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DETERMINATION OF 13-*cis*-RETINOIC ACID AND ITS MAJOR METABOLITE, 4-OXO-13-*cis*-RETINOIC ACID, IN HUMAN BLOOD BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatography (HPLC) method for the quantitation of 13-*cis*-retinoic acid (13-*cis*-RA) and its major metabolite, 4-oxo-13-*cis*-RA, in human blood has been developed. The method includes extraction of 1 ml of blood with diethyl ether at pH 6 and the analysis of the extract by reversed-phase HPLC with solvent programming and detection at 365 nm. The quantitation ranges for 13-*cis*-RA and 4-oxo-13-*cis*-RA are 10—2000 and 50—2000 ng/ml of blood, respectively. The method also provides estimates of the concentrations of all-*trans*-RA and 4-oxo-all-*trans*-RA. The mean intra- and inter-assay variabilities for all four compounds were 6% or less. The method separates 13-*cis*-RA and 4-oxo-13-*cis*-RA from 9-*cis*-RA, all-*trans*-RA, 4-oxo-all-*trans*-RA, and some other possible metabolites, such as hydroxy and epoxy retinoic acids. The method has been successfully applied to the analyses of over 1200 blood samples from four 13-*cis*-RA clinical studies.

INTRODUCTION

13-*cis*-Retinoic acid (13-*cis*-RA, Table I) is undergoing clinical trials for the treatment of cystic acne and certain disorders of keratinization [1—6]. There is also interest in the prophylactic and therapeutic effects of 13-*cis*-RA and other retinoids in in vitro and in vivo carcinogenesis studies [6—11]. Several analytical methods for quantitation of 13-*cis*-RA in blood, plasma, or serum have been published, including a colorimetric method by Wang et al. [12]; a normal-phase high-performance liquid chromatography (HPLC) method by Puglisi and De Silva [13]; and reversed-phase HPLC methods by Frolik et al. [14], by Besner et al. [15], and by Wang et al. [16]. However, none of these methods measure 4-oxo-13-*cis*-RA which we have shown to be the major drug-related component in the blood of patients on chronic

13-*cis*-RA therapy [17]. Therefore, a reversed-phase HPLC method with gradient elution was developed to determine 13-*cis*-RA and 4-oxo-13-*cis*-RA in human blood. The method also provided estimates of the concentrations of all-*trans*-RA and 4-oxo-all-*trans*-RA which were useful in evaluating the stability of the 13-*cis* isomers.

EXPERIMENTAL

Laboratory precautions

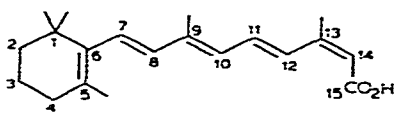
All handling of reference compounds and biological samples was performed in a darkened room illuminated with yellow light. Whenever possible, amberized containers were used.

Analytical standards

Reference compounds (Table I) were provided by Drs. M. Rosenberger

TABLE I

HPLC DATA FOR REFERENCE COMPOUNDS

13-*cis*-RA 

HPLC retention times were determined from the analyses of methanolic solutions of the reference compounds. The analyses of all compounds except retinol were done with the same batch of mobile phases to minimize variations. Elution time of a non-retained compound (t_o) was 1.6 min. The column and gradient elution conditions are described in the Experimental section.

Compound	Retention time (min)
Retinoic acids	
all-*trans*-RA	16.8
9-*cis*-RA	16.3
13-*cis*-RA	15.6
9,13-di-*cis*-RA	15.8
11,13-di-*cis*-RA	15.4
4-Oxo retinoic acids	
4-oxo-all-*trans*-RA	8.3
4-oxo-13-*cis*-RA	7.4
Hydroxy retinoic acids	
4-hydroxy-all-*trans*-RA	9.5
4-hydroxy-13-*cis*-RA	8.6
5-hydroxymethyl-all-*trans*-RA	9.8
Epoxy retinoic acids	
5,6-epoxy-all-*trans*-RA	10.2
5,6-epoxy-13-*cis*-RA	9.5
Internal standard	11.0
Retinol	17.6

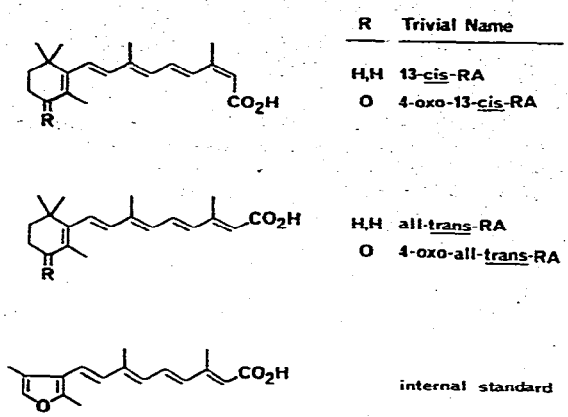


Fig. 1. Structures and trivial names of the reference standards. The IUPAC names for these compounds are given in the Experimental section.

and W. Scott, Hoffmann-La Roche. The reference standards (Fig. 1) were 13-*cis*-RA [Ro 4-3780, (*Z*,*E*,*E*,*E*)-3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4,6,8-nonatetraenoic acid, MW = 300.4], all-*trans*-RA [Ro 1-5488, (*E*,*E*,*E*,*E*)-3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4,6,8-nonatetraenoic acid, MW = 300.4], 4-oxo-13-*cis*-RA [Ro 22-6595, (*Z*,*E*,*E*,*E*)-3,7-dimethyl-9-(2,6,6-trimethyl-3-oxo-1-cyclohexen-1-yl)-2,4,6,8-nonatetraenoic acid, MW = 314.5], 4-oxo-all-*trans*-RA [Ro 12-4824, (*E*,*E*,*E*,*E*)-3,7-dimethyl-9-(2,6,6-trimethyl-3-oxo-1-cyclohexen-1-yl)-2,4,6,8-nonatetraenoic acid, MW = 314.5], and the internal standard [Ro 22-2363, (*E*,*E*,*E*,*E*)-3,7-dimethyl-9-(2,4-dimethyl-3-furyl)-2,4,6,8-nonatetraenoic acid, MW = 272.3].

The reference compounds were stored at —17°C under nitrogen and protected from light.

Preparation of standard solutions

Stock and standard solutions were prepared in amberized volumetric flasks as described in Table II. Flasks containing stock solutions were flushed with nitrogen and stored at —17°C. Standard solutions D—K, M, and N were stored at 4°C. Both sets of solutions were stable for five months.

Reagents and HPLC mobile phases

All reagents were of analytical reagent grade. Solvents used were methanol (Burdick & Jackson Labs., Muskegon, MI, U.S.A.; distilled in glass), anhydrous diethyl ether (Mallinckrodt, St. Louis, MO, U.S.A.), and glacial acetic acid (J.T. Baker, Phillipsburg, NJ, U.S.A.; Ultrex).

Phosphate buffer (1 *M*, pH 6) was prepared by titrating 1 *M* KH_2PO_4 (J.T. Baker, 68 g/500 ml) with 1 *M* $K_2HPO_4 \cdot 3H_2O$ (Mallinckrodt, 114 g/500 ml) until pH 6.0 was reached.

Mobile phase A [methanol—0.02 *M* ammonium acetate (50 : 50, v/v)] was prepared by dissolving 1.54 g of ammonium acetate (Mallinckrodt) in 1000 ml of deionized water and adding 1000 ml of methanol. Acetic acid (60 µl) was added to bring the pH to 6.65 ± 0.05. Mobile phase B [methanol—0.10 *M* ammonium acetate (90 : 10, v/v)] was prepared by dissolving 1.54 g of am-

TABLE II

STANDARD SOLUTIONS

Approximately 12.5 mg of each compound was weighed accurately and diluted with methanol in amberized 25-ml volumetric flasks to give stock solutions 1—5, each having an approximate concentration of 500 μg/ml. The volumes of stock solutions 1—5 used to prepare the other solutions were determined exactly so that the final concentrations listed for stock solution A and the standard solutions were achieved.

Stock solution	Preparation	Final volume (ml)	Concentration (μg/ml)				
			4-oxo-13-*cis*-RA	4-oxo-all-*trans*-RA	13-*cis*-RA	all-*trans*-RA	Internal standard
1	ca. 12.5 mg of 4-oxo-13-*cis*-RA	25	ca. 500	0	0	0	0
2	ca. 12.5 mg of 4-oxo-all-*trans*-RA	25	0	ca. 500	0	0	0
3	ca. 12.5 mg of 13-*cis*-RA	25	0	0	ca. 500	0	0
4	ca. 12.5 mg of all-*trans*-RA	25	0	0	0	ca. 500	0
5	ca. 12.5 mg of internal standard	25	0	0	0	0	ca. 500
A	ca. 5 ml of 1, ca. 1 ml of 2, ca. 5 ml of 3, ca. 1 ml of 4	25	100	20.0	100	20.0	0
Standard solution			Concentration (ng/100 μl)*				
D	2.00 ml of A, ca. 0.2 ml of 5	10	2000	400	2000	400	1000
E	1.00 ml of A, ca. 0.2 ml of 5	10	1000	200	1000	200	1000
G	400 μl of A, ca. 0.2 ml of 5	10	400	80.0	400	80.0	1000
H	150 μl of A, ca. 0.2 ml of 5	10	150	30.0	150	30.0	1000
I	50.0 μl of A, ca. 0.2 ml of 5	10	50.0	10.0	50.0	10.0	1000
J	20.0 μl of A, ca. 0.2 ml of 5	10	20.0	4.0	20.0	4.0	1000
K	10.0 μl of A, ca. 0.2 ml of 5	10	10.0	2.0	10.0	2.0	1000
M	ca. 0.5 ml of 5	25	0	0	0	0	1000
N	ca. 0.2 ml of 1, ca. 0.2 ml of 3, ca. 0.2 ml of 5	10	1000	0	1000	0	1000

*Since 100 μl of these solutions were added to 1 ml of control blood, these concentrations also represent the concentrations of the calibration samples (ng/ml of blood).

monium acetate in 200 ml of deionized water and adding 1800 ml of methanol. Acetic acid (0.80 ml) was added to bring the pH to 6.65 ± 0.05. Both mobile phases were degassed by stirring under vacuum for 5 min. Fresh solutions were prepared every two days.

HPLC apparatus and procedures

A Whatman Partisil PXS 10/25 ODS column (10 μm particle size, 25 cm × 4.6 mm I.D.) preceded by a guard column (7 cm × 2.1 mm I.D.) packed with Co:Pell ODS (30 μm, Whatman, Clifton, NJ, U.S.A.) was used. The guard column packing was changed about once a month or when significant broadening of the external standard peaks was observed.

A Waters Model 204 high-performance liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.) equipped with a Model 660 solvent programmer (M-45 pump), a WISP Model 710B automatic injector, a Model 440 absorbance detector (365 nm) with a dual-channel accessory, and a Hewlett-Packard Model 7132A dual-channel recorder (Hewlett-Packard, Palo Alto, CA, U.S.A.) (10 mV/full scale deflection, chart speed, 0.625 cm/min) was used. The 365-nm absorbance was monitored from one cell at 0.01 and 0.05 a.u.f.s. simultaneously with two detector channels.

The solvent programmer was set for initial solvent composition of 20% B (80% A), final solvent composition of 90% B (10% A), gradient curve No. 7 (shallow concave), and a run time of 14 min. The automatic injector was programmed for an equilibration delay of 6 min and a run time of 18 min which resulted in a 4-min hold at the final solvent conditions. Total time between injections was 25 min. The chromatographic system was operated at ambient temperature and a flow-rate of 2.0 ml/min. Typical retention times for 4-oxo-13-*cis*-RA, 4-oxo-all-*trans*-RA, internal standard, 13-*cis*-RA, and all-*trans*-RA were 7.4, 8.3, 11.0, 15.6 and 16.8 min, respectively.

The sample containers for the automatic injector consisted of 0.3-ml polyethylene micro test tubes (Brinkman Instruments, Westbury, NY, U.S.A.) inserted into amberized 1-dram screw-cap autosample vials (Waters Assoc.). The vials were capped with open screw caps fitted with self-sealing septa. A 40-μl aliquot (out of a total volume of 200 μl) of each sample was injected. Between each set of approximately 48 samples, the column was usually flushed with methanol for 30—60 min at a flow-rate of 2.0 ml/min.

Blood collection and extraction procedures

Whole blood samples from volunteers were collected in vacutainers (BD Vacutainer tubes, 10 ml, containing 20 mg potassium oxalate and 25 mg of sodium fluoride were usually used, Becton-Dickinson, Rutherford, NJ, U.S.A.). The blood was immediately transferred to polypropylene tubes with friction-fit caps (Arthur H. Thomas Co., Philadelphia, PA, U.S.A.) for freezing and storage at —17°C.

The extraction procedure of Puglisi and De Silva [13] was used with slight modifications. Frozen blood samples were thawed at room temperature and gently shaken. One ml of blood was transferred to a 15-ml centrifuge tube containing 100 μl of the appropriate standard solution. After mixing for 5 sec with a vortex action mixer, 2.5 ml of 1 *M* phosphate buffer (pH 6) was added and the sample was again mixed for 5 sec. Six ml of diethyl ether (from a container that had not been open for more than 3 days) were added. The tube was stoppered tightly with a PTFE stopper and shaken horizontally for 20 min in an Eberbach reciprocating shaker (Eberbach, Ann Arbor, MI, U.S.A.) at 180 strokes per min. The sample was then centrifuged for 20 min at 2000—2400 rpm (about 350 *g*) at room temperature (International Equipment Co., Model HN-S, rotor 263, Needham, MA, U.S.A.). Five ml of the ether layer were transferred to a 15-ml centrifuge tube. The ether was evaporated just to dryness in a 30°C water bath (N-EVAP, Organomation Assoc., Shrewsbury, MA, U.S.A.) with a stream of nitrogen. Methanol (200 μl) was immediately added to the residue and, after mixing for 5 sec, as much as

TABLE III

EFFECT OF STORAGE AT −17°C ON CONTROL BLOOD SPIKED WITH REFERENCE COMPOUNDS

Spiked blood samples I–IV were prepared by mixing control blood with known amounts of the reference compounds. Two 1-ml aliquots of each sample were analyzed immediately. Five 1.5-ml aliquots of each sample were frozen at −17°C and 1-ml aliquots of each sample were analyzed after 2, 5, 11, 17, and 22 weeks of storage. Data given are found concentrations (ng/ml).

Length of storage (weeks)	I	II		III	IV			
	13-*cis*-RA	13-*cis*-RA	all-*trans*-RA*	4-oxo-13-*cis*-RA	4-oxo-13-*cis*-RA	4-oxo-all-*trans*-RA*	13-*cis*-RA	all-*trans*-RA*
	Concentration added (ng/ml)							
	50	500	0	200	1000	200	1000	200
0	41, 44	485, 496	15, 15	170, 181	876, 940	189, 201	1020, 1090	216, 221
2	46	532	24	169	929	156	991	177
5	37	432	23	193	902	161	888	154
11	38	418	23	162	812	169	845	151
17	36	330	71	150	781	186	799	164
22	34	392	60	159	697	180	693	166
Mean	39	441	33	169	848	177	904	178
S.D.	4.4	69.0	23	14.3	88.8	16.2	138	28.7
Coefficient of variation (%)	11	16	69	8.5	11	9.1	15	16
Change (%)**	−23	−20	+300	−10	−23	−8	−34	−24

*Concentrations of *trans* compounds are estimates.

**Percent change was calculated from the concentrations found at time 0 (mean values) and 22 weeks:

$$\frac{[22\ \text{weeks}] - [\text{time } 0]}{[\text{time } 0]} \times 100.$$

possible of the methanol was transferred to an injection vial.

Calibration and isomerization samples were prepared by mixing 100-μl aliquots of standard solutions D—K, M, and N with 1-ml aliquots of control blood obtained from human volunteers, a local blood bank, or patients prior to dosing. Calibration and isomerization samples were analyzed with every set of unknown samples.

If an unknown blood sample was found to have concentrations of drug or metabolites which exceeded the calibration range, an aliquot of the blood (0.2—0.5 ml) was diluted to 1 ml with control blood and reassayed.

Recovery experiments

Recoveries of the five reference compounds from control blood were determined by comparing the HPLC responses of the calibration samples (corrected for diethyl ether aliquot) to the HPLC responses of the standard solutions injected as matrix samples. Each matrix sample was prepared by dissolving the extract residue of 1 ml of control blood in 120 μl of methanol and mixing 100 μl of this solution with 100 μl of standard solution (D—K). This method for determining percent recovery was required because the HPLC responses of the standard solutions were enhanced when they were co-injected with residue of extracted control blood as compared to the injection of standard solutions in methanol alone. This enhancement which was mainly due to improved peak shape, i.e., narrower, higher peaks, was substantially less in the absence of the guard column.

Calculations

The ratios of the peak heights of 13-*cis*-RA, all-*trans*-RA, 4-oxo-13-*cis*-RA, and 4-oxo-all-*trans*-RA to the peak height of the internal standard were determined from the chromatograms. Peak height ratios were corrected for any background interferences observed in the control blood. The peak height ratios (y) and concentrations (x) of the calibration samples were fitted to an exponential function ($y = ax^b$) by least squares regression analyses. The computer-generated exponential parameters were used to convert peak height ratios of unknown samples to concentrations.

If the ratio for the lowest calibration point was not greater than twice the background ratio, this point was not included in the calibration curve. If the peak height of the internal standard in any unknown sample was not within ±20% of the mean peak height for that subject's samples, the data for that sample were not used.

Stability studies

The first stability study evaluated the effect of storage at −17°C on control blood spiked with the reference compounds. Four spiked blood samples (I—IV) were prepared by mixing 11-ml aliquots of fresh oxalated control blood from a human volunteer with the appropriate reference compounds to give the final concentrations listed in Table III. After thorough mixing, seven 1.5-ml aliquots of I—IV were transferred to 10-ml polypropylene screw-cap tubes. One-ml aliquots were removed from two tubes of each set and immediately analyzed. The remaining tubes were frozen at −17°C and one

tube from each set was analyzed after 2, 5, 11, 17, and 22 weeks of storage.

A second study determined the effect of freezing and thawing on a pooled blood sample from a normal volunteer administered 80 mg of 13-*cis*-RA. The pooled blood sample was immediately analyzed in triplicate and the remaining blood was refrozen at −17°C. During the following month, the sample was thawed, reanalyzed in triplicate, and refrozen five times.

In a third study, eleven blood samples collected from another volunteer given 80 mg of 13-*cis*-RA were analyzed after one month of storage at −17°C and reanalyzed after three additional months of storage at −17°C.

RESULTS AND DISCUSSION

Chromatography, specificity and recovery

Chromatographic conditions for complete separation of 13-*cis*-RA and 4-oxo-13-*cis*-RA from each other, their *trans* isomers, the internal standard, and the endogenous blood substances could not be achieved in a time frame that was practical for the routine analyses of hundreds of blood samples. As shown in Fig. 2, the column, mobile phases, and gradient elution conditions chosen gave baseline separation of all components except the two 4-oxo

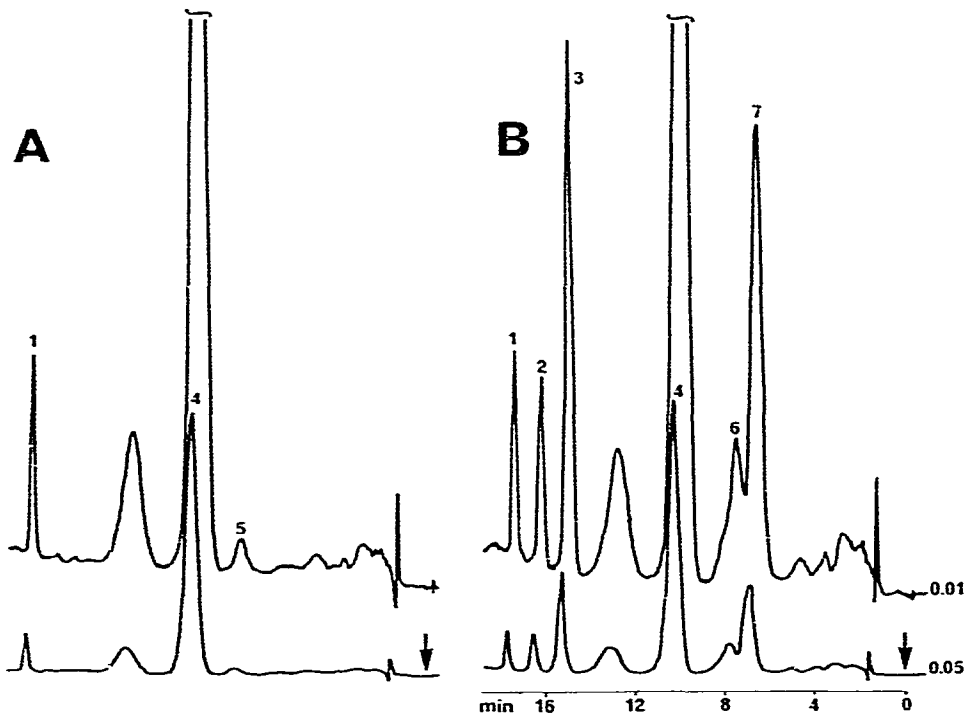


Fig. 2. Chromatograms of extracts of control blood spiked with internal standard (A) and with standard solution G (B). Absorption at 365 nm was monitored at two attenuations (0.01 and 0.05 a.u.f.s.). Peaks: 1 = endogenous retinol, 2 = all-*trans*-RA (80 ng/ml), 3 = 13-*cis*-RA (400 ng/ml), 4 = internal standard (1000 ng/ml), 5 = isomerization product of internal standard, 6 = 4-oxo-all-*trans*-RA (80 ng/ml), and 7 = 4-oxo-13-*cis*-RA (400 ng/ml).

compounds. 4-Oxo-all-*trans*-RA eluted immediately after 4-oxo-13-*cis*-RA.

No significant background interference was observed at the retention time of the internal standard in the chromatograms of control or patient blood. Chromatograms of control blood frequently showed a very small peak with the same retention time as all-*trans*-RA (< 5 ng/ml). Probably this peak is endogenous all-*trans*-RA, a metabolite of retinol [18]. Only occasionally was a small peak observed in control blood at the same retention time as 13-*cis*-RA (< 3 ng/ml). Peak height ratios of the four compounds in the calibration samples and patient samples were corrected for any background interferences observed in calibration blood and patient pre-dose blood, respectively.

The specificity of the method was investigated with respect to other geometric isomers, hydroxy retinoic acids, and 5,6-epoxy retinoic acids. The 4-hydroxy compounds have been identified as in vitro metabolites of all-*trans*-RA [19] and 13-*cis*-RA [20]. 5-Hydroxy metabolites have been identified as fecal metabolites of all-*trans*-RA in rats [21]. 5,6-Epoxy-all-*trans*-RA has been identified as an in vitro [22] and in vivo metabolite [23, 24] of all-*trans*-RA in rats.

Chromatographic studies, including co-injection of the reference compounds, indicated that 13-*cis*-RA was adequately separated from all compounds listed in Table I except its 9,13-di-*cis* and 11,13-di-*cis* isomers. In another study, we isolated the major metabolite of 13-*cis*-RA from human blood using a similar HPLC method and unequivocally identified it as 4-oxo-13-*cis*-RA by nuclear magnetic resonance spectroscopy [17]. 4-Oxo-all-*trans*-RA was also identified, but no significant amount of any other isomer was detected. In addition, only metabolites with 13-*cis* and all-*trans* geometry were identified in in vitro metabolic studies of 13-*cis*-RA with 9000 *g* rat liver supernatant [20]. Therefore, it is unlikely that any significant amounts of 9,13-di-*cis* or 11,13-di-*cis* metabolites will be encountered in 13-*cis*-RA clinical blood samples.

The average percent recoveries of 13-*cis*-RA, all-*trans*-RA, 4-oxo-13-*cis*-RA, 4-oxo-all-*trans*-RA, and the internal standard from control blood were 94, 104, 100, 131, and 91%, respectively (Table IV). The higher percent recoveries for all-*trans*-RA and 4-oxo-all-*trans*-RA compared to their 13-*cis* isomers resulted from ca. 5% isomerization occurring during the isolation procedure. This isomerization is discussed below.

Calibration curve and limits of quantitation

The assay was validated for calibration ranges of 10—2000, 50—2000, 10—400, and 80—400 ng/ml of blood for 13-*cis*-RA, 4-oxo-13-*cis*-RA, all-*trans*-RA, and 4-oxo-all-*trans*-RA, respectively. When the calibration data (peak height ratios and concentrations) for 13-*cis*-RA and 4-oxo-13-*cis*-RA were fitted with a linear least squares regression analysis, deviations of greater than 10% from the fitted line were frequently obtained. Exponential least squares regression analysis gave good results with the deviations nearly always being less than 10%. The exponential parameters for the inter-assay variability study are summarized in Table V. The mean intra-assay and inter-assay variabilities for each compound were 6% or less (Table IV).

TABLE IV

RECOVERY AND INTRA- AND INTER-ASSAY VARIABILITY DATA

One-ml aliquots of control blood were spiked with 100 µl of standard solutions D–K (Table II) and extracted as described in the Experimental section. Recoveries were determined from HPLC peak heights from one set of spiked samples. Intra-assay variability was determined from three sets of spiked samples that were extracted and analyzed in one day. Inter-assay variability was determined from three sets of spiked samples that were extracted and analyzed on three different days.

Compound	Amount added to 1 ml of blood (ng)	Recovery (%)	Inter-assay variability			Intra-assay variability		
			Amount found (ng/ml)		Coefficient of variation (%)	Amount found (ng/ml)		Coefficient of variation (%)
			mean	S.D.		mean	S.D.	
13-*cis*-RA	10.0	85	10.3	1.06	10.3	10.1	0.21	2.08
	20.0	96	18.7	1.04	5.56	19.6	0.79	4.03
	50.0	104	51.6	2.70	5.23	48.1	3.36	6.99
	150	85	156	12.7	8.14	161	5.51	3.42
	400	90	394	7.81	1.98	413	25.7	6.22
	1000	93	1019	96.2	9.44	981	34.6	3.53
	2000	107	1954	24.2	1.24	1952	45.6	2.33
	mean ± S.D.:	94 ± 9		mean:	5.98		mean:	4.09
4-oxo-13-*cis*-RA	50.0	109	48.4	3.06	6.32	49.1	0.91	1.85
	150	91	158	1.73	1.09	155	4.62	2.98
	400	98	400	3.61	0.90	397	3.06	0.77
	1000	99	982	10.0	1.02	990	13.6	1.37
	2000	104	1999	63.3	3.17	2004	9.64	0.48
	mean ± S.D.:	100 ± 7		mean:	2.50		mean:	1.49
all-*trans*-RA	10.0	104	9.79	0.87	8.89	9.34	0.67	7.17
	30.0	92	31.2	2.35	7.53	32.5	2.66	8.18
	80.0	104	78.8	2.34	2.97	83.5	5.22	6.25
	200	107	204	20.0	9.80	197	7.51	3.81
	400	114	394	4.51	1.14	388	11.3	2.91
	mean ± S.D.:	104 ± 8		mean:	6.07		mean:	5.66
4-oxo-all-*trans*-RA	80.0	135	80.3	3.42	4.26	79.9	0.67	0.84
	200	129	198	3.06	1.55	201	3.46	1.72
	400	130	402	18.7	4.65	399	4.62	1.16
	mean ± S.D.:	131 ± 3		mean:	3.49		mean:	1.24
Internal standard	1000							
	mean ± S.D.: (n = 8)	91 ± 6						

TABLE V

CALIBRATION PARAMETERS FOR INTER-ASSAY VARIABILITY STUDY

The peak height ratios (y) and concentrations (ng/ml, x) of reference compounds added to control blood were fitted to an exponential function: $y = ax^b$. The data are from the inter-assay variability study where, on three separate days, control blood was spiked with standard solutions D—K (Table II) and analyzed.

Compound	Parameter a	Parameter b	Coefficient of correlation	Average deviation (%)
13-*cis*-RA	0.00059	1.007	0.99985	2.1
	0.00055	1.003	0.99937	5.2
	0.00065	0.970	0.99920	6.3
mean	0.00060	0.993	0.99947	4.5
4-oxo-13-*cis*-RA	0.00075	0.996	0.99999	0.3
	0.00071	1.102	0.99955	2.8
	0.00075	0.995	0.99977	2.4
mean	0.00074	1.031	0.99975	1.8
all-*trans*-RA	0.00050	1.044	0.99984	2.2
	0.00063	1.007	0.99894	4.8
	0.00057	1.001	0.99588	11
mean	0.00057	1.017	0.99822	6.0
4-oxo-all-*trans*-RA	0.00044	1.124	0.99964	1.7
	0.00058	1.093	0.99888	2.7
	0.00049	1.108	0.99997	0.5
mean	0.00050	1.108	0.99950	1.6

Comparison of normal-phase and reversed-phase HPLC methods

Blood samples obtained from a patient on chronic therapy at 0, 1, 2, 3, 4, 6, 8, 12, and 24 h after a final dose of 13-*cis*-RA were analyzed by the reversed-phase HPLC method. Linear least squares regression analysis of the 13-*cis*-RA concentrations found for these time points (y = 314, 352, 322, 327, 309, 197, 178, 110, and 32 ng/ml) and the 13-*cis*-RA concentrations determined previously by the normal-phase method of Puglisi and De Silva [13] (x = 245, 323, 354, 339, 296, 215, 170, 100, and 28 ng/ml) resulted in an equation of the form $y = 0.97x + 14.7$. Although this comparison was done with limited data and few low-concentration samples, the slope (close to unity) and the correlation coefficient (r = 0.966) indicated good agreement between the two methods.

Isomerization

About 3—6% of 13-*cis*-RA and 4-oxo-13-*cis*-RA isomerized to their all-*trans* isomers when control blood spiked with the 13-*cis* compounds was extracted and analyzed. The internal standard also isomerized (2—5%), presumably to its 13-*cis* isomer. The isomerization of all-*trans*-RA and 4-oxo-all-*trans*-RA was checked in a few spiked samples and found to be ca. 2%. Very

little isomerization of the 13-*cis* compounds occurred when they were extracted from buffer or plasma. This suggests that the blood cells may play a role in the isomerization.

The isomerization introduced negligible effects on the calibration curves for 13-*cis*-RA and 4-oxo-13-*cis*-RA since the amount of isomerization was small and a similar amount of isomerization also occurred with the internal standard. Whether or not the isomerization also occurs in the unknown samples, the maximum error expected for the 13-*cis* compounds would be 6%.

In contrast, the isomerization had a significant effect on the calibration curves for all-*trans*-RA and 4-oxo-all-*trans*-RA since 5% isomerization of the 13-*cis* compounds in the calibration samples (which contain *cis*/*trans* ratios of 5 : 1) resulted in a 25% increase in the peak heights of the *trans* isomers. Therefore, the concentrations found for the *trans* isomers in the unknown samples were considered as estimates only.

Stability studies

Table III summarizes the results of storage at —17°C on control blood spiked with 13-*cis*-RA and/or metabolites. The concentrations of 13-*cis*-RA and 4-oxo-13-*cis*-RA decreased by 20—34% and 10—23%, respectively, during the 22 weeks of storage. This decrease can only be partially explained by isomerization of these two compounds to their *trans* isomers since only about 10% isomerization of 13-*cis*-RA was observed in solution II during the 22 weeks. Another phenomenon must be contributing to the decreasing concentrations of all four compounds.

In a second stability study, a pooled blood sample from a volunteer given 13-*cis*-RA was analyzed, frozen, and thawed 5 times during a 4-week period. The coefficients of variation for the concentrations found for 13-*cis*-RA and 4-oxo-13-*cis*-RA were 8.0% and 5.2%, respectively. There appeared to be a slight decrease (12%) in the concentration of 13-*cis*-RA which agrees well with the decreases observed for 13-*cis*-RA (12—15%) after 5 weeks of storage in the spiked blood study (Table III). Therefore, freezing and thawing were not having a significant effect on the stability of 13-*cis*-RA and 4-oxo-13-*cis*-RA.

Re-analysis of eleven 13-*cis*-RA blood samples after three months of storage at —17°C showed mean decreases in the concentrations of 13-*cis*-RA and 4-oxo-13-*cis*-RA of 15 and 7% respectively, when compared to the original analysis.

These three studies suggested that the concentration found for 13-*cis*-RA may be 10—15% low after 1—2 months of storage at —17°C and up to 30% low after 5-months of storage. The decrease in the concentration of 4-oxo-13-*cis*-RA on storage was about half that of 13-*cis*-RA. The samples may be more stable at —70°C, but this has not been investigated.

Application of the method to clinical blood samples

Chromatograms of extracts of blood samples collected from a patient receiving 13-*cis*-RA for treatment of cystic acne are shown in Fig. 3. The major component in the extract of blood obtained 3 h after a single 80-mg dose was 13-*cis*-RA (Fig. 3B), whereas the major component in the extract

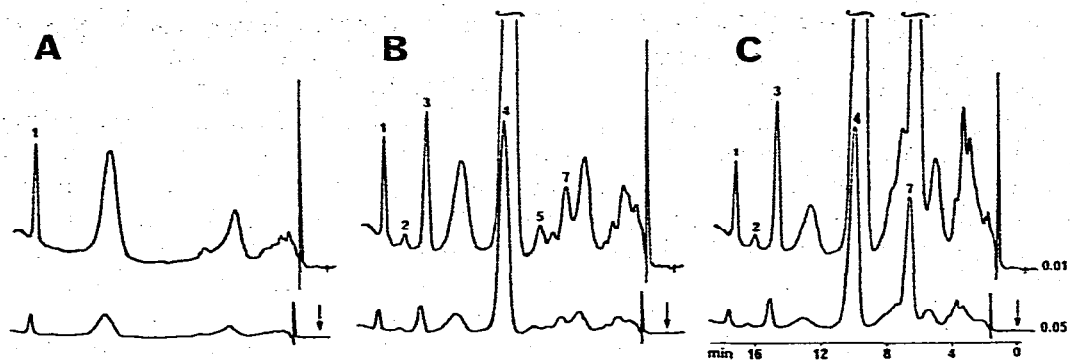


Fig. 3. Chromatograms of extracts of blood from a patient (subject JC) receiving 13-*cis*-RA for treatment of cystic acne: (A) day 1, predose; (B) day 1, 3 h after a single 80-mg dose; and (C) day 18, after 13 days of 40 mg b.i.d. dosing. Peak numbering is the same as in Fig. 2. Concentrations found for 13-*cis*-RA in B and C were 143 and 167 ng/ml, respectively. Concentration of 4-oxo-13-*cis*-RA in B was below the limit of quantitation (50 ng/ml) and in C it was 734 ng/ml.

of blood obtained during chronic administration was 4-oxo-13-*cis*-RA (Fig. 3C). The blood concentration versus time curves for two other patients also receiving 13-*cis*-RA for treatment of cystic acne are shown in Fig. 4.

Over 1200 human blood samples have been analyzed by this reversed-phase HPLC method. In general, 13-*cis*-RA was the major drug-related component in the extracts of blood obtained up to 4—6 h after a single dose

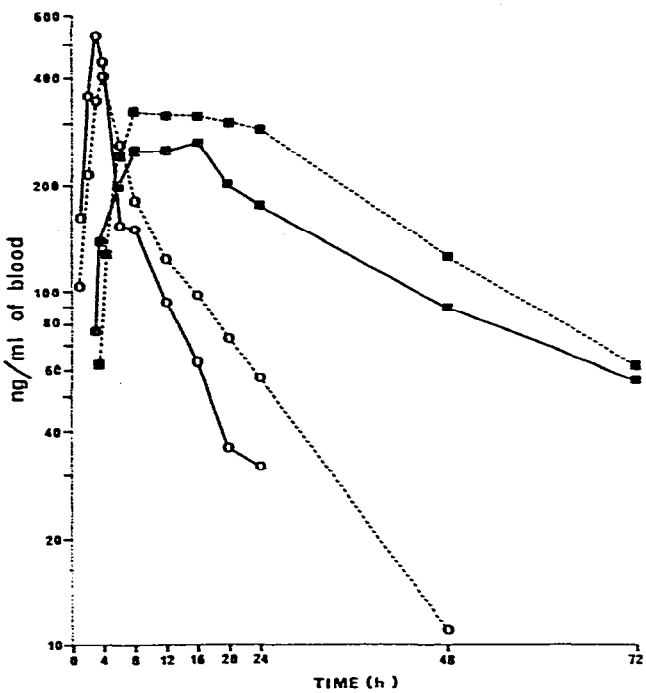


Fig. 4. Blood concentrations of 13-*cis*-RA (○) and 4-oxo-13-*cis*-RA (■) in subject MD (——) and subject DM (— — —) following a single 80-mg dose of 13-*cis*-RA on day 1.

of 13-*cis*-RA, but thereafter and during chronic dosing 4-oxo-13-*cis*-RA was the major drug-related component.

The ratios of 13-*cis*-RA and 4-oxo-13-*cis*-RA to their all-*trans* isomers in the human blood samples varied from 3 : 1 to over 30 : 1. Serial blood samples from individual subjects also showed large ranges in the *cis/trans* ratios with no discernible patterns. In general, higher ratios were found in blood from volunteers receiving a single dose of 13-*cis*-RA than in blood from patients on chronic therapy. The *cis/trans* ratios in most of the blood samples from volunteers after a single dose were the same as that expected from 3—6% isomerization (*cis/trans*) during the extraction procedure. This suggests that the all-*trans*-RA and 4-oxo-all-*trans*-RA in these samples were more likely formed by artifactual isomerization than by metabolism of 13-*cis*-RA. However, the all-*trans*-RA and 4-oxo-all-*trans*-RA in the blood from patients on chronic therapy may in part be metabolic products of 13-*cis*-RA.

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