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Note

Analysis of retinoids by high-performance liquid chromatography using programmed gradient separation

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Vitamin A (retinol), its metabolites and analogues have been heavily focused upon due to their clinical value and utility. Retinol can be used as a reflection of dietary or nutritional status, while the retinol analogues (isotretinoin, tretinoin, etretinate) are being proven as potent dermatologic and anti-tumor agents. High-performance liquid chromatography (HPLC) has been demonstrated to be applicable to the measurement of these compounds in blood. HPLC assays have been reported for retinol [1—4], 13-*cis*-retinoic acid [5, 6], all-*trans*-retinoic acid [7, 8], etretinate [9, 10], plus retinol and retinal isomers [11—15]. Several assay systems have been reported for the isocratic separation of mixtures of retinoids [13—15]. Of these, the methods of Frolick et al. [14] and McClean et al. [15] allow for the measurement of numerous retinoids in biological specimens through the use of single isocratic systems. Using these methods the required time for separation of compounds can run as long as 36 min [14]. The natural and synthetic retinoids, plus their respective major metabolites, have differences in polarity that make chromatographic separation difficult in a short time period. During the long separation times the later eluting peaks become broad and require an integrator. To counteract this

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problem solvent programming has been successfully applied to the analysis of 13-*cis*-retinoic acid and 4-oxo-13-*cis*-retinoic acid in blood samples [5].

In this report we describe a programmed gradient HPLC system for the analysis of multiple retinoids, including retinol, retinal, 13-*cis*-retinoic acid (isotretinoin) and its metabolite, all-*trans*-retinoic acid (tretinoin), etretinate and its metabolites. The use of gradient elution significantly shortens the analysis time, and results in sharp symmetrical peaks which can be measured without an integrator or data module.

EXPERIMENTAL

Reagents

All reagents were of analytical-reagent grade. Acetonitrile was from Burdick and Jackson Labs. (Muskegon MI, U.S.A.). Glacial acetic acid was from Mallinckrodt (Paris, MO, U.S.A.). Retinol, all-*trans*-retinoic acid, retinal, and retinyl acetate were purchased from Sigma (St. Louis MO, U.S.A.). 13-*cis*-Retinoic acid, etretinate, and Ro 10-1670 were gifts from Hoffmann-La Roche (Nutley NJ, U.S.A.).

Chromatography

Measurements were made using a Varian Model 5000 liquid chromatograph equippped with a UV-50 variable-wavelength detector. A Whatman 10-μm Partisil PXS 10/25 ODS-2 column (Whatman, Clifton, NJ, U.S.A.) was used. The programmed mobile phase consisted of a combination of two solutions. Solution A consisted of 0.5% (v/v) acetic acid in acetonitrile. Solution B consisted of 0.5% acetic acid in water. The instrument parameters and program are outlined in Table I. A linear gradient was used between $T_{4\ min}$ and $T_{6\ min}$. The total run time was 11 min, resulting in a 5-min hold at the final conditions. A 5-min equilibration time was used between injections.

TABLE I

INSTRUMENTAL PARAMETERS

Flow-rate	3.0 ml/min	
Temperature	ambient	
Absorbance	360 nm (UV), 0.05 a.u.f.s.	
Mobile phase	$T_{0\ min}$	80% A, 20% B
	$T_{4.0\ min}$	80% A, 20% B
	$T_{6.0\ min}$	90% A, 10% B
	$T_{11.0\ mm}$	90% A, 10% B

Specimen preparation

The extraction procedure used was similar to that outlined by Puglisi and de Silva [13], with a single modification. A 1-ml volume of blood or serum was placed in a 15-ml screw-top tube. To this 50 μl of retinyl acetate, 10 mg/l in acetonitrile, were added as internal standard. After vortexing, 2.5 ml of 1 *M* phosphate buffer, pH 6.0, were added. Following a short vortex, 6 ml of diethyl ether were added. The tube was capped and placed on an Eberbach

mechanical shaker (Eberbach, Ann Arbor, MI, U.S.A.) for 15 min at 180 strokes per min. Following centrifugation the ether layer was then removed and evaporated to dryness under nitrogen. The residue was dissolved in 100 μl of solution A. A 50-μl aliquot was injected for analysis.

Calculations

Three blood or serum standards of 250, 500, and 1000 ng/ml (500, 1000, 2000 ng/ml for retinol) were prepared for each compound using 500 μg/ml stock standards in acetonitrile. Calibration curves were made by determining peak height ratios relative to the internal standard.

RESULTS AND DISCUSSION

Typical chromatograms for standard solutions and patient specimens are shown in Fig. 1. As can be observed from Fig. 1, excellent separation of the

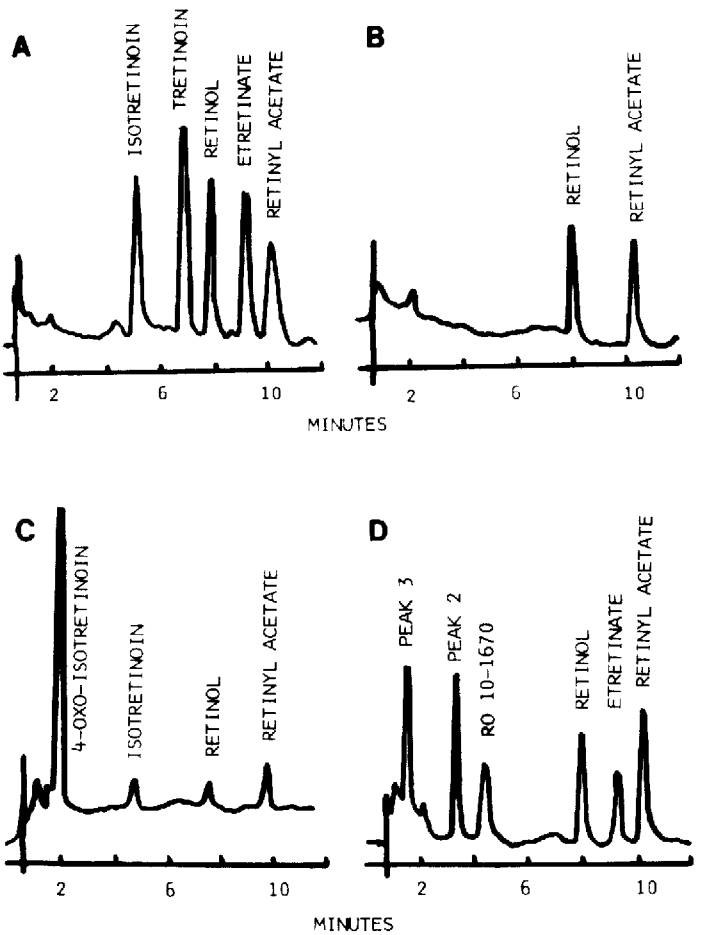

Fig. 1. Reversed-phase chromatograms for whole blood specimens containing retinoids; (A) blood spiked with standards (all-*trans*-retinoic aicd and etretinate, 500 ng/ml; 13-*cis*-retinoic acid and retinol, 2000 ng/ml), (B) normal individual, (C) blood from patient receiving multiple-dose isotretinoin, (D) blood from patient receiving multiple-dose etretinate therapy. Absorbance setting is 0.05 a.u.f.s. for A, B and D, 0.10 for C.

TABLE II

RETENTION TIMES AND RECOVERY FOR RETINOIDS

Compound	Retention time (min)	Recovery* (%)
Ro 10-1670	4.1	78 ± 5
13-*cis*-Retinoic acid	5.1	98 ± 5
All-*trans*-retionic acid	6.9	94 ± 7
Retinol	7.9	86 ± 13
Etretinate	9.1	77 ± 11
Retinyl acetate	9.9	70 ± 5
Peak 2	3.2	—
Peak 3	1.6	—

*Average based on 22 extractions and analyses of a 500 ng/ml blood standard of each compound. This concentration approximates a low-normal value for retinol [15], and a therapeutic level for etretinate [16].

compounds can be achieved using the chromatographic system described here. Although retinal will overlap with etretinate to some extent, this will cause no problem since the normal concentration in blood specimens is too minimal to cause any interference in the determination of etretinate levels. The actual retention times and analytic recoveries are summarized in Table II.

In addition to the presence of etretinate in the blood of patients receiving this drug, at least two metabolites are also found. One of these is Ro 10-1670, the acid metabolite of the parent compound [16, 17] that is also potentially active. However, as has been observed by Hänni et al. [10] and McClean et al. [15], a second metabolite is also evident. This metabolite is Ro 13-7652, the 13-*cis* isomer of Ro 10-1670. The peak height of this second metabolite was consistently larger than the Ro 10-1670 peak in blood samples from patients on chronic etretinate therapy. The exact clinical importance of this metabolite is currently under study. In addition to these two previously observed metabolites, a third metabolite can also be observed. This compound, labelled as peak 3 in Fig. 1D, seems to parallel the level of the other two metabolites in blood from individuals receiving this drug. This peak was not observed in blood from control individuals, nor in specimens taken from individuals receiving placebos during therapeutic trials. When individuals are removed from etretinate therapy, this peak disappears. This metabolite is most probably the 4-hydroxyphenyl retinoic acid analogue, Ro 12-7310, an active major metabolite of etretinate [18].

In patients receiving isotretinoin (13-*cis*-retinoic acid) this assay clearly separates and defines both the parent compound and metabolite, 4-oxo-isotretinoin [19]. The concentration of the metabolite exceeds that of the parent compound 6 h after a single dose [16] or during chronic drug therapy (Fig. 1). McClean et al. [15] have also been able to observe 4-oxo-isotretinoin in patient specimens using HPLC, although the metabolite elutes on the down-side of the solvent front in their system. This could potentially make quantitation difficult. The large solvent front is probably due to the extraction system used.

Although not as rapid as the extraction procedure just discussed above, the method of Puglisi and De Silva [13], as utilized in our assay procedure, results in a small solvent front. This allows for an accurate quantitation of 4-oxo-isotretinoin. In addition the results obtained are unaffected by variations in specimen type. The method presented here works equally well on whole blood, serum, hemolyzed specimens, or plasma collected with any common anticoagulant. The inter- and intra-assay variability (coefficient of variation) for all compounds was less than 10%.

Due to photodecomposition of retinoids, care must be taken to protect samples from direct contact with light. In our laboratory, extraction and analysis are performed in a darkened room with only a minimum of diffuse light allowed. Extraction tubes were wrapped with aluminum foil so as to protect the retinoid compounds.

The assay described here is optimized for the determination of multiple retinoids. The selective determination of retinol in biological specimens can be best performed at 324 nm, at which retinol has maximal absorbance. With this method proposed here retinol levels can be accurately determined using as little as 200 μl of serum, making this method applicable to pediatric specimens.

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