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DETERMINATION OF PLASMA LEVELS OF TWO AROMATIC RETINOIC ACID ANALOGUES WITH ANTIPSORIATIC ACTIVITY BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method is described for the quantitative analysis in plasma of Ro 10-9359, an aromatic retinoic acid analogue, ethyl all-*trans*-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoate and its major metabolite Ro 10-1670, all-*trans*-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,3,6,8-nonatetraenoic acid. The compounds are extracted from patient plasma with diethyl ether, separated on a reversed-phase column and detected and quantified by their UV absorption. The experimental error is below 9% in the concentration range 42—445 ng/ml. The detection limit is about 10 ng/ml. The method was applied to the analysis of plasma levels in healthy volunteers, receiving 75 mg orally.

INTRODUCTION

The compound ethyl all-*trans*-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoate (Ro 10-9359) (Fig. 1) has been shown to have a therapeutic and prophylactic effect against epithelial tumors in mice [1—4]. Later, it has been shown to have an antipsoriatic activity in man [5] and its clinical usefulness for this purpose is currently being investigated. To examine the pharmacokinetics of the compound in man an analytical method with high specificity, sensitivity and capacity is required.

The thermal instability of the retinoids [6, 7] excludes gas chromatographic procedures for the determination of these substances. Previously described methods for the determination of retinol and retinoic acid were based on spectrofluorometry or spectrophotometry [8—11]. However, these methods were not appropriate for the determination of the retinoids in plasma due to

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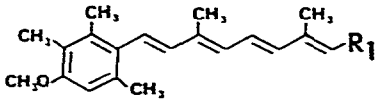
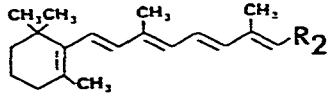


R_1	Compound
$-C(=O)-O-C_2H_5$	Ro 10-9359
$-COOH$	Ro 10-1670
$-C(=O)-O-CH(CH_3)-CH_3$	Ro 11-0249



R_2	
$-CH_2-OH$	Retinol
$-COOH$	Retinoic acid
$-C(=O)-O-NH-C_2H_5$	Ro 8-4968

Fig. 1. Chemical structures of the retinoids (Ro 10-9359, Ro 10-1670 and Ro 11-0249), retinol and retinoic acid.

their low specificity and sensitivity. Liquid—gel partition chromatography [12] could probably solve the problem of specificity and sensitivity but it is a slow and time consuming method. High-performance liquid chromatography (HPLC) has previously been used for the analysis of vitamin A, its acid, its isomers and esters. Liquid—solid adsorption or reversed-phase chromatography [13—23] have been utilized in these cases.

In this paper it was examined whether HPLC could be used also for the simultaneous analysis in plasma of Ro 10-9359 and its major metabolite, the corresponding free acid Ro 10-1670. In the preliminary studies reversed-phase chromatography was found to be most appropriate to the problem and the method was based on this principle. In order to optimize the chromatographic separation the retention properties of the retinoids and some related compounds were studied. The UV absorption and fluorescence characteristics of the drug and its metabolite were also studied to achieve highest specificity and sensitivity of the detection system.

EXPERIMENTAL

Equipment

The HPLC system consisted of a Spectra-Physics Model 3500B high-pressure pump, a Valco sample injector equipped with a 50-μl injection loop and a Laboratory Data Control Spectromonitor II UV detector, operated at 350 nm. The columns used for reversed-phase chromatography were μBondapak C_{18} (300 × 4 mm I.D.) (Waters Assoc., Milford, MA, U.S.A.) and a Hibar LiChrosorb RP-18, particle size 7 μm (250 × 3 mm I.D.) (Merck, Darmstadt, G.F.R.). For liquid—solid adsorption chromatography a Hibar LiChrosorb Si-60, particle size 7 μm (300 × 3 mm I.D.) column was used.

Chemicals

The water was taken from a Milli-Q™ Reagent-Grade Water System (Milli-

pore, Bedford, MA, U.S.A.). Ro 10-9359, Ro 10-1670, retinol and retinoic acid were supplied by Hoffmann-La Roche (Basle, Switzerland). All solvents and chemicals used were of p.a. quality (Merck). Acetonitrile, HPLC-grade, was purchased from Rathburn Chemicals (Walkerburn, Great Britain), and 2,2-di-*tert.*-butyl-*p*-cresol (BHT) from Sigma (St. Lous, MO, U.S.A.).

Solutions

The retinoids easily undergo photoisomerization and oxidation, which makes it necessary to handle and store all substances and solutions in yellow dark-room light. In order to prevent oxidation BHT was added in an amount of about 50 μg/ml [12] to all solutions and all bottles and tubes were flushed with nitrogen when stored and when the plasma samples were extracted.

Solutions of retinol, retinoic acid and the retinoids were prepared by dissolving about 10 mg of the substance in 100 ml of acetonitrile. For the determination of Ro 10-9359 and Ro 10-1670 in plasma, standard plasma samples were prepared by serial dilutions in plasma to give ten concentrations in the range of 5—1000 ng/ml. Solutions of the internal standard, Ro 11-0249, were instable and prepared just before use. All buffer solutions used were prepared from Na_2HPO_4 and NaH_2PO_4 to the desired pH value in a concentration of 0.1 *M*. For routine analysis the mobile phase was a mixture of acetonitrile—water (80:20, v/v) and 1% of acetic acid.

Procedure

Plasma samples were stored under nitrogen at —70°C pending analysis. Into a 15-ml tube, 1 ml of plasma, 3 ml of buffer solution (pH 6) and 40 μl of internal standard solution were introduced. The tubes were flushed with nitrogen and the mixture was extracted with 6 ml of diethyl ether on a reciprocating shaker for 15 min. After centrifugation at 2000 *g* for 10 min, the supernatant was transferred into another 15-ml tube with a conical bottom and evaporated under a stream of nitrogen. The residue was dissolved in 100 μl of acetonitrile and 10—20 μl were injected into the chromatographic system.

A calibration curve was prepared by taking the standard samples through the procedure (Fig. 2). The peak heights were used for the quantitation.

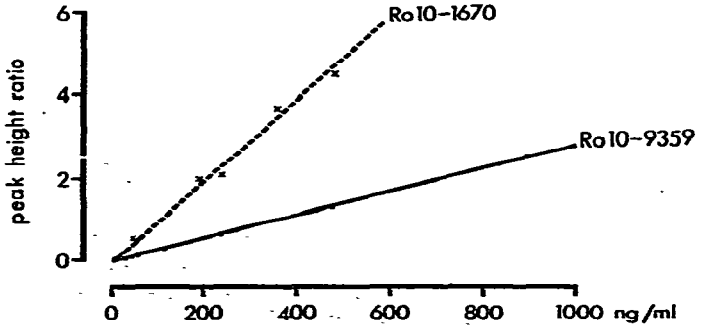


Fig. 2. Standard curves for the quantitation of Ro 10-9359 and Ro 10-1670 in plasma. Peak height ratios of Ro 10-9359 and Ro 10-1670 to Ro 11-0249 versus concentrations of Ro 10-9359 and Ro 10-1670 added to plasma.

RESULTS AND DISCUSSION

Liquid—solid adsorption HPLC systems have been reported to separate isomers and esters of vitamin A with high selectivity [6, 13, 14]. In this study it was found that this approach was successful also for the separation of the retinoids [19]. A typical chromatogram from a plasma extract is shown in Fig. 3A. The separation was achieved using a mixture of hexane—tetrahydrofuran (95:5, v/v). One per cent of acetic acid was used as deactivator of the column as otherwise severe peak tailing occurred with the carboxylic acids. Due to unstable retention times for the most retained peaks after injection of a series of plasma samples, a reversed-phase chromatographic system was studied. The chromatographic properties of the retinoids on a reversed-phase system were investigated as regards the pH and the per cent organic modifier of the mobile phase. First the retention, in terms of capacity factor (k'), was

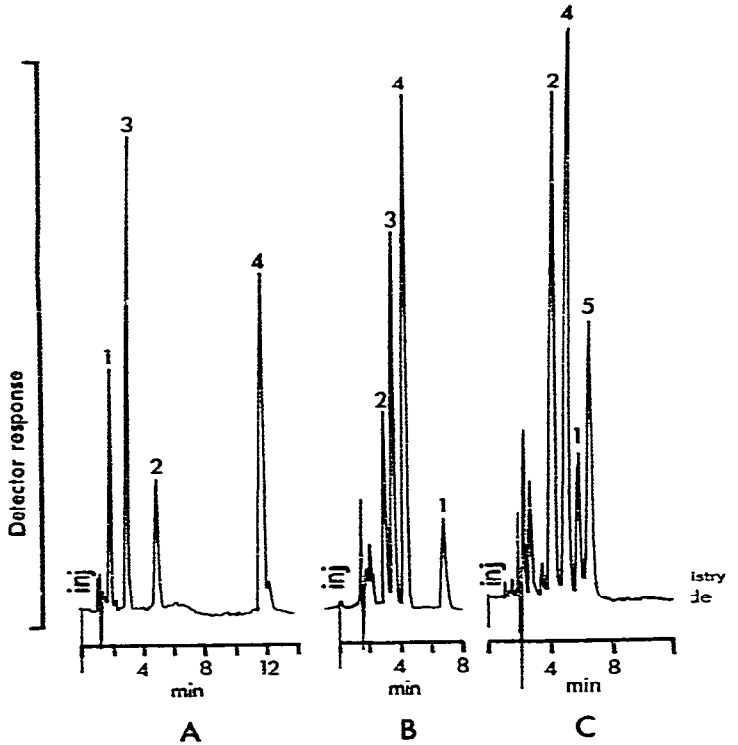


Fig. 3. Chromatograms from the analysis of plasma samples. (A) Liquid—solid adsorption chromatography. Mobile phase: hexane—tetrahydrofuran (95:5, v/v) containing 1% acetic acid. Flow-rate: 1.5 ml/min. Column: Hibar LiChrosorb Si-60. Peaks: 1, Ro 10-9359 (280 ng/ml); 2, Ro 10-1670 (224 ng/ml); 3, retinoic acid (internal standard, 250 ng/ml); 4, endogenous retinol. (B) Reversed-phase chromatography. Mobile phase: methanol—water (90:10, v/v) containing 1% acetic acid. Flow-rate: 2 ml/min. Column: μBondapak C_{18}. Samples are the same as in (A). (C) Reversed-phase chromatography. Column: Hibar LiChrosorb RP-8. Mobile phase: acetonitrile—water (80:20, v/v) containing 1% acetic acid. Flow-rate: 1 ml/min. Plasma sample from a patient 2.5 h after administration of a 75-mg oral dose of Ro 10-9359. Peaks: 1, Ro 10-9359 (174 ng/ml); 2, Ro 10-1670 (112 ng/ml); 4, endogenous retinol; 5, Ro 11-0249 (internal standard, approx. 400 ng/ml).

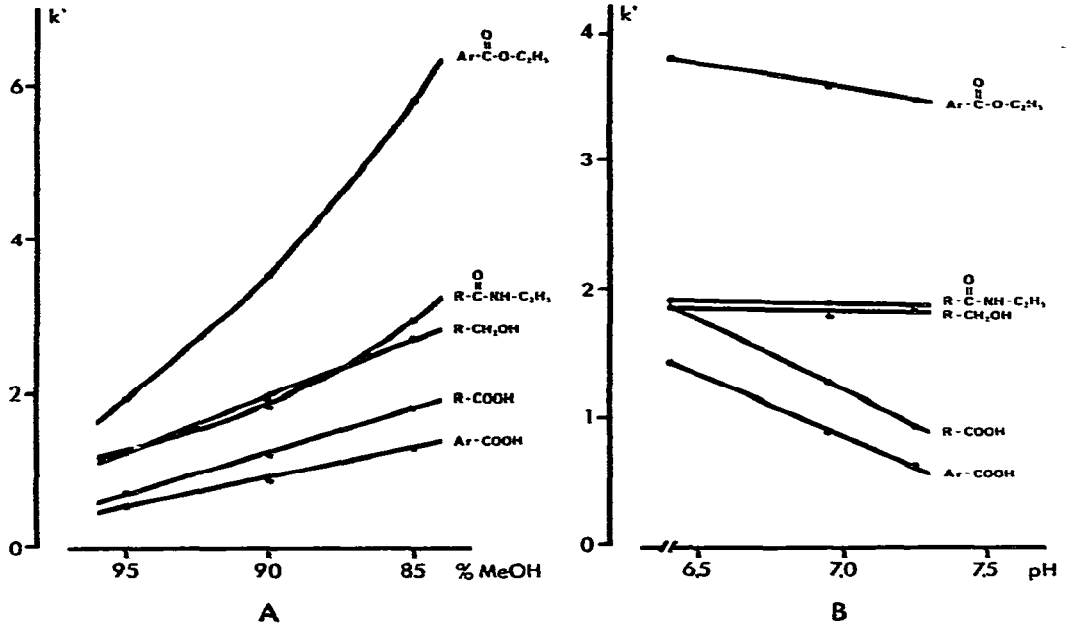


Fig. 4. (A) Effect of concentration of organic modifier on k' values. Mobile phase methanol—water, pH = 7.0. Stationary phase μBondapak C_{18}. (B) Effect of pH of mobile phase on k' values. Mobile phase: methanol—water (90:10, v/v) containing 1% acetic acid, pH = 6.40—7.37. Stationary phase μBondapak C_{18}.

measured for the retinoids as a function of the amount of methanol in the mobile phase at pH 7.0. k' decreased for all substances as the proportion of methanol was increased (Fig. 4A). The influence of pH on the retention at constant methanol—water composition was also studied. As expected the degree of ionization of the carboxylic acids greatly influenced the retention (Fig. 4B). A chromatogram from a plasma sample analysed at pH 7.0 and methanol—water (90:10, v/v) is shown in Fig. 3B. Acetonitrile as organic modifier had a peak-sharpening effect and was used in the routine analysis. A typical chromatogram from the routine analysis, when Ro 11-0249 was used as internal standard, is shown in Fig. 3C.

Ro 11-0249 was found to be the most suitable internal standard to obtain correction for losses during the analytical procedure. However, the internal standard could not eliminate the extraction procedure as a source of error, probably due to exposure of the substances to air and light during the work. The standard deviation was in the range of 7—14% when Ro 10-9359 and Ro 10-1670 were added to drug-free plasma (Table I). The extraction recoveries for Ro 10-9359 and Ro 11-0249 are shown in Table II. The error of the method, determined from 16 samples with concentrations in the range of 42—445 ng/ml, was below 9% (Table III).

The fluorescence properties of the retinoids were investigated for HPLC fluorescence detection, but the relatively weak fluorescence was not preferable to the highly selective and sensitive UV detection obtained at 350 nm. The detection limit in plasma was about 10 ng/ml for Ro 10-9359.

TABLE I

ASSAY REPRODUCIBILITY

Data represent mean ± S.D. from 5 measurements, when Ro 10-9359 and Ro 10-1670 were added to drug-free plasma.

Amount added (ng/ml)		Amount found (ng/ml)	
Ro 10-9359	Ro 10-1670	Ro 10-9359	Ro 10-1670
103	113	107 ± 7.9	117 ± 4.6
206	226	198 ± 21	217 ± 20
310	339	314 ± 45	342 ± 40

TABLE II

EXTRACTION RECOVERIES FROM PLASMA

The internal standard was added after the extraction procedure. Recovery was determined by comparison with a non-extracted methanol standard series. Each figure represents the mean of two observations.

Amount of Ro 10-9359 (ng/ml)			Amount of Ro 11-0249 (ng/ml)		
Added	Found	Recovery (%)	Added	Found	Recovery (%)
35	26	74	36	25	69
70	46	66	72	40	57
104	58	56	89	46	52
174	102	59	143	81	56
348	188	54	268	125	47

TABLE III

EXPERIMENTAL ERROR FOR THE DETERMINATION OF Ro 10-9359 IN PLASMA

Compound I in plasma was determined with duplicate analysis. S.D. = $\sqrt{(2d^2)/2n}$, where d is the difference between duplicate analyses.

Range (ng/ml)	n	mean	S.D.	% of mean
42—445	16	227	19.7	8.7

The time course for the amount of the drug and the major metabolite in plasma was examined after oral administration of 75 mg of Ro 10-9359 to a volunteer (Fig. 5). The peak levels were reached after about 5 h. The plasma levels can readily be detected 24 h after administration which indicates that the method will be appropriate for systematic pharmacokinetic studies in man.

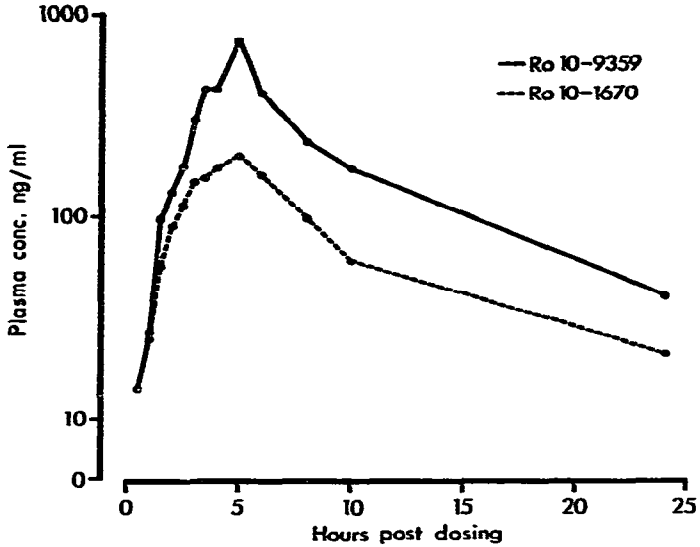


Fig. 5. Plasma levels of Ro 10-9359 and Ro 10-1670 in a patient after oral administration of 75 mg of Ro 10-9359.

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