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DETERMINATION OF RETINOIC ACID (13-*cis*- AND ALL-*trans*-) AND AROMATIC RETINOIC ACID ANALOGS POSSESSING ANTI-TUMOR ACTIVITY, IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

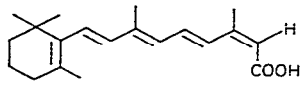
A sensitive and specific high-performance liquid chromatographic assay was developed for the determination of 13-*cis*- and all-*trans*-retinoic acid in blood or urine with an overall recovery of 90 $\pm$ 5.0% and a limit of detection of 10–20 ng/ml of sample. The method provides for rapid and simple quantitation of the compounds using 1 ml of blood.

The assay was applied in the determination of blood levels of 13-*cis*-retinoic acid in the dog following intravenous and oral administration of 9.5 mg/kg and 2.0 mg/kg doses, and in man following a single 100-mg oral dose and following divided daily doses totalling 2 mg per kg of body weight. The assay is also applicable with minor modifications to the determination of a series of aromatic retinoic acid analogs of clinical interest as anti-tumor agents.

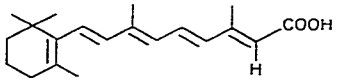
INTRODUCTION

Biochemical interest in vitamin A acid was initially stimulated by the discovery that the compound can be substituted for vitamin A (retinol) in its general tissue functions as a growth promoter in vitamin A-deficient rats[1], but not in its role in vision[2]. Thus, rats supplemented with vitamin A acid grew as normally as those fed on vitamin A (ref. 3), but became blind, indicating that retinoic acid was not reduced to either retinol (vitamin A) or retinal (aldehyde) which are precursors in the biosynthesis of rhodopsin, an essential visual pigment[4].

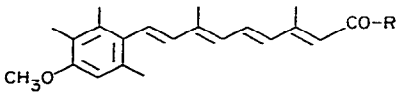
Current clinical interest in retinoic acid analogs[5] is directed to the fact that 13-*cis*-retinoic acid (I in Fig. 1) (Ro 4-3780) administered orally has shown encouraging systemic therapeutic effect on chemically induced benign and malignant epithelial tumors (skin papillomas and carcinomas) in animals[6], and in certain lung and bladder carcinomas in man[7,8], whereas all-*trans*-retinoic acid (II) (Ro 1-5488) is the active agent in Airol® (Roche) used in acne therapy. The aromatic retinoic acid analog, compound III is of clinical interest as an anti-tumor agent in epithelial tissues in mice[9–11] and as a systematically effective anti-psoriatic in man[12]. Similarly, compound



13-CIS-RETINOIC ACID [I]
(Ro4-3780)



TRANS-RETINOIC ACID [II]
(Ro1-5488)



AROMATIC RETINOIC ACID ANALOGS
R = $-OC_2H_5$ = Ro10-9359 [III]
= -OH = Ro10-1670 [IV]
= $-NHC_2H_5$ = Ro11-1430 [V]

Fig. 1. Chemical structures of 13-*cis*-, all-*trans*-retinoic acids and some aromatic retinoic acid analogs.

V is also active as an anti-tumor agent in mice[13] and as a topically effective anti-acne agent in man[14].

The need to monitor clinically effective blood levels of patients undergoing oral therapy and the pharmacokinetic profile of 13-*cis*-retinoic acid required the development of a suitable chemical assay which is both sensitive, specific and amenable to rapid routine analysis. Although spectrophotometric assays have been reported for the determination of vitamin A acid in plasma[15,16], they are nonspecific, relatively insensitive and cumbersome to perform on a routine basis. The classical column chromatographic–spectrophotometric/fluorometric assays for the determination of carotenoids are time-consuming, nonspecific, and unreliable due to endogenous interferences[17]. Gas chromatographic analysis was used following derivatization with silylating reagents, but is also prone to error due to thermal instability of the derivatives[18,19].

High-performance liquid chromatography (HPLC) appeared to be the method of choice, since these compounds can be analyzed at ambient temperatures without chemical derivatization and exploiting their ultraviolet (UV) absorption spectrophotometric properties for quantitation. This HPLC technique has been successfully used in the analysis of vitamin A in bulk chemical products and pharmaceutical formulations[18], in the analysis of phytoene (an orally administered sunscreen) in blood[20], retinol in serum[21], and of retinal isomers[22], using either adsorption (silica gel) or reversed-phase (ODS) chromatography.

Biotransformation of retinoic acid is complex due to the endogenous contribution of naturally occurring carotenoids (retinol, retinal) which produce retinoic acid, coupled with that fraction that is orally administered as such[23–26]. The complex metabolic pathways of *trans*-retinoic acid were elucidated by Hanni *et al.*[27,28] who verified the formation of short-chain metabolites by oxidative decarboxylation and cyclization to form furanone structures and the formation of several polar metabolites by ring

oxidation to form cyclohexanone derivatives and oxidation of the 5-methyl group to a primary alcohol. All these metabolites were isolated as conjugates in urine only and have not as yet been identified in blood as directly extractable moieties which could impair the specificity of a chemical assay. The aromatic retinoid III undergoes analogous biotransformation in man[29].

The method presented herein quantitates both 13-*cis*- and *trans*-retinoic acids using their UV absorbance at 365 nm to advantage, since neither β-carotene ($\lambda_{max.}$ = 445 nm) nor vitamin A (retinol) ($\lambda_{max.}$ = 325 nm) absorb significantly at 365 nm. Furthermore, they are all chromatographically resolved, thus imparting further specificity to the assay. A series of aromatic retinoic acid analogs, compounds III, IV, and V in Fig. 1, can also be assayed using minor modifications (Fig. 2).

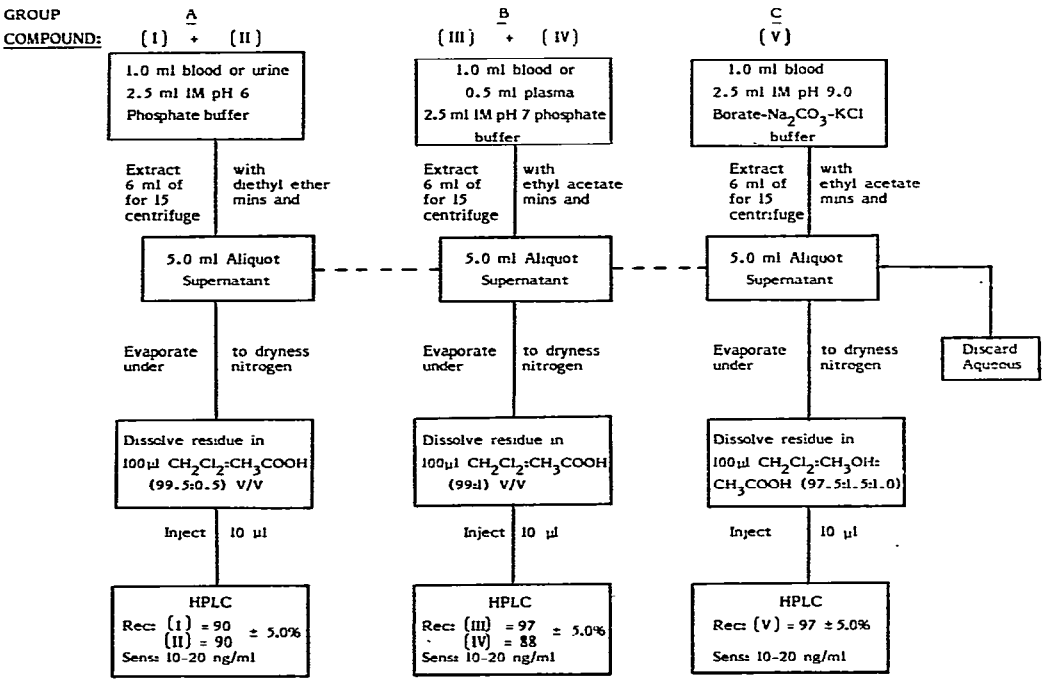


Fig. 2. Flow diagram for the analysis of compounds I, II, III, IV and V.

EXPERIMENTAL

Column

The column used was a prepacked 0.25 m × 4.6 mm I.D. stainless-steel column containing 10-μm Partisil silica gel, generating 25,500 plates per m (Whatman, Clifton, N.J., U.S.A.).

Instrumental parameters

A DuPont Model 830 high-pressure liquid chromatograph equipped with a Model 835 multiwavelength UV detector using a medium-pressure mercury lamp.

operated at 365 nm, and a Model U6K loop injector (Waters Assoc., Milford, Mass., U.S.A.) was used. The isocratic mobile phase used was a mixture of methylene chloride–glacial acetic acid (99.5:0.5) at a constant flow-rate of 2.2 ml/min. The column was operated at ambient temperature. Under these conditions the retention times of I and II were 2.7 and 3.5 min, respectively. The detector sensitivity was 4×10^{-2} a.u.f.s., and the chart speed on the 1.0-mV Honeywell recorder (Model 194) was 30 in./h. Under these conditions 20 ng of compounds I and II per 10 μl injected give nearly full-scale pen response. The minimum detectable amount of each compound is 10–20 ng/ml of blood or urine. The isocratic mobile phase for compounds III and IV was a mixture of methylene chloride–glacial acetic acid (99:1), and the mobile phase for compound V was a mixture of methylene chloride–methanol–glacial acetic acid (97.5:1.5:1.0). The instrumental parameters are the same for all compounds.

Analytical standards

All analytical standards were of pharmaceutical grade purity (>99%). These included compound I (13-*cis*-retinoic acid), 2-*cis*-9(2,6,6-trimethylcyclohexene-1-yl)-3,7-dimethyl-2,4,6,8-nonatetraenoic acid, $C_{20}H_{28}O_2$, M.W. = 300.42 (Ro 4-3780); compound II (all-*trans*-retinoic acid), 2-*trans*-9(2,6,6-trimethylcyclohexene-1-yl)-3,7-dimethyl-2,4,6,8-nonatetraenoic acid, $C_{20}H_{28}O_2$, M.W. = 300.42 (Ro 1-5488); compound III, ethyl-all-*trans*-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoate, $C_{23}H_{30}O_3$, M.W. = 354, m.p. = 88–89° (Ro 10-9359); compound IV, all-*trans*-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoic acid, $C_{21}H_{26}O_3$, M.W. = 326, m.p. = 225–227° (Ro 10-1670); compound V, all-*trans*-N-ethyl-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenamide, $C_{23}H_{31}O_2N$, M.W. = 353.57, m.p. = 179–180° (Ro 11-1430).

Preparation of standard solutions

All analyses must be performed in a darkened room under yellow lighting and all glassware used must be amberized (low actinic) to prevent photoisomerization of these compounds.

Weigh out 10 mg of compounds I–V in separate 10-ml volumetric flasks and dissolve in 10 ml of methylene chloride to yield a stock solution A containing 1 mg/ml. Make serial dilutions of solution A to yield separate series of working solutions B_1–B_4 containing the concentrations of each pair of compounds I and II, III and IV, and V per 0.1 ml of their respective mobile phases as indicated in Table I.

TABLE I

PREPARATION OF STANDARD SOLUTIONS OF COMPOUNDS I–V FOR HPLC ANALYSIS

Standard solution	*ng of compound per 0.1 ml of solution*				
	Group A		*Group B*		*Group C*
	I	*II*	*III*	*IV*	*V*
B_1	25	25	50	100	25
B_2	50	50	100	200	50
B_3	75	75	150	300	75
B_4	100	100	200	400	100

Aliquots (10 μl) of these solutions are injected as external standards for establishing the LC parameters. Aliquots (0.1 ml) of the same solutions are added to blood as the internal standard calibration curve for the determination of the concentration in the unknowns and for the determination of percent recovery.

Calibration of compounds I–V by HPLC

A calibration (external standard) curve of either the peak height (manual) or peak area (digital integration) *versus* concentration of compounds I–V per 10 μl of standard solution is prepared as given in Table I. The linear range of quantitation is from 5 to 1000 ng of each compound. A fresh calibration curve of the external standards and of the recovered internal standards is prepared for each day of analysis to establish the reproducibility of the HPLC system.

Reagents

All reagents must be of analytical reagent grade (>99% purity). The inorganic reagents used are 1.0 *M* phosphate K_2HPO_4–KH_2PO_4 buffers of pH 6.0 and 7.0 and 1.0 *M* borate–Na_2CO_3–KCl buffer pH 9.0 prepared as follows.

(1) 1.0 *M* (pH 6.0) phosphate buffer is prepared by titrating 1 *M* KH_2PO_4 (136.09 g/l) with 1 *M* anhydrous K_2HPO_4 (174.18 g/l) until pH 6.0 is reached. Mix well and check final pH with a pH meter.

(2) 1.0 *M* (pH 7.0) phosphate buffer is similarly prepared as the pH 6.0 phosphate buffer but is titrated to pH 7.0. Mix well and check final pH with a pH meter.

(3) 1.0 *M* H_3BO_3–Na_2CO_3–KCl buffer (pH 9.0) is prepared as follows: Dissolve 61.8 g of boric acid (H_3BO_3) and 74.6 g of KCl per liter of distilled water Dissolve 106 g of anhydrous Na_2CO_3 per liter of distilled water. To 630 ml of the boric acid–KCl solution, add 370 ml of the Na_2CO_3 solution to make a liter of buffer solution. Shake well and check pH, buffer it up to pH 9.0, if necessary, with the Na_2CO_3 solution and mix well. Store at 37° to prevent crystallization of the salts.

The organic solvents used are methylene chloride (Mallinckrodt, St. Louis, Mo., U.S.A.; nanograde) methanol (Fisher Scientific, Pittsburgh, Pa., U.S.A.), and glacial acetic acid (J. T. Baker, Phillipsburg, N.J., U.S.A.), diethyl ether (Mallinckrodt; absolute) and ethyl acetate (Mallinckrodt; nanograde).

Procedure

The flow diagram of the extraction procedure for compounds I–V is shown in Fig. 2. Into a 15-ml amberized centrifuge tube (PTFE no. 16 stoppered), add 1.0 ml of whole blood, 2.5 ml of the respective buffer, and mix well on a vortex action mixer. Extract the mixture with 6 ml of diethyl ether for I and II, or ethyl acetate for III, IV, or V, by shaking for 15 min on a reciprocating shaker (Eberbach, Ann Arbor, Mich., U.S.A.) at 80–100 strokes per min. Along with the samples process a specimen of control blood and four 1.0-ml specimens of control blood containing 0.1 ml of the respective standard solutions B_1, B_2, B_3, or B_4 for each assay, respectively. Centrifuge the samples at 2500 rpm (1500 *g*) in a refrigerated centrifuge (Model PR-J, rotor 253; Damon/IEC Corp., Needham, Mass., U.S.A.) at 5° for 10 min and transfer a 5.0-ml aliquot of the upper organic layer into another amberized 15-ml conical centrifuge tube. Evaporate the organic layer to dryness at 30° in a N-EVAP evaporator (Organo-

mation Assoc., Worcester, Mass., U.S.A.) under a stream of clean, dry nitrogen. Dissolve the residues in a 100-μl aliquot of the respective mobile phases used in each assay and inject a 10-μl aliquot. Typical chromatograms of blood extracts for the respective assays are shown in Fig. 3.

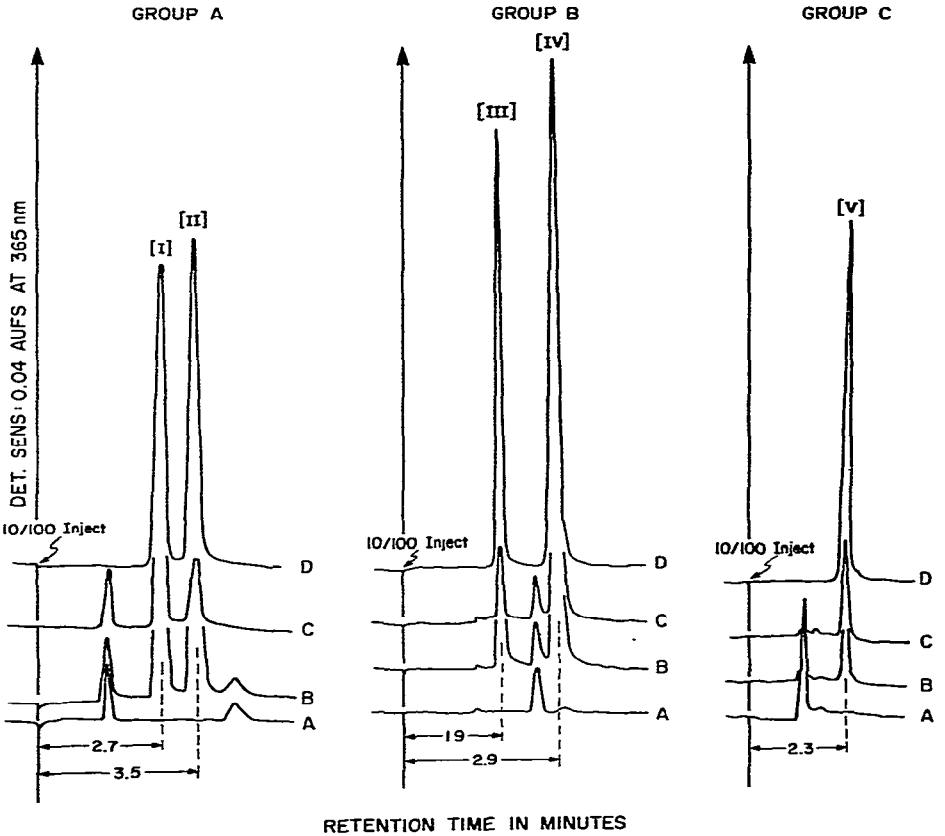


Fig. 3. Chromatograms of the HPLC analysis of 13-*cis*- and all-*trans* retinoic acids, compounds I and II, and of the aromatic retinoic acid analogs, compounds III, IV, and V. A = control blood, B = control blood extract containing recovered authentic standards, C = *in vivo* post dosing specimen extracts, and D = authentic standards.

Calculations

The concentration of each compound in the unknowns is determined by interpolation from the respective calibration curve of the internal standards processed along with the unknowns, using the direct calibration (peak height or area *versus* concentration) technique. The percent recovery of the internal standards is determined by comparing the slope value per ng of compound of the internal standard curve to that of the external standard curve.

RESULTS AND DISCUSSION

A sensitive and specific HPLC assay was developed for the determination of 13-*cis*- and all-*trans*-retinoic acid and aromatic retinoic acid analogs in blood using the principle of adsorption chromatography. The extraction procedure used is a modification of a previously published assay for phytoene[20] and provides for rapid and simple quantitation of the retinoic acids using 1 ml or less of blood.

Compounds I–V have sufficiently good UV absorption at 365 nm which can be utilized for their quantitation in the low nanogram (10–20 ng/ml) range in blood by HPLC analysis. Adsorption chromatography was selected in preference to reversed-phase chromatography due to the shorter analysis time per sample and the fact that the resolution of specific pairs of compounds was desired. Since it was not our intention to resolve the vitamin A precursors in addition to these five retinoic acid analogs in a single analysis, adsorption chromatography on 10-μm silica gel was more expeditious, hence preferred. The specificity of the assay is achieved by using relatively polar mobile phases to separate the respective pairs of compounds (Fig. 2) and a high-efficiency silica gel column generating 25,500 plates per meter, under isocratic conditions. The extraction pH and solvents used were selective for the quantitative extraction of the respective compounds of interest, as was also evidenced by the absence of endogenous interfering peaks in the retention areas of interest in the respective chromatograms (Fig. 3).

The use of amberized glassware and working in darkened rooms under yellow lights is essential to prevent photoisomerization, especially of compounds I and II. It was therefore of interest to be able to determine metabolically produced isomers, since it was reported that the 13-*cis* isomer was found in the livers of rats fed all-*trans*-retinoic acid[23]. It was also of interest to know whether the converse was true and especially if these isomers can be isolated in plasma or whole blood. Thus, the analysis of Group A compounds was specifically designed for the chromatographic resolution of compounds I and II (Fig. 3).

The aromatic retinoic acid analogs III, IV, and V are reported to be anti-tumor agents[9–11,13]. Compound III is an ester and is readily converted enzymatically to the carboxylic acid IV which probably reflects the extensive esterase activity present in the gut wall, liver and blood itself. Thus, the assay procedure for Group B was specifically designed for the resolution of the ester III from the acid IV (Fig. 3). Compound V, an amide, is relatively stable although it could be hydrolyzed to the acid analog IV, which has not yet been shown to occur *in vivo*. Hence, the assay for group C is that for a single compound V, although the chromatographic system could resolve IV should it be present *in vivo* on chronic administration.

Recovery and sensitivity limits of the assay

The overall recovery of compounds I–V in their respective groups is quantitative ($90 \pm 5.0\%$) with fairly similar limits of sensitivity of the order of 10–20 ng/ml of blood using a 1-ml specimen per assay. The chromatograms are devoid of extraneous endogenous peaks which could interfere with the baseline resolution of the compounds of interest or their accurate quantitation.

Application of the method to biological specimens containing compounds I and II

Studies in the dog. Blood levels of I were determined in a single dog following the administration of single 2- and 9.5-mg/kg doses of I by intravenous (solution) and oral (soft shell gelatin capsule) routes, Table II. Following intravenous administration, blood concentrations were 35 μg/ml at 1 min and declined to 20 ng/ml at 30 h after the 9.5-mg/kg dose, and were 10 μg/ml at 1 min and declined to 20 ng/ml at 24 h after the 2-mg/kg dose. Following oral administration of the 9.5-mg/kg dose, a blood level plateau of about 1.5 μg/ml was observed between 45–120 min post administration, declining to nonmeasurable amounts at 48 h. Following the 2-mg/kg oral dose, the blood level maxima of 0.4 μg/ml occurred at 2 h and declined to nonmeasurable levels by 24 h. Analysis of the 0–24-h urine following both dosing regimens did not reveal significant excretion of either I or II in the directly extractable (unconjugated) fraction. The analysis of the conjugated fraction was not attempted.

TABLE II

BLOOD LEVELS OF I (μg/ml) IN A DOG FOLLOWING THE ADMINISTRATION OF SINGLE 9.5- AND 2.0-mg/kg INTRAVENOUS AND ORAL DOSES OF 13-*cis*-RETINOIC ACID

NST = No sample taken; NM = nonmeasurable, <10 ng/ml blood or urine; ND = not detectable, no peak observed.

Time	*9.5 mg/kg dose*		*2.0 mg/kg dose*	
	Intravenous	*Oral*	*Intravenous*	*Oral*
0 min	ND	ND	ND	ND
1 min	35.10	NST	10.00	NST
2.5 min	19.20	NST	4.72	NST
6 min	14.44	NST	2.88	NST
10 min	11.83	ND	2.37	ND
15 min	10.11	NST	1.92	NST
20 min	9.85	0.07	1.92	ND
25 min	8.17	NST	1.58	NST
30 min	7.24	0.49	1.59	ND
45 min	6.05	1.63	1.23	0.02
1 h	5.28	1.63	1.11	0.10
1.5 h	4.23	1.51	1.14	0.25
2 h	4.83	1.71	0.99	0.41
3 h	3.55	1.30	0.85	0.37
4 h	3.13	1.06	0.56	0.30
6 h	1.81	0.38	0.38	0.13
7 h	0.72	0.26	0.29	0.10
9 h	NST	0.12	NST	NST
10 h	NST	NST	0.16	0.04
12 h	0.27	NST	NST	NST
24 h	0.04	0.03	0.02	ND
30 h	0.02	NST	NM	ND
48 h	NM	ND	ND	ND
72 h	ND	ND	ND	ND

Studies in man. Blood levels in a single subject following a single 100-mg oral dose (equivalent to 2.0 mg/kg) reached a maximum concentration of 300 ng/ml at 6 h post dosing, declining to nonmeasurable amounts at 24 h.

Blood levels of I were also determined in a single male subject (weight 61.4 kg) during one day of a chronic oral administration regimen of 2.1 mg/kg/day of I in four unequally divided doses as indicated in Fig. 4. Blood concentrations ranged from a low value of 0.24 μg/ml to a high value of 1.6 μg/ml. Such levels indicate ability to monitor therapeutic doses of I. It is interesting to note that relatively low levels of *trans*-retinoic acid were also measured at sporadic time points (with no clearly defined profile), suggesting possible *in vivo cis–trans* isomerase activity in man. A typical chromatogram is shown in Fig. 3.

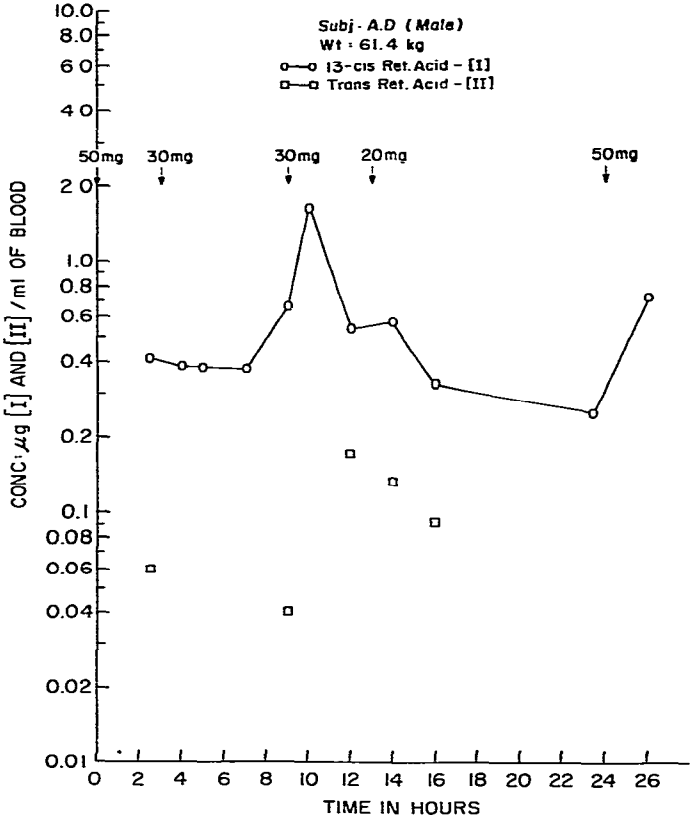


Fig. 4. Blood level profile in man following the chronic administration of 2.1 mg/kg/day of 13-*cis*-retinoic acid I in divided oral doses.

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