Journal of Chromatography, 162 (1979) 615—621
Biomedical Applications

CHROMBIO. 286

Note

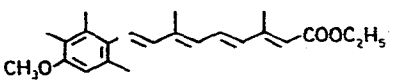
Determination of an aromatic retinoid and its main metabolite by high-performance liquid chromatography

RALPH HÄNNI*, DOMINIQUE HERVOUET and ARTHUR BUSSLINGER

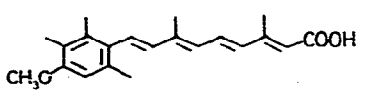
Biological Pharmaceutical Research Department, F. Hoffmann-La Roche & Co., Ltd., Basle (Switzerland)

(Received August 16th, 1978)

Many therapeutic successes have been achieved with the retinoic acid analogue, Ro 10-9359 [ethyl all-*trans*-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoate, Fig. 1], in psoriasis patients. The compound



Ro 10-9359



Ro 10-1670

Fig. 1. Retinoid Ro 10-9359 and metabolite Ro 10-1670.

is both therapeutically effective in the mouse and prophylactically effective on epithelial tumors [1—3]. The need to determine the bioavailability of different oral formulations of Ro 10-9359, to monitor clinically-effective plasma levels of patients undergoing oral retinoid therapy and to determine the pharmacokinetic parameters necessitated the development of a sensitive and specific assay for the determination of Ro 10-9359 and its pharmacologically-active main metabolite Ro 10-1670 in plasma (Fig. 1).

*To whom correspondence should be addressed at the following address: Mittlerer Rainweg 3, CH-4414 Füllinsdorf, Switzerland.

MATERIALS AND METHODS

All work with the retinoids and the internal standard retinoic acid must be carried out in darkened rooms, and all glassware must be amberized to prevent photoisomerisation of these compounds.

Reagents

All reagents must be of analytical reagent grade (>99% purity). The organic solvents were purchased from E. Merck (Darmstadt, G.F.R.).

Columns

The columns were 0.25 m × 3.0 mm I.D. stainless steel, containing 5 μl silica gel (Partisil, Whatman, Clifton, N.J., U.S.A. or LiChrosorb, Merck) and generating 20,000—40,000 plates per m. The temperature of the columns was 20°.

Instrumental parameters

Pump: Milton-Roy 5000. Injector: Altex high-pressure sample injection valve. Detector: Cecil 212 UV detector at 360 nm.

Mobile phases

The isocratic mobile phases were mixtures of: (A) hexane—tetrahydrofurane —glacial acetic acid (98:1.5:0.6, v/v/v) and (B) hexane—methylbenzoate—propionic acid (87.5:12.5:0.35, v/v/v). The mixtures were boiled before use.

Analytical standards

All analytical standards were of pharmaceutical grade purity (> 99%). These included Ro 10-9359 (ethyl all-*trans*-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoate, $C_{23}H_{30}O_3$), Ro 10-1670 (all-*trans*-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoic acid, $C_{21}H_{26}O_3$) and the internal standard (all-*trans*-retinoic acid, $C_{20}H_{28}O_2$).

Preparation of standard solutions

Place 10.0 mg of the retinoids or the retinoic acid in separate 100-ml volumetric flasks and dissolve in 100.0 ml of tetrahydrofuran to obtain stock solutions containing 100 ng/μl. These stock solutions could be stored without alteration for 3 months at - 20°.

To prepare the standard solutions, aliquots of 10.0 ml of the stock solutions were adjusted to 100.0 ml with hexane. Volumes (10.0 ml)of these solutions containing 10 ng/μl were diluted to 100.0 ml with hexane in volumetric flasks, forming the standard solutions of the retinoids and the internal standard containing 1 ng/μl. These standard solutions could be stored undecomposed for 2 weeks at 5°.

Procedure

To a 20 ml amberized centrifuge tube, add 0.5 ml of plasma, 50 μl of the internal standard solution (= 50 ng retinoic acid), and 4.5 ml of a 0.1 *M* buffer pH 6 solution (citrate—NaOH, Titrisol; Merck). The plasma samples thus

prepared could be stored for 1 week at -20°. Extract the aqueous phase with 10 ml of hexane by shaking for 10 min on a reciprocating shaker (Heidolph) at 60 rpm. Along with 10 samples, process two samples of 0.5 ml of control plasma, containing 50 µl Ro 10-9359 and Ro 10-1670 standard solutions, respectively (= 50 ng retinoid). Centrifuge the samples at 2000 *g* at room temperature for 4 min. Transfer approx. 9 ml of the upper organic layer into another amberized 20-ml conical centrifuge tube. Evaporate the organic layer to dryness at 40° under a stream of clean, dry nitrogen. Dissolve the residues, cooled to 4°, in two 100-µl aliquots of the mobile phase and filter through a small swab of cotton wool into a microtube. Inject approx. 100 µl. Plasma samples which were not analysed immediately after collection could be stored without alteration for 3 months at -20°.

Calibration curves

Calibration curves of the peak height ratios (retinoid:internal standard) versus the concentration of the retinoids were prepared, analysing 0.5 ml of the plasma samples, each containing 100 ng/ml of the internal standard and different concentrations of the retinoids (Fig. 2).

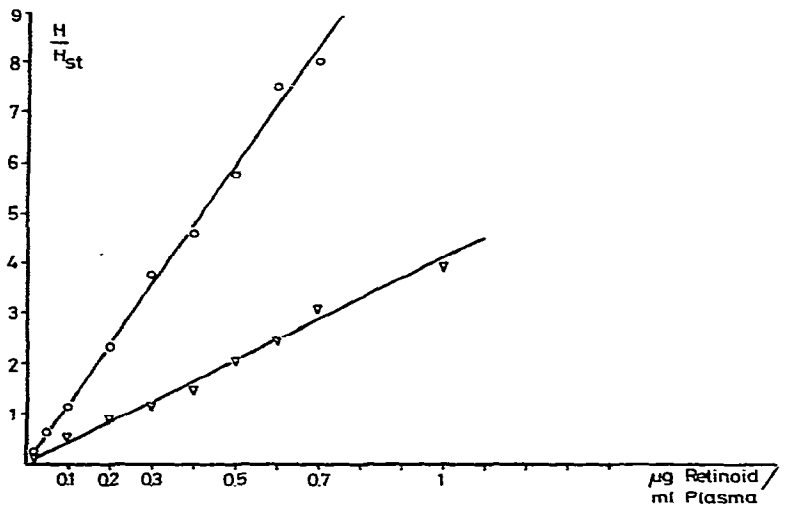


Fig. 2. Calibration curves for Ro 10-9359 (o) and the metabolite Ro 10-1670 (v). Mobile phase: mixture A.

RESULTS

The assay allows the simultaneous determination of the drug Ro 10-9359 and its main metabolite Ro 10-1670 after extraction from plasma, using their UV absorbance at the absorption maximum of 360 nm. So far, more than one thousand plasma samples from rats, rabbits, dogs and humans have been analysed using this assay.

Using the mobile phase A and a flow-rate of 1.2 ml/min, the retention times of Ro 10-9359, of Ro 10-1670 and of the internal standard were 2.6,

6.0 and 4.1 min, respectively. Under these conditions, a new metabolite of Ro 10-9359, found only in human plasma [4] after multiple dosing of Ro 10-9359, is not separated from the peak of the main metabolite Ro 10-1670 in the chromatogram (Fig. 3).

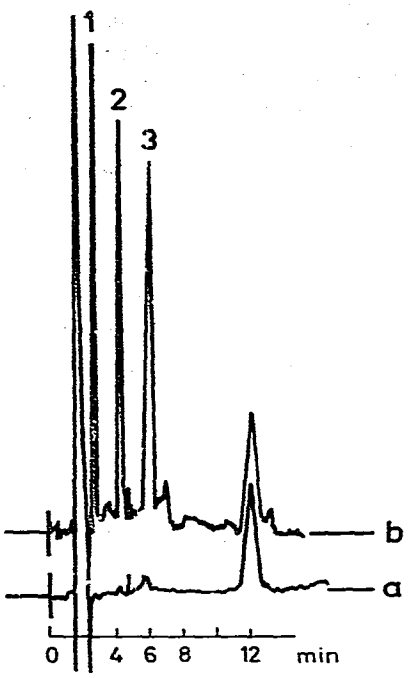


Fig. 3. Chromatograms of the HPLC analysis of: (a) 0.5 ml control plasma and (b) 0.5 ml plasma containing 105 ng/ml Ro 10-9359 (peak 1), 100 ng/ml internal standard (retinoic acid, peak 2) and 210 ng/ml Ro 10-1670 (peak 3). Stationary phase: LiChrosorb SI 60 (Merck); mobile phase: hexane—tetrahydrofurane—glacial acetic acid (98:1.5:0.6, v/v/v) at 360 nm.

Using the mobile phase B and a flow-rate of 1.2 ml/min, the retention times of Ro 10-9359, Ro 10-1670 and the internal standard were 3.2, 8.1 and 4.2 min, respectively. Under these conditions, the peak of the new metabolite, not detectable in Fig. 4, is separated sufficiently from the Ro 10-1670 peak and appears 7 min after injection (Fig. 4).

The calibration curves for Ro 10-9359 and Ro 10-1670 were linear from 10 to 1000 ng/ml of the retinoid, using the mobile phase A, and from 20 to 500 ng/ml of the retinoid, using the mobile phase B.

The relative standard deviation of the whole assay ranges from ± 10 to ± 20% for the unaltered drug and the metabolite Ro 10-1670, within the range of concentration of 50 to 1000 ng/ml and to ± 10 to ± 25% within 10—50 ng/ml of the retinoid. These relatively high values are due to bad extractability specific to these substances and the low concentrations to be determined.

The concentration of each retinoid is determined from the slope of the calibration curves and the peak height ratio, retinoid:internal standard (i.s.) as

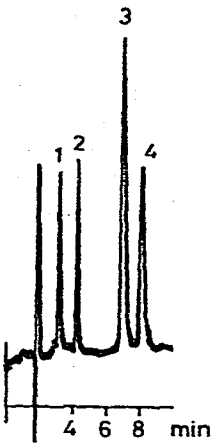


Fig. 4. Chromatogram of the HPLC analysis of 0.5 ml human plasma following multiple dosing of Ro 10-9359, containing 80 ng/ml Ro 10-9359 (peak 1), 100 ng/ml internal standard (retinoic acid, peak 2), 240 ng/ml of the metabolite Ro 10-1670 (peak 4) and an unknown amount of a new metabolite (peak 3). Stationary phase: LiChrosorb SI 60 (Merck); mobile phase: hexane—methyl benzoate—proprionic acid (87.5:12.5:0.35, v/v/v) at 360 nm.

follows:

$$\text{ng/ml Ro 10-9359} = \frac{\text{Peak height Ro 10-9359 (mm)}}{\text{Peak height i.s. (mm)}} \cdot f$$

$$\text{ng/ml Ro 10-1670} = \frac{\text{Peak height Ro 10-1670 (mm)}}{\text{Peak height i.s. (mm)}} \cdot f$$

Average factors (f_A) for Ro 10-9359 and Ro 10-1670, using the mobile phase A, were 84 ng/ml (S.D. = 9 ng/ml) and 235 ng/ml (S.D. = 31 ng/ml), respectively. Using the mobile phase B, they were 90 ng/ml (S.D. = 10 ng/ml) for Ro 10-9359 and 260 ng/ml (S.D. = 34 ng/ml) for Ro 10-1670.

As already mentioned, along with 10 samples, two samples of control plasma containing 50 ng internal standard, 50 ng Ro 10-9359 and 100 ng Ro 10-1670, were analysed in order to control the calibration curves. From these control samples the daily factors (f_D) were calculated and compared with the average factors. If the factors were within the limits given by the standard deviation of the assay, the average factors were used for calculation. If they were within twice the standard deviation, the actual factors were put into the equation, and if there was a large difference between the daily and the average factors, the assays had to be repeated.

The overall recoveries of Ro 10-9359 and Ro 10-1670 determined with the radioactive compounds, were 75 ± 10% and 65 ± 10%, respectively.

The same recoveries were obtained when more polar organic solvents were used, e.g. ethyl acetate, but the chromatograms contained fewer impurities after extraction with hexane.

The limits of detection are in the order of 10—20 ng/ml for Ro 10-9359 and Ro 10-1670, using a 0.5-ml aliquot of plasma.

DISCUSSION

The retinoids Ro 10-9359 and Ro 10-1670 have a high UV absorption at 360 nm, which was utilized for their quantification in the low nanogram range. Both compounds can be analysed either in human or animal plasma (rat, rabbit, dog) using the same procedure and calibration curves. As was shown in an assay with radioactive Ro 10-9359 and Ro 10-1670, the formation of the retinoid—protein complex in plasma is reversible, and the retinoid is extracted by hexane.

Adsorption chromatography was selected in preference to reversed-phase chromatography because of better sensitivity (columns with 20,000—40,000 plates per m could be used) and the fact that the resolution of the *cis*-isomers of the all-*trans* compounds, produced by exposure to light or by biotransformation, was achieved.

The assay presented is based on the comparison of the peak heights of the retinoid with that of an internal standard. For this analytical procedure it is essential to use an adequate internal standard.

Retinoic acid is an ideal internal standard because of the similar chemical and physical behaviour. Irregularities in extracting the compounds from the plasma and the loss of compounds due to absorption on to the glass walls are taken into account. Retinoic acid absorbs enough UV light at 360 nm, the UV absorption maximum of Ro 10-9359 and Ro 10-1670, and is always available in a pure form*.

The disadvantage of this internal standard is its sensitivity to light. Since Ro 10-9359 and Ro 10-1670 are also very sensitive to light the whole analytical procedure has to be done in a darkened room.

Care must be taken in slowly thawing the frozen plasma samples in order to avoid the formation of solid protein particles. After careless thawing, the plasma might not be homogenous and, since the lipophilic retinoids are bonded to a great extent to these proteins, the determination can be inaccurate.

Ro 10-9359 has been shown previously [5] to be highly metabolized and to form at least 21 metabolites. These were found in urine, bile and blood. Most have a shortened tetraen side chain, with a UV adsorption maximum near 260 nm, and do not impair the assay.

The oxygenated metabolites of Ro 10-9359, with an intact tetraen side chain found in rat bile and which show an absorption maximum near 260 nm, are separated sufficiently well with this high-performance liquid chromatographic assay to prevent their interference.

The attempt to perfect a quantitative method of determination on thin-layer plates and a gas chromatographic assay failed because of the extreme photosensitivity and the thermal instability of the retinoids Ro 10-9359 and Ro 10-1670.

Application of the assay to plasma samples containing the retinoids Ro 10-9359 and Ro 10-1670

Typical plasma level curves of the drug and the main metabolite after oral

*Retinoic acid is the active principle of AIROL Roche.

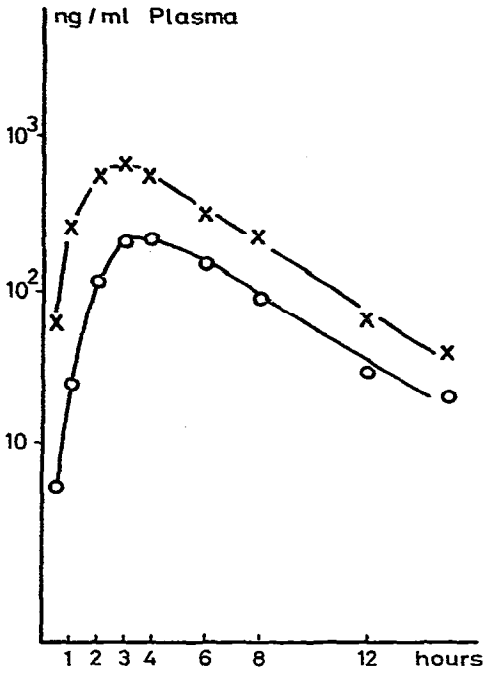


Fig. 5. Plasma levels of Ro 10-9359 (o) and Ro 10-1670 (x) after oral administration of 2 mg/kg of Ro 10-9359 to an adult male.

administration of 100 mg of the drug to an adult male are shown in Fig. 5. The drug reaches its maximum concentration of 230 ng/ml in plasma, 3—4 h following oral administration, showing a relatively slow absorption. After the same period, the metabolite reaches its maximum concentration of 700 ng/ml. Twenty-four hours after administration the concentrations declined to immeasurable amounts.

REFERENCES

1 W. Bollag, Eur. J. Cancer, 10 (1974) 731.
2 W. Bollag, Eur. J. Cancer, 11 (1975) 721.
3 W. Bollag, Chemotherapy (Basel), 21 (1975) 236.
4 R. Hänni, A. Busslinger and M. Klaus, Eur. J. Drug Metab., in press.
5 R. Hänni, F. Bigler, W. Vetter, G. Englert and P. Loeliger, Helv. Chim. Acta, 60 (1977) 2309.