All-*trans*-retinoic acid: measurement of reference values in human serum by high performance liquid chromatography

André P. De Leenheer,[1] Willy E. Lambert, and Ivan Claeys

Laboratoria voor Medische Biochemie en voor Klinische Analyse, Harelbekestraat 72, B-9000 Gent,[2] and Kliniek voor Inwendige Ziekten, Afdeling Endocrinologie, De Pintelaan 135, B-9000 Gent, Belgium[3]

Abstract A quantitative determination of physiological levels of all-*trans*-retinoic acid (vitamin A acid) in human serum has been developed. A double-phase extraction of 3.5 ml of serum followed by a specific and sensitive high performance liquid chromatography procedure allowed measurement of levels down to 1 ng/ml. Serum concentrations in 37 fasting volunteers ranged from 2.7 to 4.2 ng/ml and fitted a normal-Gaussian distributional shape with a mean value of 3.5 ng/ml and SD of 0.4 ng/ml, as demonstrated by the Kolmogorov-Smirnov test.—**De Leenheer, A. P., W. E. Lambert, and I. Claeys.** All-*trans*-retinoic acid: measurement of reference values in human serum by high performance liquid chromatography. *J. Lipid Res.* 1982. **23:** 1362–1367

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All-*trans*-retinoic acid (RA, vitamin A acid), a physiological metabolite of retinol (1), retains the growth-promoting and epithelial-differentiating activity of retinol (2, 3); however it cannot maintain reproduction (4) or vision (5). A highly specific retinoic acid binding protein (CRABP) isolated from rat tissues (6, 7) and chicken embryo skin (8, 9) was demonstrated to be elevated in human lung and breast tumors (10–12). This suggests a biochemical function for RA in the cell proliferating process (13). Furthermore, retinoic acid and its aromatic analogs inhibit both the incidence and the severity of chemically induced tumors (14–16). Up to now, a thorough study of the biochemical role of retinoic acid suffered from the lack of suitable analytical techniques (17). Consequently, retinoic acid was measured in biological materials only following administration of massive amounts of metabolic precursors, e.g., retinol (18, 19), retinyl acetate (20, 21) or retinoic acid itself (22, 23). We present a highly sensitive quantitative determination for RA in human serum. Reference values range from 2.7 to 4.2 ng/ml and fit a normal-Gaussian distribution curve.

EXPERIMENTAL

Materials

All-*trans*-retinoic acid was purchased from Fluka AG (Buchs, Switzerland). All-*trans*-13-demethylretinoic acid was supplied by Hoffmann-La Roche Inc., Nutley, NJ. All-*trans*-[15-^{14}C]retinoic acid (sp act 59 μCi/mg) was obtained from Amersham, Buckinghamshire, England. Reagents and solvents of analytical grade were purchased from Merck AG, Darmstadt, West Germany and were used without further treatment. The scintillation cocktail used was RIA Luma from Lumac, The Netherlands.

Laboratory precautions

All handling of reference compounds and biological samples was performed in a darkened room illuminated with yellow light. Whenever possible, amberized containers were used. Extraction and storage of the organic layer before evaporation were carried out at 4°C. Oxidative degradation was prevented by addition of butylated hydroxytoluene (0.025%) to the *n*-hexane used for extraction.

Isolation of all-*trans*-retinoic acid from serum

The assay required 3.5 ml of serum and consisted of a pre-extraction and a simple double-phase extraction at different pH values. Before extraction, 25 μl of an ethanolic solution of the internal standard (IS) all-*trans*-

Abbreviations: HPLC, high performance liquid chromatography; SD, standard deviation; RA, retinoic acid; CRABP, cellular retinoic acid binding protein; IS, internal standard; FFAP, free fatty acid phase; GLC–MS, gas–liquid chromatography–mass spectrometry; AUFS, absorption units full scale.

[1] To whom correspondence should be addressed.

[2] A. P. De Leenheer and W. E. Lambert.

[3] I. Claeys.

13-demethylretinoic acid (1.78 μg/ml) was added to the sample. After addition of 3.5 ml of ethanol and 1.5 ml of 2 N NaOH, the neutral and basic lipophilic constituents were extracted with *n*-hexane (7.0 ml) for 10 min at 4°C on a rotary mixer (Cenco Instruments, Breda, The Netherlands). Centrifugation separated the two phases and the organic layer was discarded. The aqueous layer was acidified with 3.0 ml of 2 N HCl and reextracted for 10 min at 4°C with 7 ml of *n*-hexane. After evaporation of the *n*-hexane layer under reduced pressure (Rotary Evapo-Mix, Buchler Instruments, Fort Lee, NJ), the residue was redissolved in 100 μl of the chromatographic solvent. Finally a 50-μl aliquot was injected on the HPLC column.

Peak identification

Repetitive injections of a serum extract with detection at different wavelength settings allowed the reconstruction of a UV spectrum of the peak of interest. Additional evidence was sought in rechromatography of the collected peak, both on a reversed phase system and on the straight phase system after diazomethane treatment. Finally, the collected serum peak was treated with diazomethane and analyzed by GLC–MS. An electron impact LKB 9000S apparatus with multiple ion detection device was equipped with a 1% FFAP (1.80 m × 2 mm) column coated on Gas Chrom Q (100–120 mesh). Temperatures were 210°C for the injection port, 190°C for the oven, 265°C for the separator, and 270°C for the ion source. Electron energy was 20 eV and trap current was 60 μA. The multiple ion (MID) detection device was focused on the ion *m/z* 314 (M^+, methylretinoate) at an acceleration voltage of 3500 V. Helium at a flow rate of 30 ml/min was used as carrier gas.

HPLC determination of retinoic acid

The samples were analyzed on a high performance liquid chromatographic system consisting of a Pye Unicam LC_3-XP (Cambridge, England) pump, a sampling valve (Model CV-6-UHPa-N60, Valco Instruments Co., Houston, TX) with a 50-μl loop and a Pye Unicam LC_3-UV variable wavelength detector set at 350 nm and used at the maximum sensitivity (0.005 AUFS). A 15 × 0.32-cm ID column was home-packed with 5μm RSIL (RSL, St.-Martens-Latem, Belgium). Elution was performed with a mixture of petroleum ether–acetonitrile–acetic acid 99.5:0.2:0.3 (v/v) at a flow rate of 0.75 ml/min.

In the reversed phase HPLC system, the column (10 × 0.2 cm ID) was filled with Lichrospher RP-8 (Merck AG, Darmstadt, West Germany) and was eluted with a mixture of methanol–water–acetic acid 89.7:10:0.3 (v/v) at a flow rate of 0.2 ml/min (24), or with a mixture of acetonitrile–water–acetic acid 79.6:20:0.4 (v/v) at a flow rate of 20 ml/hr.

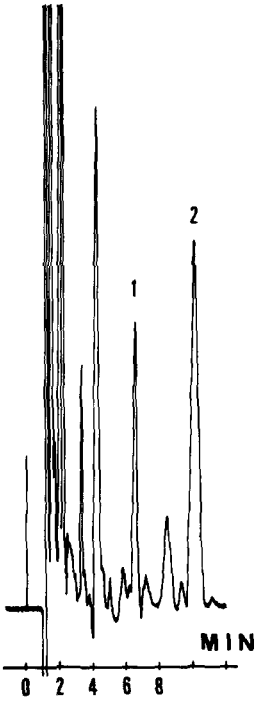

Fig. 1. High performance liquid chromatographic profile of a serum extract under following conditions: column dimensions, 15 cm × 3.2 mm ID; packing material, RSIL 10 μm; eluent: petroleum ether–acetonitrile–acetic acid 99.5:0.2:0.3 (v/v); flow rate, 0.75 ml/min; detection wavelength, 350 nm; temperature, ambient; sensitivity setting, 0.005 AUFS. Peak identity: 1), all-*trans*-retinoic acid; 2), all-*trans*-13-demethylretinoic acid.

Calibration

Calibration was performed by analyzing 3.5-ml aliquots of a serum pool, supplemented with known amounts of all-*trans*-retinoic acid (1.26, 2.40, 4.10, 5.80, and 8.77 ng/ml). Linear standard curves were constructed by plotting the peak height ratios (RA/IS) versus the amount of retinoic acid.

RESULTS AND DISCUSSION

Peak identification

The primary aim of this investigation was a systematic analysis of a number of serum samples from healthy volunteers in order to estimate the normal physiological serum levels of retinoic acid. Two major problems always hampered the development of quantitative assays for retinoic acid, i.e., the extremely low levels and the susceptibility of the compound towards isomerization and oxidative degradation during its manipulation. A very sensitive HPLC procedure with a high separating power (**Fig. 1**) allowed this quantitative work.

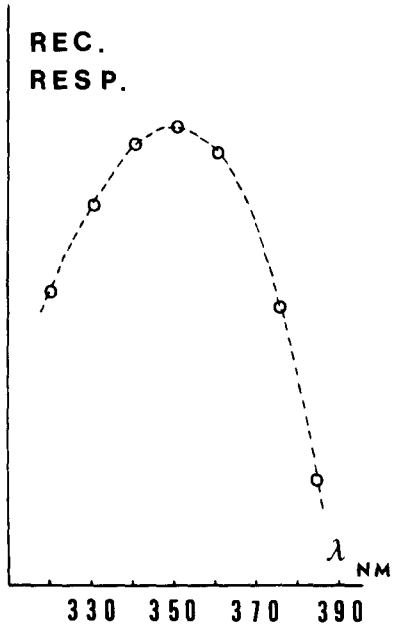

Fig. 2. Recorder response as obtained from repetitive injections of a serum extract on the RP 8 column eluted with methanol–water–acetic acid 89.7:10:0.3 (v/v), and detected at different wavelength settings (λ in nm).

Positive evidence for the identity of the compound was obtained by several techniques. The reconstructed UV spectrum as obtained from repetitive injections of a serum extract showed a maximum at 350 nm, the absorption maximum of retinoic acid (**Fig. 2**).

Fig. 3. HPLC recording of the fraction collected from the silica system and rechromatographed on the RP 8 column eluted with acetonitrile–water–acetic acid 79.6:20:0.4 (v/v) at a flow rate of 20 ml/hr, detection at 350 nm, and sensitivity at 0.005 AUFS. The retention time of the "unknown" corresponded to the retention time of all-*trans*-retinoic acid standard.

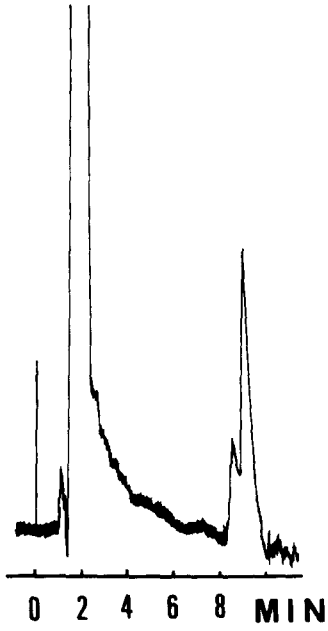

Fig. 4. HPLC recording of the fraction collected from the silica system and treated with diazomethane. The chromatographic conditions were as in Fig. 3. The retention time of all-*trans*-methylretinoate standard was 9.1 min.

By rechromatography of the collected peak on the reversed-phase system again, the "unknown" compound displayed retention characteristics identical with a retinoic acid reference standard (**Fig. 3**). Alternatively, treatment of the trapped peak with diazomethane resulted in a pronounced shift of its retention time, which now corresponded to that of a methyl retinoate standard (**Fig. 4**).

Finally, the electron impact mass spectrum (20 eV) displayed the molecular ion of pure methylretinoate (*m/z* 314) and fragment ions at *m/z* 299 and 255 $(M\text{-}CH_3)^+$ and $(M\text{-}COOCH_3)^+$ (**Fig. 5**). From the above experiments it is clear that there is no doubt about the identity of the peak ascribed to retinoic acid. However, the identity of the peak eluting after 4 min is still unknown. This compound has no direct relationship to retinoic acid since this peak remained quantitatively unaffected in serum supplemented with all-*trans*-retinoic acid. Furthermore this peak is very well separated from all-*trans*-retinoic acid and does not interfere in the analysis.

Internal standard

All-*trans*-13-demethylretinoic acid is used as internal standard and compensates for possible losses caused by evaporation or spilling. This compound behaves as an ideal internal standard during sample pretreatment and chromatography, as predicted from its close structural analogy with retinoic acid. Physicochemical properties of both substances are indeed remarkably similar and

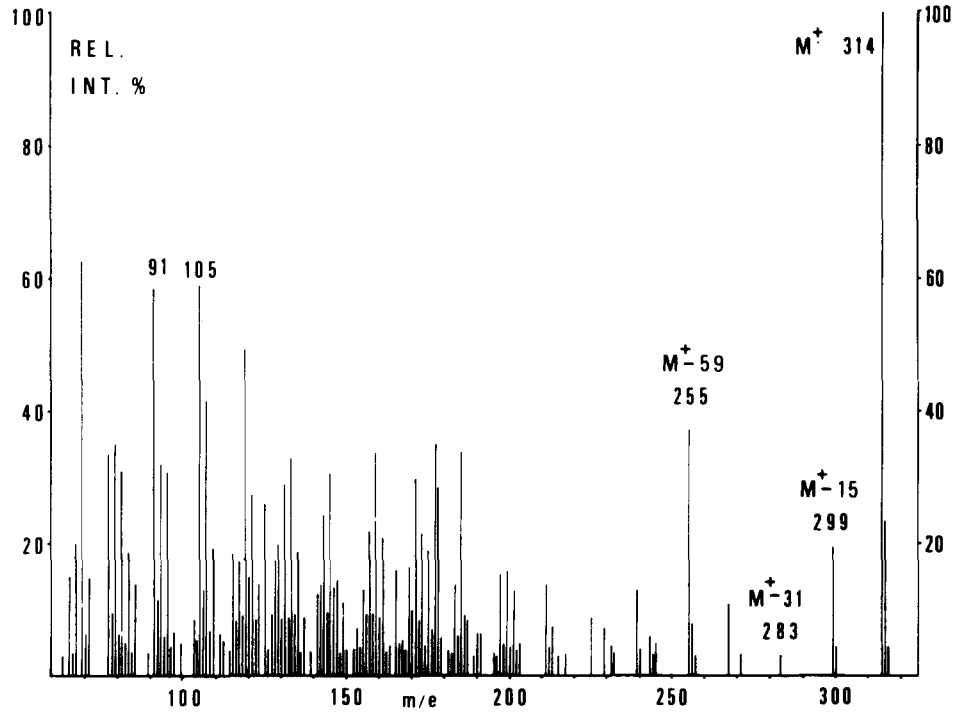

Fig. 5. Electron impact mass spectrum (20 eV) of the diazomethane-treated fraction from the silica system.

the compounds are well resolved in the present chromatographic system. No serum components were found to interfere with the elution position of the demethylated homolog.

Degradation

In the absence of special precautions, peaks of 13-*cis*, 9-*cis*, and 11,13-*cis,cis* isomers appeared in the chromatogram. They were all readily separable from all-*trans*-retinoic acid as indicated in **Table 1** but did not occur under the present conditions. Also during chromatography in the acidic eluent, no formation of isomers was observed as demonstrated by several injections of pure all-*trans*-retinoic acid resulting in one single peak of the all-*trans* isomer. Other vitamin A analogs, including 5,6-epoxyretinoic acid, a potential degradation product of retinoic acid, did not interfere.

Linearity, recovery and precision

A linear relationship was found up to 10 ng/ml. In a typical experiment, the equation of the regression line was $y = 0.0848x + 0.2309$, $r = 0.9984$. The significant intercept of the standard curve is attributable to the endogenous all-*trans*-retinoic acid present in the serum pool. Analyzing 3.5 ml of serum, quantitation even down to 1 ng/ml is possible, whereas the detection limit is estimated at 300 pg/ml. In view of the complexity of the biological matrix, the sensitivity of the present system approaches the ultimate limits of HPLC with on-line UV-detection. The overall recovery was determined by addition of known amounts (2.4, 3.6, 4.7, and 5.8 ng/ml) of [15-^{14}C]retinoic acid to 3.5-ml aliquots of a serum pool. Two samples of each concentration were analyzed. After chromatographic separation the all-*trans*-retinoic acid peaks were collected in a counting vial; subsequently the organic phase was evaporated and the residue was redissolved in 7.5 ml of a suitable scintillation mixture. After counting on a Packard Tri-Carb scintillation counter (Model 3390), an overall recovery of 69.0 ± 5.2% (n = 8) was found. Within-day reproducibility (CV, coefficient of variation) of the method was evaluated by nine replicate analyses of the same serum sample and averaged 6.9% ($\bar{x}$ = 2.7 ng/ml). Day-to-day precision (CV) over a period of 10 days was 10.2% ($\bar{x}$ = 2.9 ng/ml). These data are quite acceptable considering the extremely low levels of the compound

TABLE 1. Chromatographic parameters of related compounds

Compound	*k'*	Retention time
		min
Beta-carotene	<1	<1.5
13-*cis*-Retinoic acid	3.34	5.1
9-*cis*-Retinoic acid	3.86	5.6
All-*trans*-retinoic acid	4.25	6.5
11,13-*cis,cis*-Retinoic acid	7.60	7.2
All-*trans*-13-demethyl retinoic acid	11.75	10.2
5,6-Epoxyretinoic acid	12.89	15.8
Retinol	30.10	33.0

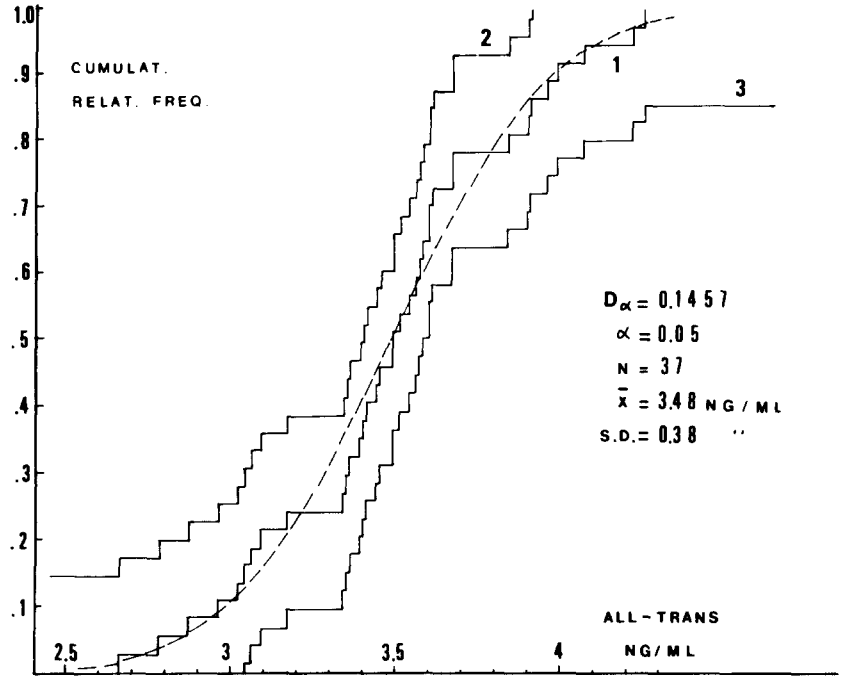

Fig. 6. Kolmogorov-Smirnov test for the results of the retinoic acid analysis. The broken curve represents the theoretical cumulative distribution, stepfunction 1 represents the sample cumulative distribution; the highest allowable deviation (D_α) between these two distributions is given by stepfunctions 2 and 3.

to be assayed. Serum levels in 37 fasting healthy volunteers ranged from 2.7 to 4.2 ng/ml, with a mean value of 3.5 ng/ml. These data fitted a normal-Gaussian distribution curve within 95% confidence limits, determined by the Kolmogorov-Smirnov test (25, 26) as shown in **Fig. 6**. This test is an efficient study of the distribution of a population when the chi-square test is not applicable due to the lack of sufficient data.

Improvement of our earlier presented method (24) by incorporation of an internal standard, a linearity study in the low nanogram range, and modification of the extraction procedure, allowed quantitation of physiological serum levels of all-*trans*-retinoic acid and also the separation of different *cis*-isomers. In a recent paper (17), Chiang described a GLC–MS procedure for low levels of retinoic acid. However, possible incomplete derivatization in the serum extract itself resulted in the failure to detect retinoic acid under physiological conditions. Other workers in this field also failed to meet this analytical challenge. They were dealing with levels arising from the administration of massive pharmacological doses of either 13-*cis* or all-*trans*-retinoic acid. Measurement of normal endogenous levels of this compound is extremely important as it enables future fundamental research on the biochemical mode of action of vitamin A. In addition, the chromatographic system shows great potential for the analysis of various other biological samples. Minor modifications in the extraction procedure could make the method useful in the study of retinoic acid levels both in serum and tumor tissues of patients with different stages of neoplastic diseases. The well documented biological activity of retinoic acid and the low circulating levels, kept within a narrow range, as clearly demonstrated by our results, suggest a possible hormonal function for this compound. In addition, the existence of a binding protein specific for retinoic acid supports the same hypothesis. More experimental work is needed to further elucidate the mode of action and the nature of the target tissues involved.

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