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Determination of isotretinoin or etretinate and their major metabolites in human blood by reversed-phase high-performance liquid chromatography

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Abstract: A method is described for the quantitative analysis of isotretinoin and its 4-oxo metabolite, or of etretinate and its principal metabolites, in human blood in the range 10–2000 ng/ml. Following a simple one-step extraction, the compounds are determined by reversed-phase high-performance liquid chromatography (HPLC) with gradient elution and detection at 365 nm. This highly specific method separates the *cis* and *trans* isomers of the parent compounds and their metabolites. Examples are given of the application of this method to clinical studies of these two therapeutically important retinoids.

Keywords: *Isotretinoin; etretinate; reversed-phase HPLC; separation of* cis/trans *isomers; retinoids; metabolites.*

Introduction

Isotretinoin (Accutane®) (13-*cis*-retinoic acid, Ro 4-3780, **I**) is an oral dermatological agent used in the treatment of severe recalcitrant cystic acne [1–3]. Etretinate (Ro 10-9359, **VII**) is undergoing clinical evaluation for the treatment of severe psoriasis [1–3]. The comparative pharmacokinetics of these drugs have been reviewed [4].

Both compounds are analogues of retinoic acid. The structures of **I** and **VII** and their principal metabolites are shown in Table 1. The principal metabolite of **I** in human blood is 4-oxo-13-*cis* retinoic acid (Ro 22-6595, **II**) [5]. Small amounts of 4-hydroxy-13-*cis*-retinoic acid (**III**), all-*trans*-retinoic acid (**IV**) and 4-oxo-all-*trans*-retinoic acid (**V**) were also identified in human blood after administration of **I** [5] and were shown to be formed from **I** in studies *in vitro* with rat liver preparations [6]. The principal metabolites of **VII** found in human blood after multiple-dose administration of this aromatic retinoid ester are the all-*trans* acid (Ro 10-1670, **VIII**) [7, 8] and the 13-*cis* acid (Ro 13-7652, **X**) [8]. All of the above metabolites from both compounds contain an intact tetra-ene side chain which permits their detection by measurement of UV absorption at 365 nm.

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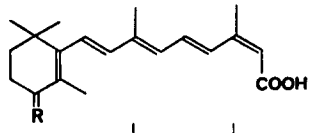
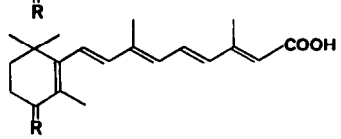
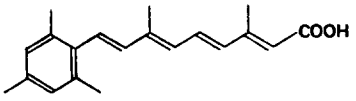
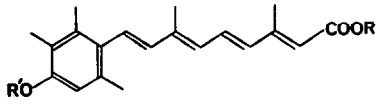
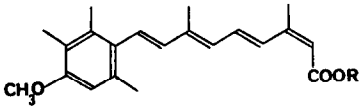
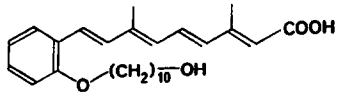
Table 1
Structures and HPLC retention times of reference compounds

	R	Compound		HPLC retention time (min)*
	H,H	13-*cis*-RA	**I**	11.3
	O	4-oxo-13-*cis*-RA	**II**	6.8
	H,OH	4-hydroxy-13-*cis*-RA	**III**	6.4
	H,H	All-*trans*-RA	**IV**	12.1
	O	4-oxo-all-*trans*-RA	**V**	6.5
		Internal standard	**VI**	10.1
	R′			
	CH_3	CH_2CH_3	**VII**	13.5
	CH_3	H	**VIII**	9.7
	H	H	**IX**	5.9
	H		**X**	9.4
	CH_2CH_3		**XI**	13.1
		Internal standard	**XII**	11.3

* Retention time of a non-retained compound (t_0) was 0.75 min, as estimated by the injection artifact. A 0.3-min separation in retention times yields complete resolution.

Several analytical methods for the determination of **I** and/or **VII** and some of their metabolites in blood, plasma or serum have been published. These include normal-phase HPLC methods by Puglisi and de Silva [9], Hänni *et al.* [10], and Paravicini and Busslinger [11]; and reversed-phase methods by Frolik *et al.* [12], Wang *et al.* [13], Besner *et al.* [14, 15], Palmskog [16], McClean *et al.* [17], Vane *et al.* [18], Shelley *et al.* [19], Goodman *et al.* [20], Kerr *et al.* [21] and Annesley *et al.* [22]. These methods are time-consuming because of the need for a cumbersome extraction followed by evaporation of the organic layer, or do not separate all the metabolites or potential metabolites of each drug, or do not determine the major metabolites with sensitivity in the low nanogram range.

An analytical procedure has been developed for the determination of **I** or **VII** and their metabolites in human, dog and rat blood or plasma by combining a modification of the simple and rapid extraction technique of McClean *et al.* [17] with a sensitive and specific gradient elution HPLC method. In the present report the assay procedure is described and results of the analysis of some clinical samples are recorded and discussed.

Experimental

Materials

All reagents were of analytical grade. Acetonitrile and 1-butanol were UV grade (Burdick & Jackson Laboratories, Inc., Muskegon, MI). Acetic acid was glacial "Ultrex" grade (J.T. Baker Chemical Co., Phillipsburg, NJ). All reference retinoids (Table 1) were provided by Hoffmann–La Roche Inc. and were stable at 4°C in sealed amber glass ampoules. The quality of the material from opened ampoules could be conveniently determined by measuring the peak area response factor with HPLC. Yellow lighting was used to minimize photodecomposition.

Stock and standard solutions of reference compounds

Approximately 10 mg of each of the compounds in the isotretinoin series (**I** and **II**) or the etretinate series (**VII**, **VIII** and **X**) were weighed accurately into individual amber volumetric flasks with Teflon stoppers and were dissolved in methanol–acetonitrile (50:50, v/v) with ultrasonication. These stock solutions were stable for several months at −20°C.

Intermediate stock solutions containing 40.0 μg/ml of retinoids were prepared by diluting with acetonitrile calculated volumes of stock solutions of the isotretinoin series or etretinate series. The intermediate stock solutions were used to prepare standard solutions containing 5, 10, 35, 140, 500 and 1000 ng/25 μl of the respective retinoids in acetonitrile. These solutions were prepared every two months and were stored at 4°C in amber Teflon-stoppered volumetric flasks.

Stock solutions of **VI** (internal standard for **I** and **II**) or **XII** (internal standard for **VII**, **VIII** and **X**) were diluted with acetonitrile to give a working concentration of 200 ng/25 μl.

Blood samples

Heparinized blood samples were obtained from a healthy volunteer after a single 240-mg dose of Accutane (**I**). Blood samples were also obtained from a psoriatic patient after a single 100-mg dose and then after multiple dosing for 8 weeks with etretinate (**VII**). Heparinized control blood was collected from healthy volunteers.

Extraction procedure

Blood samples were thawed at room temperature and then mixed by gentle shaking.

The standard solution (25 μl) containing 5–1000 ng of the reference compound being measured (or 25 μl of acetonitrile) was placed in a 10 × 75 mm disposable culture tube with the appropriate internal standard solution (25 μl). Control or experimental blood (0.5 ml) was added and the contents of the tube were briefly mixed with a vortex mixer. A solution of 1-butanol–acetonitrile (50:50, v/v) (350 μl) was added and the contents of the tube were mixed thoroughly. A saturated solution of K_2HPO_4 (about 1 kg/l) (300 μl) was added and the contents were again mixed thoroughly. After the tubes had been centrifuged at 3000 rpm for 10 min at 4°C, the organic layer (about 200 μl) was transferred to a disposable polyethylene micro test tube (Cat. No. 22-36-440-5, Brinkmann Instruments Inc., Westbury, NY) that had been inserted into an amber 1-dram screw-cap auto-sample vial (Waters Associates, Milford, MA). The vials were closed with open screw caps fitted with self-sealing septa. A 35-μl sample of each extract was injected onto the chromatograph.

HPLC

The equipment consisted of a 150 × 4.6 mm i.d. reversed-phase column packed with Zorbax ODS (Dupont Instruments, Wilmington, DE) in a liquid chromatograph equipped for gradient elution and automatic injection. Models M-6000 and M-45 pumps, model 660 solvent programmer, model 440 absorbance detector, and model 710 B automatic injector (Waters Associates, Milford, MA) were used. The retinoids were detected at 365 nm. A computing integrator (Model SP4100, Spectra-Physics Inc., Santa Clara, CA) was used to plot the chromatograms at an attenuation of 16 (= 0.016 a.u.f.s.) and to calculate the ratios of the peak heights of the reference compounds to those of the internal standards.

Mobile phase A was a solution of ammonium acetate (1.54 g) in deionized distilled water (1000 ml) to which was added acetonitrile (1000 ml) and acetic acid (10 ml). Mobile phase B was a solution of ammonium acetate (1.54 g) in water (100 ml) to which was added acetonitrile (1900 ml) and acetic acid (0.8 ml). The mobile phases were degassed by stirring under vacuum for 5 min. The linear gradient profile adopted was: $t = 0$, $\%B = 0$; $t = 10$ min, $\%B = 100$ and the flow rate was 1.5 ml/min. The final solvent composition was kept constant for 3 min after which the column was equilibrated with the initial solvent composition for 6 min before the next sample injection. The total run time between injections was 21 min.

Calculations

The peak height ratios (y) (calculated by the computing integrator through an overlay modification to its BASIC program) of reference compounds **I** and **II** to their internal standard **VI**, or of **VII**, **VIII** and **X** to their internal standard **XII** and the concentrations (x) of the standard solutions were fitted to a power function $y = ax^b$ by least squares regression analysis. The computer-generated parameters were used to convert peak height ratios of the experimental samples to concentrations.

Results and Discussion

Isotretinoin (**I**)

The retention times of drug and related reference compounds are given in Table 1. Good separation of **I** and **II** from their respective all-*trans* isomers **IV** and **V** and from **III** and the internal standard **VI** was achieved (Fig. 1C). Chromatograms of control blood and control blood spiked with a standard solution are shown in Figs 1A and 1B, respectively. The major peak observed in the control blood extract (Fig. 1A) is due to endogenous retinol which does not interfere with this assay.

A chromatogram of an extract of blood from a volunteer who had received a single oral dose of **I** is shown in Fig. 1C. **I** and the major metabolite **II** are present; in addition small amounts of **IV** and incompletely resolved **III** and **V** were identified by their retention times. This confirms the results of a previous report [5]. The extent to which **IV** and **V** are present in the blood is not known due to slight artifactual isomerization of **I** and **II** (see below). The identity of the small broad peak at about 5 min is not known. Figure 3A shows the graph of blood concentration against time for this subject after administration of a single dose. **I** is the major drug-related component up to approximately 6 h, whereas **II** is not only the major component after 6 h but is also the major component in blood during multiple dosing [23].

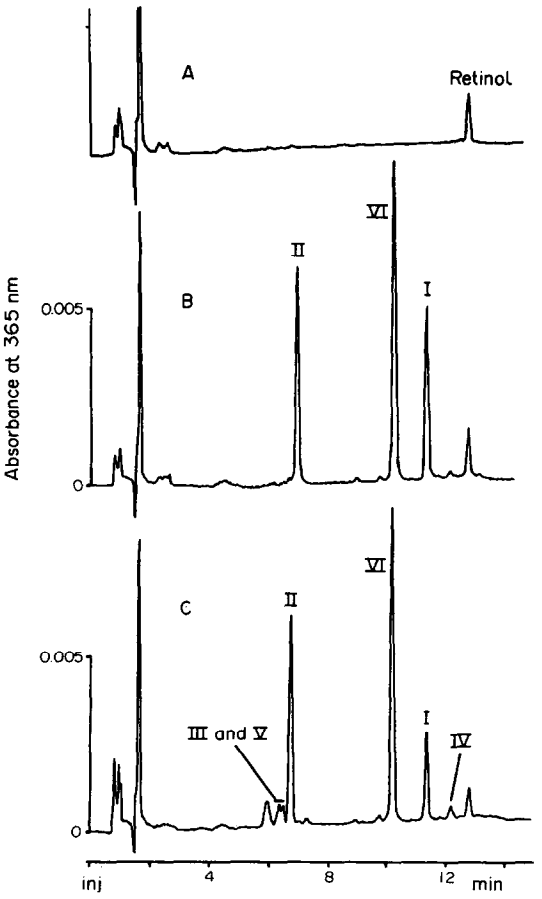


Figure 1
Chromatograms of blood extracts in the analysis of **I** and metabolites. (A) Control blood alone; (B) control blood spiked with 25 μl of standard solution containing 140 ng each of **I** and **II**; and (C) blood from volunteer (P.M.J.) 10 h after administration of a single 240-mg dose of **I**. The blood samples (0.5 ml) in (B) and (C) were spiked with internal standard **VI** and were extracted as described in the text. The concentrations determined in (C) for **I** and **II** were 219 and 296 ng/ml, respectively; the 0–72 h blood concentration-time graph for this subject is shown in Fig. 3A.

Etretinate (**VII**)

The same chromatographic conditions that were used for compound **I** resulted in the separation of **VII**, **VIII**, **X** and their internal standard **XII** from each other and from endogenous blood substances (Table 1 and Fig. 2A). Two metabolites of **VII** in the rat, **IX** (phenolic analogue of **VIII**), identified as a conjugate in bile [7], and **XI** (13-*cis* isomer of **VII**), detected in plasma (unpublished data), were separated from the compounds of interest in this series.

A chromatogram of an extract of blood from a psoriatic patient who had received multiple doses of **VII** is shown in Fig. 2B. **VII** and the major metabolites **VIII** and **X** are present; however, the rat metabolites **IX** and **XI** were not detected. The small peak at about 5.5 min did not co-elute with reference sample **IX**. The single-dose (Fig. 3B) and multiple-dose (Fig. 3C) blood concentration graphs with time are shown for this subject.

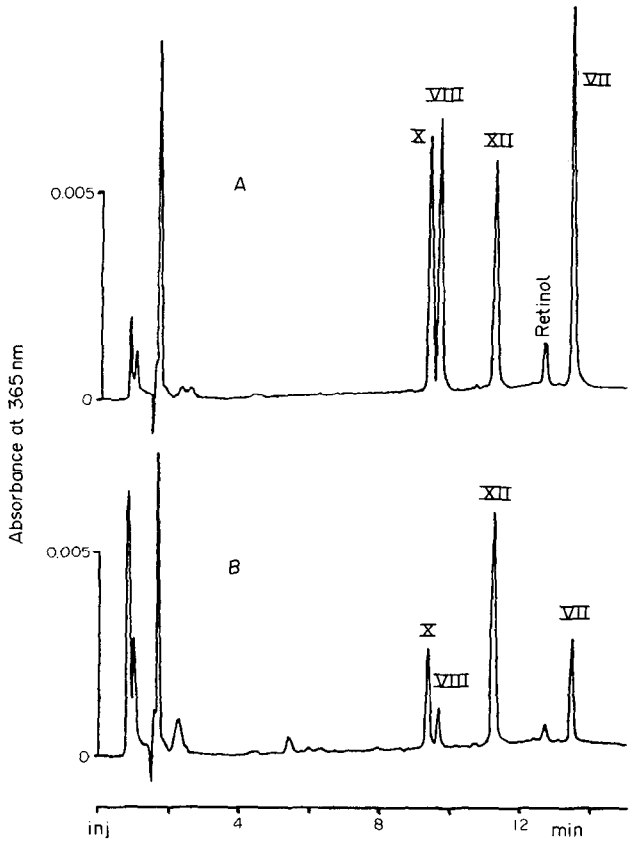


Figure 2
Chromatograms of blood extracts in the analysis of **VII** and metabolites. (A) Control blood spiked with 25 μl of standard solution containing 140 ng each of **VII**, **VIII** and **X**; and (B) blood from a psoriatic patient (R.S.) 8 h after a morning dose of 25 mg of **VII** during the 8th week of multiple-dose treatment. The blood samples (0.5 ml) were spiked with internal standard **XII** and were extracted as described in the text. The concentrations determined in (B) for **VII**, **VIII** and **X** were 69.1, 41.1 and 117 ng/ml of blood, respectively; the 0–12 h blood concentration–time graph for this patient is shown in Fig. 3C.

Following single-dose administration, the major component in the first 12 h was the metabolite **VIII**; thereafter metabolite **X** was the major component observed. During multiple-dose treatment **VII**, **VIII** and **X** were all major components; however, the concentrations of **VIII** declined rapidly leaving **X** as the major metabolite after 6 h with concentrations that remained high. The graphs for this patient were found to be typical for other patients undergoing similar treatment.

Although the presence of **VIII** has been previously reported in human plasma [7, 8, 10, 11], the present report is the first of **X** in the blood of subjects after administration of a single dose. **X** may not have been observed previously owing to insufficient resolution of the *cis* and *trans* isomers by the HPLC methods employed by earlier investigators.

General

The assays for **I**, **II**, **VII**, **VIII** and **X** were validated for concentration ranges of 10–2000 ng/ml in blood. The peak height/noise ratios of these compounds in the 10 ng/ml

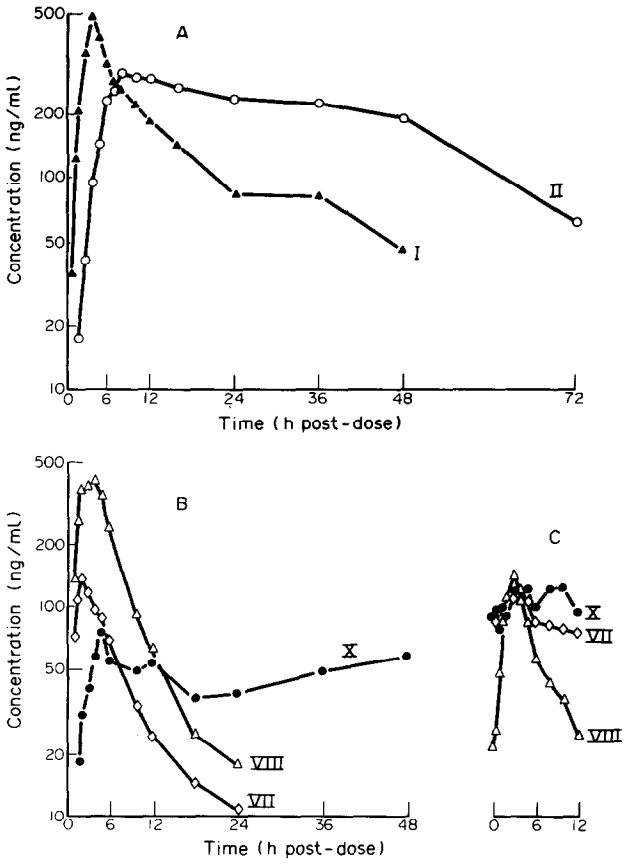


Figure 3
Blood concentration–time graphs of **I** and **VII** and their metabolites: (A) after a single 240 mg oral dose of **I**; (B) after a single 100 mg oral dose of **VII**; and (C) after a morning dose of 25 mg of **VII** during the 8th week of treatment with 25 mg twice daily. The subjects were the same as those in Figs 1 and 2.

standard solutions were 2.5 or greater. Typical values of parameter a obtained by fitting the calibration data for each reference compound to the equation $y = ax^b$ were 0.0008 (**I**), 0.0024 (**II**), 0.0073 (**VII**), 0.0041 (**VIII**) and 0.0036 (**X**). Parameter b values for the same curves were 1.117, 0.986, 1.003, 1.011 and 1.030, respectively. The correlation coefficients for all calibration curves for both sets of compounds were 0.999 or greater. To evaluate the fit of the calibration data to the least squares regression line, the calibration curve ratios were resubstituted into the derived equation and treated as unknowns ('amount found'). The intraday (n = 3) and interday (n = 3) relative standard deviations between the 'amount found' and the 'amount added' for all compounds at all concentrations were less than 6%.

Recoveries were determined by comparing the peak heights of the reference compounds in the extracts of the standard solutions with those of the standard solutions injected directly onto the chromatograph based on an extraction solvent volume of 0.4 ml. The mean percentage recoveries (and percentage relative standard deviations) for each of the compounds **I**, **II**, **VII**, **VIII** and **X** (10–2000 ng/ml) from control blood were 69.1 (11.1), 95.9 (7.7), 92.3 (13.7), 63.5 (5.0) and 61.4 (4.5), respectively (n = 6). The

corresponding values for the internal standards **VI** and **XII** (400 ng/ml) were 70.0 (2.2) and 76.2 (4.1), respectively (n = 19).

Aliquots of control blood were spiked with 140 ng of **I** and **II**, or 500 ng of **VII**, **VIII** or **X**, together with 200 ng of their respective internal standards **VI** or **XII** to determine the stability of the compounds during the assay procedure with respect to possible isomerization and/or hydrolysis. There was about 2.9% isomerization of **I** to **IV** and 3.7% isomerization of **II** to **V** and about 1.1% isomerization of **VI** to its 13-*cis* isomer. In the etretinate series, there was no detectable hydrolysis (less than 0.5%) of **VII** to **VIII**; there was about 0.8% isomerization of **VII** to its 13-*cis* isomer **XI**, about 2.0% isomerization of **VIII** to **X** and about 4.5% isomerization of **X** to **VIII**. The internal standard **XII** showed 1.6% decomposition to produce an unknown, non-interfering peak. These values were based on peak height measurements; equivalent response factors were assumed for the standard and isomerization product.

The mean concentrations of **VII**, **VIII** and **X** determined in a pooled patient blood sample after exposure for 1 h to white fluorescent light at room temperature were not significantly different from the pre-exposure values (data not shown). McClean *et al.* [17] have shown that only 52% of **VII** was recovered when serum was exposed to white fluorescent light for 1 h. Therefore, whole blood provides better protection against photodecomposition than does serum.

The reproducibility of the chromatograms produced by this gradient elution HPLC procedure was tested by successively injecting 35 μl of a sample containing 1250 ng/ml each of **VII**, **VIII**, **X** and **XII**. The relative standard deviations of the peak heights of all four compounds were each less than 3.6% (n = 6); these values were 1.5% or less when ratios of **VII**, **VIII** and **X** to **XII** were used.

This assay differs from those of other workers [8–22] in that it uses a rapid one-step extraction followed by gradient elution HPLC which allows for specificity and high sensitivity (10 ng/ml in blood) in the simultaneous determination of **I** and **II** or **VII**, **VIII** and **X**. In the authors' experience, the use of a column containing a high-efficiency C_{18} packing material with residual exposed Si-OH sites (i.e. not 'end capped' with TMS) in combination with gradient elution is an important factor in achieving resolution of the *cis* and *trans* isomers of these retinoids.

The method has been successfully applied to the analysis of human blood samples; it is expected that the calibration range of 10–2000 ng/ml for all the compounds will adequately cover the blood concentrations found in human subjects after therapeutic doses of **I** or **VII** without the need for dilution of blood samples. In addition, the method has also been used successfully for the determination of these retinoids in human plasma, in the plasma and blood of the dog and rat, and in metabolism studies *in vitro* of these retinoids with rat liver preparations.

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