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Note

Simultaneous microassay for etretinate and its active metabolite, etretin, by reversed-phase high-performance liquid chromatography

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Etretinate (Ro 10-9359), a synthetic aromatic retinoid, has been well accepted in Europe as an effective drug for the treatment of severe psoriasis and several hereditary keratotic disorders [1]. It has recently been approved in the United States for similar indications. After oral dosing in patients, both etretinate and its active metabolite, etretin (Ro 10-1670), have been identified in the blood or plasma [2–5]. Etretinate may be metabolized to etretin both in the gastrointestinal wall and in the liver [6], but the relative importance of these metabolic sites in man is unknown. In order to study the presystemic metabolism of etretinate, rats were used as an animal model. However, the previously published assays for the simultaneous quantitation of etretinate and etretin in blood or plasma required a sample volume of not less than 0.5 ml [7–15]. The objective of this study was to develop a reliable and sensitive analytical method for the simultaneous quantitation of etretinate and etretin using sample volumes as small as 100 μl, to allow serial blood sampling in the rat.

EXPERIMENTAL

Laboratory precautions

In order to minimize the photodegradation of etretinate and etretin, all animal experiments and procedures with reference compounds and biological samples were carried out in an inner laboratory fitted with yellow lights. Whenever possible, amberized containers were used including amber injections vials for the

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high-performance liquid chromatography (HPLC) autosampler. Butylated hydroxytoluene (BHT) was added as an antioxidant at a final concentration of 50 μg/ml to stock solutions of etretinate, etretin and retinyl acetate (the internal standard). Blood samples taken from rats were also collected in tubes containing BHT.

Analytical standards and chemicals

Etretinate and etretin were generously supplied by Hoffmann-La Roche (Nutley, NJ, U.S.A.) and were stored upon receipt, wrapped in aluminum foil. Retinyl acetate was obtained from Sigma (St. Louis, MO, U.S.A.).

Acetonitrile (Chrom AR™ HPLC), 1-butanol (Spectr AR™) and dipotassium hydrogenphosphate trihydrate (AR grade) were obtained from Mallinckrodt (Paris, KY, U.S.A.). Glacial acetic acid was obtained from CCI (Columbus, WI, U.S.A.) and HPLC-grade ammonium acetate was obtained from Fisher Scientific (Eden Prairie, MN, U.S.A.). BHT and methanol (Omnisolv™) were obtained from Sigma and EM Science (Cherry Hill, NJ, U.S.A.), respectively.

Preparation of calibration solutions

The solutions of etretinate and etretin used for constructing the calibration curves were prepared by serial dilution of a stock solution containing 250 μg/ml of each in a methanol–acetonitrile mixture (1:1, v/v). A series of calibration solutions containing etretinate and etretin in equal concentrations of 0.2, 0.4, 0.8, 2.0, 4.0, 8.0, 10.0, 20.0 and 50.0 μg/ml were prepared from the stock solutions every six weeks. A stock solution of retinyl acetate (the internal standard) was prepared in acetonitrile (376 μg/ml). A working solution of the internal standard was prepared every month by diluting 10 ml of the stock solution with acetonitrile to a final concentration of 37.6 μg/ml. Calibration and internal standard solutions were stored at −20°C in foil-wrapped vials.

Extraction procedure

The extraction procedure was similar to that reported by McClean et al. [9]. All frozen blood samples were thawed at room temperature in the dark just prior to analysis. The extractions were performed in 11.5 cm × 10 mm glass centrifuge tubes.

The calibration curves were prepared for each assay run by transferring 20 μl of the calibration solutions containing 4–1000 ng of etretinate and etretin into a tube containing 100 μl of blank rat blood. A volume of 20 μl of the internal standard working solution was then added to each tube. Similarly, 20 μl of acetonitrile and 20 μl of internal standard were added to the tubes containing 100 μl of blood taken from the rats that had received etretinate. The tubes were then all gently vortexed. Blood standards and samples were extracted at the same time by adding 350 μl of 1-butanol in acetonitrile (1:1, v/v) into each tube. The tubes were then vortexed for 1 min. A 300-μl volume of aqueous dipotassium hydrogenphosphate solution (1 g/ml) was added to enhance the subsequent separation of the aqueous and organic phases [9]. The tubes were again vortexed for 30 s and then centrifuged at 4°C and 1300 *g* (Clini-cool™, Damon/IEC, Needham Heights, MA,

U.S.A.) for 10 min. The organic layer was then transferred to an amber injection vial fitted with a small-volume insert in preparation for placement into the autosampler.

Chromatographic equipment and conditions

The chromatography was carried out with a Model 6000A solvent delivery system, a WISP autoinjection module, a Model 450 variable-wavelength detector and a Model 730 integrator (all from Waters Assoc., Milford, MA, U.S.A.). A 40-μl aliquot of the extracted sample was injected onto a reversed-phase column (Supelcosil LC-18, 5 μm, 15 cm$\times$4.6 mm I.D., Supelco, Bellefonte, PA, U.S.A.) which was preceded by a guard column packed with LC-18 pellicular packing (Supelco). The isocratic mobile phase was made up of acetonitrile in water (79:21, v/v) with 0.8 g of ammonium acetate and 10 ml of glacial acetic acid per 1000 ml. The flow-rate was 1.5 ml/min. Detection was carried out at 360 nm with a sensitivity of 0.02 a.u.f.s. The chart-speed was 0.5 cm/min.

Calibration and calculations

Linearity of the assay was established in the ranges 0.08–0.8 and 0.8–10 μg/ml of blood by analyzing spiked rat blood samples covering these ranges. A separate pair of calibration curves was generated for etretinate and etretin by using the least-squares regression of the peak-area ratios against the concentration of the spiked compounds. The concentrations of etretinate and etretin in the blood samples were obtained from computerized interpolation of the calibration curves.

Precision

The precision of the extraction procedure and chromatography was evaluated by analyzing replicate calibration curves on the same day (within-run precision) and on different days (between-run precision). The peak-area ratio of each compound to the internal standard for each concentration was compared over the calibration curves and the percentage coefficient of variation (C.V.) for each concentration was determined.

Recovery

The peak areas of etretinate, etretin and retinyl acetate in extracted blood were each compared with those of individual calibration solutions injected directly onto the HPLC system. The percentage recovery of each compound at each concentration was calculated as:

$$\text{recovery} = \frac{\text{peak area of extracted sample per } \mu\text{l injected}}{\text{peak area of calibration solution per } \mu\text{l injected}} \times 100\%.$$

RESULTS AND DISCUSSION

The isocratic reversed-phase HPLC conditions described allowed the separation of etretinate, etretin and the internal standard within a run time of less than 12 min. The endogenous retinol peak did not interfere with the separation. Typ-

ical chromatograms obtained from the analysis of blank rat blood containing internal standard and a blood sample from a rat that received etretinate are shown in Fig. 1. The retention times for etretin, etretinate and retinyl acetate were approximately 3.2, 8.1 and 11.1 min, respectively. When blank blood extracts without internal standard were injected, only the endogenous retinol peak was observed (data not shown).

The summaries of the within-run precision ($n=3$) and the between-run precision ($n=6$) over the calibration range appear in Tables I and II, respectively. The percentage C.V. varied randomly over the concentration range.

The lower limit of quantitation of the method was determined to be the lowest concentration in the calibration curve showing an acceptable C.V. in the within-run analysis. Therefore, the lower limit of quantitation for etretinate was 0.08 μg/ml and for etretin was 0.16 μg/ml, based on blood samples of 100 μl. It would be possible to increase the sensitivity of the assay by increasing the blood volume used.

The calibration curves for etretinate were linear over the concentration ranges 0.08–0.8 and 0.8–10 μg/ml. For etretin, linearity of the calibration curves was observed in the ranges 0.16–0.8 and 0.8–10 μg/ml.

Extraction recoveries over the range of concentrations for etretinate, etretin and retinyl acetate are shown in Table III. The recoveries of the compounds appeared to be independent of concentration, indicating an efficient extraction as well as stability of the compounds in the blood during the extraction procedure.

The method was used to determine etretinate and etretin concentrations in rat

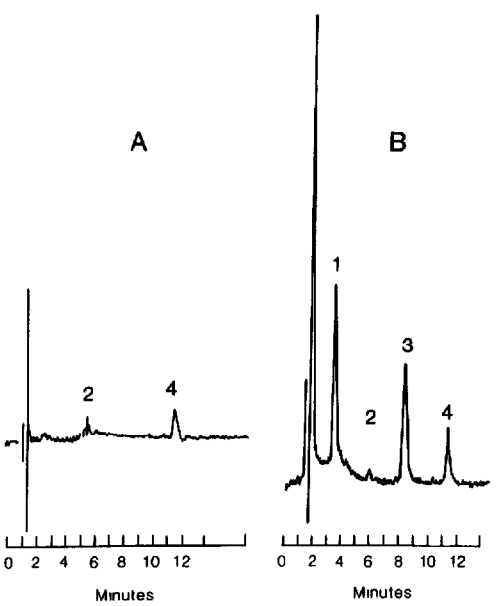

Fig. 1. HPLC profiles of etretinate and etretin. (A) Blank rat blood spiked with 500 ng retinyl acetate. (B) Blood sample from a rat that had received 10 mg/kg etretinate intravenously. Both (A) and (B) were carried through the extraction procedure; injection volume 40 μl; a.u.f.s. 0.02. Peaks: 1 = etretin; 2 = endogenous retinol; 3 = etretinate; 4 = retinyl acetate (internal standard).

TABLE I

WITHIN-RUN PRECISION FOR ETRETINATE AND ETRETIN ANALYSIS IN RAT BLOOD ($n=3$)

Concentration (μg/ml)	Etretinate		Etretin	
	Peak-area ratio (mean ± S.D.)	C.V. (%)	Peak-area ratio (mean ± S.D.)	C.V. (%)
0.08	0.045 ± 0.006	12.2	0.041 ± 0.009	22.0*
0.16	0.116 ± 0.012	9.9	0.081 ± 0.009	11.1
0.4	0.280 ± 0.016	5.8	0.240 ± 0.010	4.0
0.8	0.491 ± 0.030	6.0	0.480 ± 0.048	10.1
1.6	1.01 ± 0.080	7.9	1.01 ± 0.063	6.3
2.0	1.26 ± 0.070	5.6	1.33 ± 0.036	2.7
4.0	2.24 ± 0.056	2.5	2.38 ± 0.050	2.1
10.0	5.67 ± 0.443	7.8	6.00 ± 0.533	8.9

*Because of the high C.V., this concentration of etretin was not included in the standard curves used for determining blood sample concentrations.

TABLE II

BETWEEN-RUN PRECISION FOR ETRETINATE AND ETRETIN ANALYSIS IN RAT BLOOD ($n=6$)

Concentration (μg/ml)	Etretinate		Etretin	
	Peak-area ratio (mean ± S.D.)	C.V. (%)	Peak-area ratio (mean ± S.D.)	C.V. (%)
0.8	0.063 ± 0.009	13.9	0.032 ± 0.008	24.2*
0.16	0.126 ± 0.011	9.0	0.085 ± 0.010	12.2
0.4	0.296 ± 0.023	7.9	0.274 ± 0.026	9.6
0.8	0.546 ± 0.050	9.2	0.511 ± 0.043	8.4
1.6	1.10 ± 0.096	8.8	1.10 ± 0.100	9.1
2.0	1.30 ± 0.100	7.7	1.39 ± 0.071	5.1
4.0	2.37 ± 0.163	6.9	2.60 ± 0.266	10.2
10.0	6.36 ± 0.665	10.4	6.10 ± 0.501	8.2

*Because of the high C.V., this concentration of etretin was not included in the standard curves used for determining blood sample concentrations.

blood in the studies of the pharmacokinetics of these compounds. The concentration–time profile of etretinate and etretin following the intravenous administration of 10 mg/kg etretinate is shown in Fig. 2. Both etretinate and etretin could be detected for up to 32 h after dosing. The method is therefore appropriate for use in studies where the sample volume is limited to 100 μl of blood.

Both reversed-phase and normal phase HPLC methods have previously been reported for the simultaneous analysis of etretinate and etretin [7–15]. The most important improvement in the present method is that the extraction procedure can be performed on as little as 100 μl of whole blood. The ability to use so small

TABLE III

RECOVERY OF ETRETINATE, ETRETIN, AND RETINYL ACETATE FROM RAT BLOOD ($n=4$)

Concentration (μg/ml)	Etretinate		Etretin		Retinyl acetate	
	Recovery (mean ± S.D.)	C.V. (%)	Recovery (mean ± S.D.)	C.V. (%)	Recovery (mean ± S.D.)	C.V. (%)
0.1	88.1 ± 4.7	5.3	84.5 ± 8.5	10.1	78.7 ± 8.1	10.3
0.2	81.1 ± 5.4	6.7	82.3 ± 4.4	5.3	90.0 ± 3.1	3.4
0.8	81.0 ± 6.9	8.5	79.5 ± 5.6	7.1	81.3 ± 10.3	12.7
1.6	75.7 ± 3.1	4.1	74.0 ± 8.7	11.8	86.0 ± 7.5	8.7
2.0	74.4 ± 6.1	8.1	80.9 ± 3.9	4.8	89.3 ± 7.3	8.2
5.0	80.1 ± 7.0	8.8	86.4 ± 9.0	10.4	81.5 ± 7.6	9.3
10.0	91.1 ± 11.6	12.8	100.6 ± 3.0	3.0	83.4 ± 15.3	18.4

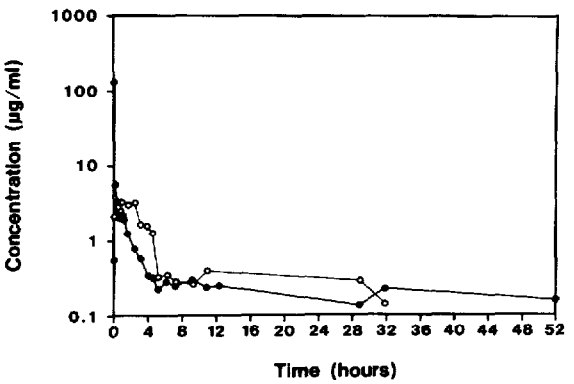

Fig. 2. Venous blood concentration–time profile of etretinate (●) and etretin (○) following intravenous administration of 10 mg/kg etretinate to a rat.

a sample allows the pharmacokinetic study of etretinate and etretin in small animal models, such as the rat. The extraction procedure modified from that of McClean et al. [9] was also less time-consuming than those previously described, and does not require an evaporation step [7,10–15]. Finally, the use of an isocratic system with a 15-cm column decreased the elution time without sacrificing resolution.

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