-RA (14.9 ng) from pooled serum (2 ml, nonfasting)

Compound	Recovery	SD	CV	n	Range
	%	%	%		%
13-*cis*-RA	102	3.0	2.9	5	99–107
All-*trans*-RA	96.4	7.7	8.0	5	87–107

TMMP (peak a in Fig. 1A) as the internal standard showed that the serum concentration of all-*trans*- and 13-*cis*-RA in 26 fasting volunteers ranged from 1.1 to 1.9 ng/ml and 1.0 to 2.2 ng/ml, respectively, with both compounds having identical mean values of 1.4 ng/ml (Table 2). The percentage of 13-*cis*-RA in total RA showed individual variation which ranged between 36% and 59%, the mean value being 50%. The analytical recoveries from serum of added 13-*cis*-RA and all-*trans*-RA are shown in **Table 3**, and the precision of the method (CV) is given in **Table 4**. The results from ten nonfasting subjects showed the percentage of 13-*cis*-RA in total RA in serum to be between 29% and 66% with a mean value of 50%. After a meal, the concentration of total RA was 36% greater compared to fasting subjects. This indicates that the level of RA in human serum is influenced by dietary intake.

DISCUSSION

In the present report, we demonstrate that 13-*cis*-RA is a naturally occurring isomer of RA in human serum. This has been equivocal since 1967 when the identification of 13-*cis*-RA was first reported in rat liver extracts; however, the isolation procedure used in that report was found to result in extensive isomerization between all-*trans*- and 13-*cis*-RA (1). In 1985, all-*trans*- and 13-*cis*-RA at levels of 2.7 and 0.9 ng/ml in rat plasma were shown to be retinyl acetate metabolites under steady state conditions; the isomerization of all-*trans*-RA to 13-*cis*-RA during manipulation of plasma was found to be less than 3% (17). Recently, it has been reported that 75% of RA in human plasma was all-*trans*-RA and that 25% was made up of other isomers of RA (14). Although the best evidence for the existence of 13-*cis*-RA is its nuclear magnetic resonance spectrum, this procedure would require 0.1 mg of purified 13-*cis*-RA from serum (needing at least 100 liters of serum), which makes use of this technique unrealistic. Based on our observations using various isolation procedures, the use of standards in both normal-phase and reverse-phase HPLC systems, the use of UV spectra, and by methylation of serum extracts and standards, we conclude that 13-*cis*-RA is a normally circulating retinoid in human blood.

TABLE 4. Analytical precision of 13-*cis*- and all-*trans*-retinoic acid (RA) in pooled serum by HPLC analysis

	n	Mean	SD	CV
		ng/ml		%
Within day				
13-*cis*-RA	5	1.8	0.15	8.3
All-*trans*-RA	5	1.9	0.16	8.4
Between days				
13-*cis*-RA	6	1.8	0.17	9.4
All-*trans*-RA	6	1.5	0.10	6.7

Since the level of RA is extremely low, to assure the precision of our analysis, TMMP was used as an internal standard to quantitate the serum level of RA. This compound has the same side chain and terminal group as RA, a fact that is most important for the isolation procedure used here (Fig. 1A, peak a).

The liquid–liquid double-phase extraction method gave levels of RA that were 90% higher than that obtained by using the solid-phase extraction procedure. This might be explained by hydrolysis of blood retinoyl β-glucuronide. It has been demonstrated that retinoyl β-glucuronide is an endogenous compound in human serum with levels ranging from 1 to 5 ng/ml (mean = 2.42 ng/ml). The extraction procedures with hexane after treatment with a strong base and acid could convert much of retinoyl β-glucuronide in the serum to RA (13). Our experiment with standard retinoyl β-glucuronide also demonstrated the same result. Thus, the levels reported for liquid–liquid double-phase extraction (Table 2) may represent the sum of RA and retinoyl β-glucuronide. Moreover, since the levels of both 13-*cis*- and all-*trans*-RA are increased under this extraction method, it is possible that there might be endogenous 13-*cis*-retinoyl β-glucuronide in human blood as well.

The presence of 13-*cis*-RA as the predominant form of RA in human urine under normal physiological conditions recently has been reported (18). A prior study has demonstrated that 13-*cis*-RA is an in vivo metabolite of all-*trans*-RA in the intestinal mucosa of rats (17). In our experiments, human intestinal mucosa homogenates were used, and the data obtained presented unequivocal evidence of in vitro formation of 13-*cis*-RA from all-*trans*-RA. Although it is reasonable to assume that all-*trans*-RA can be converted to isomers other than 13-*cis*-RA (such as 13-*cis*-, 11-*cis*-, 9,13-di-*cis*-, etc.), our studies using the human small intestine and our analysis of human serum showed that 13-*cis*-RA is the predominant *cis* isomer. This is consistent with the isomerization patterns of all-*trans*-RA that were obtained by us in both photo and catalytic isomerization experiments of all-*trans*-RA (G. Tang and R. M. Russell, unpublished results).

Reversible *cis*-trans isomerization of RA (predominantly between 13-*cis*- and all-*trans*-RA) has been observed in tissues of vitamin A-sufficient rats (19). From our analysis, 13-*cis*-RA is the only detectable endogenous isomer of all-*trans*-RA in human serum. This is unlike the transformation pattern of all-*trans*-retinol in the liver and eye of rats and frogs. For example, in the pigment epithelia of frog and rat retina, retinol isomerase (20) converts all-*trans*-retinol to 11-*cis*-retinol in the dark; the isomerase in frog liver forms 9-*cis*- and 13-*cis*-, but not 11-*cis*-retinol (21). Thus, neither of these two isomerases could be the enzyme that catalyzes the isomerization of RA in human intestine. Although 13-*cis*-RA in human serum is most likely an isomerization metabolite of all-*trans*-RA, it is possible that 13-*cis*-RA is an oxidation metabolite of 13-*cis*-retinol as well.

In the visual cycle, the unique role of 11-*cis*-retinaldehyde in the formation of visual pigment is well known. Whether the isomerization of all-*trans*-RA to its 13-*cis*- isomer plays a role in the control of epithelial differentiation or whether there is any connection between human serum levels of 13-*cis*-RA and certain skin disorders awaits further study.

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