papers and notes on methodology

Quantification of retinoic acid by gas–liquid chromatography–mass spectrometry: total versus all-*trans*-retinoic acid in human plasma

Joseph L. Napoli,[1,*] Bikash C. Pramanik,[*] John B. Williams,[*] Marcia I. Dawson, [**] and Peter D. Hobbs[**]

Department of Biochemistry, The University of Texas Health Science Center, Dallas, TX 75235[*] and Bio-Organic Chemistry Laboratory, SRI International, Menlo Park, CA 94025[**]

Abstract An assay based on negative ion chemical ionization mass spectrometry has been developed to quantify retinoic acid in plasma or serum. The lower limit of detection is 75 pg (240 fmol); normal values of retinoic acid can be determined on as little as 40 μl of human plasma. The plasma concentrations of total retinoic acid in 12 healthy male volunteers taking no medication or vitamin supplementation ranged from 2.8 to 6.6 ng/ml; the mean was 4.9 ng/ml. The assay can be manipulated to measure all-*trans*-retinoic acid alone; about 75% of retinoic acid in human plasma or rat serum is all-*trans*-retinoic acid. Both retinol and retinoic acid can be quantified on the same 0.1-ml sample; the concentration of retinoic acid in human plasma or rat serum is at least 150-fold less than that of retinol. — Napoli, J. L., B. C. Pramanik, J. B. Williams, M. I. Dawson, and P. D. Hobbs. Quantification of retinoic acid by gas–liquid chromatography–mass spectrometry: total versus all-*trans*-retinoic acid in human plasma. *J. Lipid Res.* 1985. **26:** 387–392.

Supplementary key words negative ion chemical ionization • rat serum retinoic acid • 13-*cis*-retinoic acid • HPLC • retinoic acid isomers

Retinoic acid is an endogenous retinoid that is in the pathway of retinol (vitamin A alcohol) metabolism under physiological conditions (1). Retinoic acid supports the growth and epithelial differentiation activity of vitamin A in vivo, but cannot support the visual function (2). Retinoic acid stimulates the differentiation of stem cells into mature epithelial cells in vitro, as well as the differentiation of tumorigenic cells into non-tumorigenic cells (3). Therefore, retinoic acid may be the retinoid directly responsible for mediating the differentiation function of vitamin A. It is probable that several diseases are causally related to dysfunctions in the homeostasis, metabolism, and function of naturally occurring retinoids such as retinoic acid (4); and it is possible that decreased retinoic acid synthesis, or resistance to retinoic acid action, may increase risk of disease. A variety of synthetic retinoids, structurally based on retinoic acid, are undergoing development as experimental agents for use in clinical oncology and dermatology (5).

Sensitive and specific assays for retinoic acid concentrations in biological samples are needed for continued studies of the importance of retinoic acid to vitamin A activity, its possible role in the etiology of disease, and to determine the effects of the synthetic retinoids on the homeostasis and metabolism of the naturally occurring retinoids. Only a few attempts have been made to develop such assays. Nelson, Dehority, and Teague (6) have reported a colorimetric assay sensitive to sub-microgram levels, but did not detect retinoic acid in blood under physiological conditions. De Ruyter, Lambert, and De Leenheer (7) reported, using GLC–MS techniques, that human plasma contained retinoic acid concentrations of about 2 ng/ml; but, Chiang (8) validated a GLC–MS assay for retinoic acid based on electron impact mass spectrometry, which was sensitive to 1 ng/ml of plasma on a 10-ml sample, and could not detect retinoic

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acid in human plasma. More recently, De Leenheer, Lambert, and Claeys (9) developed an assay based on HPLC that is sensitive to 1 ng/ml, provided 3.5-ml samples are used, and reported all-*trans*-retinoic acid concentrations of 3.5 ng/ml in human plasma.

This communication reports an assay for retinoic acid in plasma or serum, based on negative ion chemical ionization (NCI) mass spectrometry, that is sensitive to less than 1 ng/ml of retinoic acid with a 0.1-ml sample. Moreover, the assay can be used to detect total retinoic acids or all-*trans*-retinoic acid, itself. Finally, the assay is amenable to measurements of retinol and retinoic acid on the same 0.1 ml of plasma or serum sample.

MATERIALS AND METHODS

Retinoids

Retinoic acid and retinol were purchased from Sigma Chemical Co., St. Louis, MO. [11-^{3}H]Retinol (1.95 Ci/mmol) was prepared by alkaline hydrolysis of [11-^{3}H]retinyl acetate, which was obtained from the Chemoprevention Program, Chemical and Physical Carcinogenesis Branch, National Cancer Institute, Bethesda, MD. All-*trans*-retinoic acid-4,4,18,18,18-d$_5$ was synthesized as described (10). 13-*cis*-Retinoic acid was a gift from Hoffmann-La Roche, Inc., Nutley, NJ. Methyl-d$_3$ all-*trans*-retinoate and methyl-d$_3$ 13-*cis*-retinoate were prepared by heating the appropriate acids (27 mg) at 50°C for 1 hr with 0.8 ml of Tri-Deutero-8 (Pierce Chemical Co., Rockford, IL). The structures were confirmed by UV absorbance and MS analyses. The purity of retinoids was monitored by HPLC; they were purified by HPLC as required.

Serum and plasma

Rat blood was obtained by decapitation of Sprague-Dawley males (120–150 g, Harlan, Indianapolis, IN) fed a stock diet. Human blood was obtained at mid-morning by venipuncture with EDTA-containing Vacutainers. Serum or plasma was recovered by centrifuging blood at 7800 g_{ave} for 15 min. Samples free of retinoids were prepared by irradiating plasma or serum at 4°C for 24–36 hr with a UV lamp (Sylvania germicidal lamp, G15T8) placed 10 cm from the top of a beaker containing the samples.

Extraction

Method A: 0.2 N HCl in methanol (1 vol) was added to the sample. After thorough mixing, the sample was extracted with three 0.5-ml portions of hexane. Recovery was 83% ± 2 (± SD, n = 10). Method B: ethanol (2 vol) and 0.1 M potassium phosphate buffer, pH 5.4 (4 vol) was added to the sample (11). After thorough mixing, the sample was extracted with three 0.5-ml portions of

hexane. Recovery was 73% ± 9 (± SD, n = 9). In each case, the solvent was removed from the combined hexane phases under a stream of nitrogen.

Methylation

The residue obtained from evaporation of the hexane phase was dissolved in methanol (50 μl), placed on ice, and excess diazomethane in diethyl ether (50 μl) was added. After 5 min, the solvents were evaporated under a stream of nitrogen. All work with diazomethane was done in a well-ventilated hood.

HPLC

A Dupont Zorbax-Sil normal-phase column (0.46 × 25 cm) was connected to a Waters Associates ALC/GPC 204 liquid chromatograph. Solvents were HPLC grade. Retinoids were monitored by UV absorbance at 340 nm. Three different mobile phases were used: HPLC-1, *tert*-butyl methyl ether–hexane 2:98 (v/v); HPLC-2, toluene–hexane 45:55 (v/v); HPLC-3, *tert*-butyl methyl ether–hexane 20:80 (v/v). Flow rates were 2 ml/min.

GLC–MS

A glass column (2 mm × 3 ft) packed with 3% SP2100-DOH on Supelcoport (100/120 mesh, Supelco, Bellefonte, PA) was used with a Finnigan Model 9610 gas chromatograph connected to a Finnigan Model 4021 mass spectrometer equipped with an INCOS 2000 data system. The column was eluted with methane at a flow rate of 20 ml/min and a temperature of 230°C. Chemical ionization was performed with methane as the reagent gas at a source pressure of 10^{-5} torr.

Assay of retinoic acid

All-*trans*-retinoic acid-d$_5$ (250 pg) in ethanol (10 μl) was added to the sample (40–100 μl) as internal standard. The sample was mixed thoroughly, allowed to stand for 15 min, extracted by method A, and methylated. The sample was dissolved either in methanol (5 μl) and applied to GLC–MS or in column solvent (100 μl), applied to HPLC, and eluted with mobile phase 1 or 2. The material recovered from HPLC was quantified by GLC–MS. All procedures were conducted under yellow light, with the exception of GLC–MS, which was conducted in dim light.

Combined assay of retinoic acid and retinol

All-*trans*-retinoic acid-d$_5$ (250 pg) in ethanol (10 μl) and [^{3}H]retinol (4000 dpm, 0.26 ng) in ethanol (10 μl) were added to the sample (100–200 μl). The sample was mixed thoroughly, allowed to stand for 15 min, and extracted by method B. The sample was applied to a DEAE-Sephadex A-25 column (0.2 g, 0.5 × 1.5 cm) in 0.5 ml of methanol. Retinol was eluted with a further 5.5 ml of methanol. Retinoic acid was eluted with 4 ml of formic acid–metha-

nol 2:98. Retinol was applied to HPLC, eluted with mobile phase 3 in 22 ml, and was quantified by peak height at 0.02 AUFS. A standard curve with five points from 10 to 60 ng of retinol was linear (correlation coefficient, $r > 0.99$, slope = 1, y-intercept = 0).

RESULTS AND DISCUSSION

The electron impact mass spectrum of methyl retinoate contains well over 200 peaks, most of which have not been assigned (12). To develop a sensitive assay for retinoic acid in samples of biological origin, we relied on chemical ionization MS. Chemical ionization MS ionizes a higher proportion of sample and can provide a much simpler spectrum than electron impact MS. Both factors afford greater sensitivity. Also, the molecular ion of a chemical ionization mass spectrum of a heat-tolerant, low molecular weight lipid is usually intense and is frequently the base peak. Depending on the nature of the sample, negative ions, as well as the more familiar positive ions, can be detected and are sometimes more intense than the positive ions.

The PCI mass spectrum of methyl retinoate, obtained with methane as the reagent gas, was somewhat simpler than its electron impact mass spectrum, but the sensitivity was approximately equivalent (**Fig. 1**). Interpretable peaks were noted at m/z 343 ($M^+ + C_2H_5$), 315 ($M^+ + H$), 283 ($M^+ - CH_3O$), and 255 ($M^+ - CO_2CH_3$). In contrast, the NCI mass spectrum of methyl retinoate had only one peak at m/z 314, representing its molecular ion. In addition, at least 10-fold greater sensitivity was obtained with NCI than with PCI. Like that of methyl
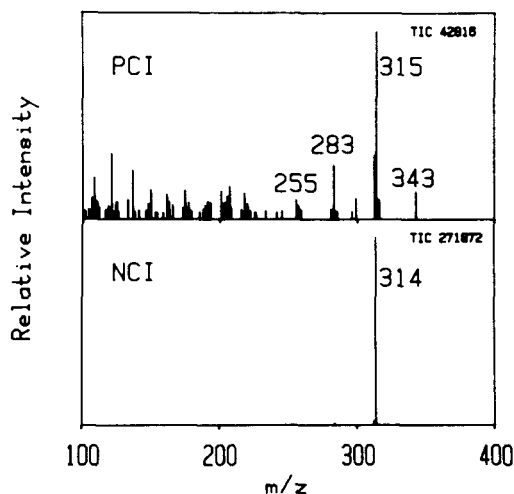

Fig. 1 Comparison of PCI and NCI mass spectra of methyl all-*trans*-retinoate: PCI, positive ion chemical ionization; NCI, negative ion chemical ionization. The samples (100 ng) were introduced into the mass spectrometer through the GLC column with methane as both GLC carrier and chemical ionization reagent gas.
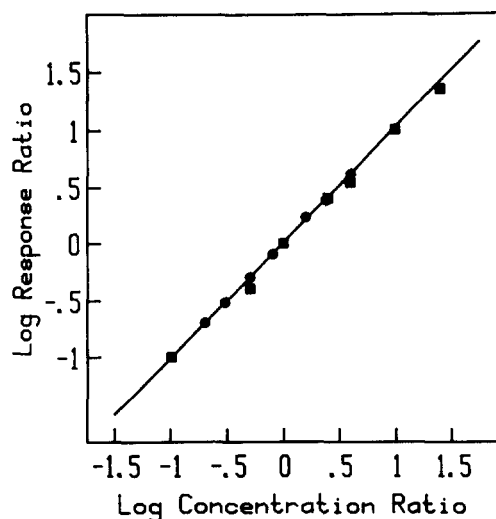

Fig. 2 Determination of the response factor. The log of the ratio methyl retinoate/internal standard detected by GLC–MS (y-axis) is plotted versus the log of the ratio methyl retinoate/internal standard injected onto the GLC–MS (x-axis). Two sets of data were obtained with two different amounts of the internal standard: 0.25 ng (●); 1.0 ng (■). Each set of data and the combined sets provided a straight line (correlation coefficient, $r > 0.99$) with a slope of 1.

retinoate, the NCI mass spectrum of the pentadeuterated internal standard had essentially one peak, but at m/z 319. Therefore, the assay was based on selective monitoring of the negative ions produced from methyl retinoate and methyl retinoate-d_5 at m/z 314 and 319, respectively.

To determine the response factor, the ratio of the amounts of methyl retinoate and the internal standard applied to GLC–MS was compared to the ratio of the areas of the peaks produced. A plot of the log of the response ratio versus the log of the concentration ratio, obtained using 250 pg of internal standard, was linear with a slope of 1 (response factor) for concentration ratios of 0.2 to 4 (**Fig. 2**). These ratios extend through the range obtained from human plasma samples assayed using 250 pg of internal standard. The response factor was also determined to be 1 for concentration ratios of 0.1 to 20 in the presence of 1 ng of internal standard, indicating that the response factor would also be valid with larger amounts of internal standard.

The accuracy of the assay was assessed by adding increasing quantities of retinoic acid and 250 pg of internal standard to aliquots of irradiated human plasma. The plasma samples were extracted by method A, methylated, and were quantified by GLC–MS. Without added retinoic acid or internal standard, no peaks at m/z 314 or 319 were observed. The values of retinoic acid detected agreed well with the amounts that had been added, as shown by a linear relationship between the two with a slope of 1 (**Fig. 3**). Fifty picograms of added retinoic acid were not detected, but 75 pg were (68 ± 7, mean ± SD, n = 4). These results demonstrate that there is no interference
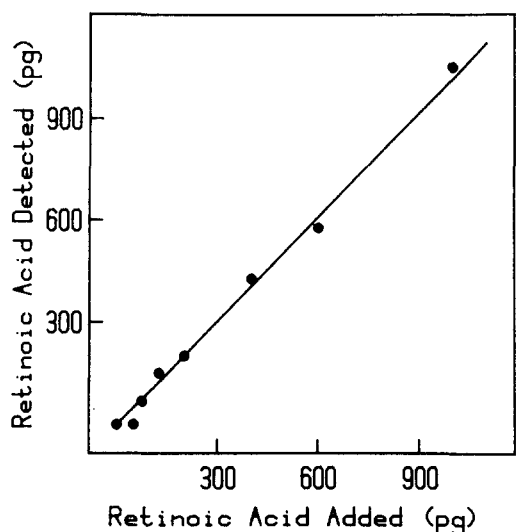
Fig. 3 Determination of accuracy. The amount of retinoic acid detected (pg) is plotted versus the amount of retinoic acid added to irradiated human plasma (pg). Experiments were done with 250 pg of internal standard. The data provide a straight line (correlation coefficient, $r > 0.99$) with a slope of 1 and a y-intercept of 0.

with the internal standard, the assay is accurate, and the lower limit of detection is 75 pg.

Representative mass fragmentograms are shown in **Fig. 4.** A mixture of 75 pg of retinoic acid and 250 pg of internal standard was methylated and applied directly to GLC–MS. The results were compared to a mixture of the same composition that had been added to irradiated human serum and processed through the direct GLC–MS assay (extraction method A, methylation, application to GLC–MS). The signal to noise ratios were high; the signal produced by 75 pg of retinoic acid that had been processed through the assay was readily measureable. Notably, these GLC conditions do not separate the isomers of methyl retinoate; for example, methyl 13-*cis*-retinoate co-elutes with the all-*trans* isomer (3.1 min). Therefore, this assay is a measure of total retinoic acid in plasma or serum.

The *intra*-assay variation (CV) for the direct GLC–MS assay was determined with a human plasma sample to be 5.3% (mean = 3.6 ng/ml, SD = 0.19, n = 10). The *inter*-assay variation (CV) was determined on a different human plasma sample in two separate experiments. In the first, the internal standard was added to aliquots of plasma, and the samples were frozen at −20°C under argon until they were assayed. Nine values were obtained over 14 days. The CV was 7.2% (mean = 5.0 ng/ml, SD = 0.36, n = 9). In the second, aliquots of the same plasma sample were stored similarly but without added internal standard; samples were also assayed over 14 days but with the addition of the internal standard on the day of assay. The CV was 6.4% (mean = 4.7 ng/ml, SD

= 0.3, n = 7). The similar values for the *inter*-assay CV in both cases and the similar mean values for the plasma retinoic acid concentrations ($P > 0.1$) indicate that, under these storage conditions, retinoic acid in plasma is stable for at least 2 weeks.

Twelve different human plasma samples were measured for total retinoic acid. They were obtained from healthy males in the age range of 24 to 35 years, who were not taking medication or vitamin supplementation. The mean value was 4.9 ng/ml and the range was 2.8 to 6.6 ng/ml.

A diagram of the methodology is shown in **Fig. 5.** An HPLC step can be incorporated into the direct GLC–MS assay to provide greater flexibility or further information. For example on HPLC with HPLC-1, the 13-*cis* isomer of methyl retinoate elutes close to the all-*trans* isomer (**Fig. 6A**). This elution pattern provides the opportunity to interject a purification step, so that the assay might be used to measure total retinoic acid in samples that might contain a higher level of contaminants, such as larger serum aliquots or extracts of cultured cells or tissues. In contrast, the 13-*cis* and all-*trans* isomers of methyl retinoate are well-resolved by HPLC with HPLC-2 (Fig. 6B). This provides the ability to measure only all-*trans*-retinoic acid, rather than total retinoic acid.

It is important to note that care must be taken during HPLC not to contaminate the sample by using either the analyte or the internal standard as chromatographic markers, which are usually injected in nanogram-sized
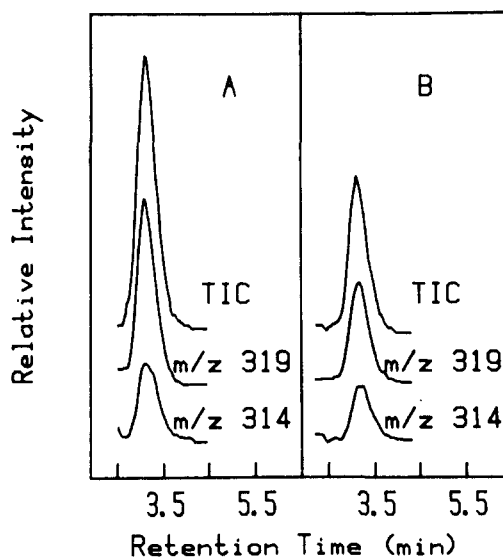

Fig. 4 GLC–MS responses of methyl retinoate and the internal standard. Mixtures of 75 pg of retinoic acid and of 250 pg of the internal standard were: A, applied directly to GLC–MS after methylation; or B, were added to irradiated human plasma (40 μl) and processed through the assay. The mass fragmentograms of methyl retinoate (m/z 314) and the internal standard (m/z 319) are shown plotted against the total ion current (TIC).

Plasma or Serum

Retinoic Acid

↓

internal standard

↓

0.2 N HCl/MeOH (1 vol.)
hexane (3 x 0.5 ml)

↓

methylate

↓

methyl retinoate

↓

GC/MS ← HPLC-1
         or
        HPLC-2

Retinoic Acid and Retinol

↓

internal standards

↓

ethanol (2 vol.)
0.1 M phosphate, pH 5.4 (4 vol.)
hexane (3 x 0.5 ml)

↓

DEAE Sephadex A-25
(0.5 x 1.5 cm)

2% HCO₂H/MeOH   MeOH

↓   ↓

retinoic acid   retinol

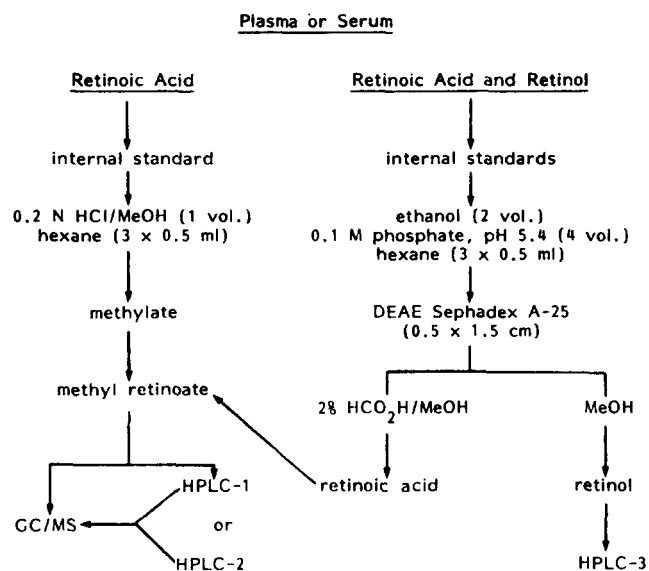   ↓

                       HPLC-3

Fig. 5 Diagram of optional analytical steps available in the assay of retinoic acid.

samples to determine elution positions on the HPLC columns. This is especially true with sensitive assays that measure picograms of analyte, because residual material left in the injector or on the column could cause inaccuracies. To avoid such contamination, the elution positions of methyl 13-*cis*-retinoate and methyl all-*trans*-retinoate were determined with trideuterated standards. The signal obtained in NCI from these species is at m/z 317. Therefore, it does not interfere with the measurement of the analyte or the internal standard.

Assays performed with added HPLC steps, using either HPLC-1 or HPLC-2, were applied to three different human plasma samples and one rat serum sample. Both the direct GLC–MS assay and the assay using HPLC-1 gave the same values for human plasma retinoic acid. Therefore, introduction of this HPLC step did not affect the results. However, introduction of HPLC-2, which separates all-*trans*-retinoic acid specifically, consistently produced values ~25% lower than either of the methods that measured total retinoic acid (**Table 1**). Approximately 75% of retinoic acid in human or rat blood was present as all-*trans*-retinoic acid; the exact isomeric composition of the remainder has not been determined, but it is possible that a substantial proportion was 13-*cis*-retinoic acid. The use of the internal standard, pentadeuterated retinoic acid, which is chemically equivalent to retinoic acid, excludes the possibility of isomerization during the assay and indicates that the blood itself contained isomers of retinoic acid.

It would be advantageous to be able to measure both retinol and retinoic acid in the same serum extract. The methodology developed for the quantification of retinoic

acid was modified so that retinol could also be measured. An internal standard for retinol, [³H]retinol, in addition to the internal standard for retinoic acid, was added to the samples. To avoid dehydrating retinol, a more mild extraction procedure, method B, was used, rather than the low pH method A. Retinol was separated from retinoic acid with a small DEAE column, and was quantified by elution from a normal-phase HPLC column with HPLC-3. Although retinoic acid could be measured by any of the previously described procedures, in this case, it was eluted through HPLC-1 and applied to GLC–MS. To illustrate the efficacy of this method, the concentrations of retinoic acid and retinol were measured in the combined sera of 15 rats and in a single human plasma sample. Data (ng/ml) are the mean ± SD of 5 to 13 replicates, and represent analytical, rather than biological variation. In the human sample, the retinoic acid concentration was 6.4 ± 0.6, whereas the retinol concentration was 741 ± 5. In the rats' sera, the retinoic acid concentration was 2.2 ± 0.2 and the retinol concentration was 736 ± 30. Note that the plasma/serum retinoic acid concentration is at least 150-fold less than the plasma/serum retinol concentration in both species. The retinol values were within the normal range (13–15).

In summary, this assay is the most sensitive one for quantifying retinoic acid reported to date. It is versatile and will allow quantification of total retinoic acids, all-*trans*-retinoic acid, or retinoic acid and retinol on the same
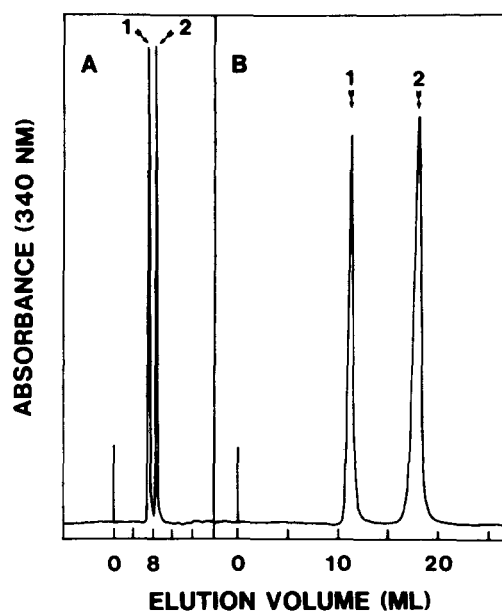

Fig. 6 HPLC of methyl retinoates. Methyl 13-*cis*-retinoate (*1*) and methyl all-*trans*-retinoate (*2*) were eluted from a normal-phase analytical column with: A, HPLC-1 (*tert*-butyl methyl ether–hexane 2:98) *1*, (7 ml), *2*, (8.5 ml); or B, HPLC-2, (toluene–hexane 45:55) *1*, (11.5 ml), *2*, (18.5 ml). Trideuterated methyl esters were used as chromatographic standards to avoid contaminating the sample or the internal standard.

TABLE 1. Concentrations of all-*trans*-retinoic acid relative to total retinoic acid in human plasma and rat serum

| Sample | Species | Total | | All-*trans* HPLC-2 |
		GLC–MS	HPLC-1	
		ng/ml		*ng/ml*
1	Human	5.3 ± 0.2[a,b]	5.9 ± 0.7[a,c]	4.1 ± 0.1[b,c]
2	Human	6.6 ± 0.2[d]	6.7 ± 0.9[d]	
3	Human	6.0 ± 0.2[e]		4.3 ± 0.1[e]
4	Rat		1.6 ± 0.1[f]	1.2 ± 0.1[f]

Separate extracts (extraction method A) of individual samples were analyzed directly by the GLC–MS assay, or were eluted through HPLC with mobile phase 1 (HPLC-1) before GLC–MS analysis to provide total retinoic acid. Alternatively, extracts were eluted through HPLC with mobile phase 2 (HPLC-2) to provide all-*trans*-retinoic acid. Values are the mean ± SD of three to five replicates.

[a,d] The two means having these superscripts were not significantly different, $P > 0.2$.

[b,c,e,f] The means having these superscripts were significantly different, $P < 0.001$.

0.1-ml sample. This sensitivity should be particularly useful where sample quantities are limited, such as in pediatric samples, or where quantification of low amounts of retinoic acid is desired. Our results indicate that human plasma has a total retinoic acid concentration of 4.9 ng/ml and an all-*trans*-retinoic acid concentration of about 75% of the total, i.e., 3.7 ng/ml and that the retinoic acid concentration is about 150-fold less than the retinol concentrations. ▮▮

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