Journal of Chromatography, 258 (1983) 314–319
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,556

Note

Determination of all-*trans*- and 13-*cis*-retinoic acids by two-phase, two-dimensional thin-layer chromatography in creams and by high-performance thin-layer chromatography in gel formulations

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(First received October 18th, 1982; revised manuscript received November 24th, 1982)

Tretinoin, all-*trans*-retinoic acid, is employed in the topical treatment of acne. It has been listed in the USP XX, NF XV (U.S. Pharmacopeia National Formulary, 1980) as cream and gel formulations. The usual concentrations are 0.05% (w/w) and 0.1% (w/w) for the cream and 0.025% (w/w) and 0.01% (w/w) for the gel formulations. Tretinoin has been shown to isomerize partially to 13-*cis*-retinoic acid quite rapidly when exposed to light (<550 nm)[1]. It is, therefore, important to be able to monitor the isomerization process with minimum handling of the samples.

Several high-performance liquid chromatographic (HPLC) methods have been developed for the resolution of tretinoin and 13-*cis*-retinoic acid both by normal-phase and by reversed-phase HPLC[2–4]. Methods have also been described for their determination in human plasma[5,6], rat serum[7] and tissue extracts[8–10]. In addition, a gas chromatographic–mass spectrometric assay for these two isomers of retinoic acid in human blood has been reported[11].

A rapid, inexpensive and semi-quantitative thin-layer chromatographic (TLC) analysis has been developed for expeditious monitoring of the all-*trans*- and 13-*cis* isomers of retinoic acid in cream and gel formulations. Although retinoic acid has been separated from other vitamin A derivatives by TLC[1,12], no resolution of the all-*trans* and 13-*cis* isomers has been reported.

Two separate TLC methods, one for gel and one for cream samples are described. A methanol extract of the gel formulation can be analyzed directly on a high-performance TLC (HPTLC) silica gel plate. The method gives fast, complete resolution of the two isomers in only 10–15 min with a detection limit of about 20 ng for both isomers. Under the conditions of the study, there was little chance for any photochemical isomerization during the analysis. Cream samples, however, contained some interfering excipients. Methanol extracts of these samples required a pre-cleaning prior to chromatography. This was accomplished conveniently on a two-phase (reversed-phase C_{18} and normal-phase silica gel), two-dimensional TLC plate, which first eliminated interfering cream excipients in the reversed-phase mode and then resolved both isomers using the normal-phase mode. The limit of detection is about 40 ng for each isomer. This method allows the resolution of tretinoin and 13-*cis*-retinoic acid with minimal sample preparation and no sample transfer between the two TLC phases.

0021-9673/83/0000–0000/$03.00

Tretinoin was USP Reference Standard, 13-*cis*-retinoic acid (13-*cis*-RA) was purchased from BASF Wyandotte (Parsippany, NJ, U.S.A.). As an alternative, the 13-*cis*-RA standard could be prepared by exposing a small aliquot (3–5 ml) of the tretinoin standard stock solution to long wavelength (365 nm) UV light for 1 h at room temperature. This procedure would give adequate isomerization (*ca.* 30%) of tretinoin to 13-*cis*-RA for reference. Tenox-BHT (butylated hydroxytoluene) was food grade (Eastman Kodak, Rochester, NY, U.S.A.). All solvents were reagent grade. All TLC plates were obtained from Whatman (Clifton, NJ, U.S.A.). High-performance plates (LHP-KF), silica gel, 10 cm × 10 cm, 200 μm thickness, with yellow-green fluorescent indicator under 254 nm UV light, and preadsorbent strip) were used for gel samples. Cream samples were run on two-phase TLC plates (Multi-K CS5, $KC_{18}F$/K5F, 20 cm × 20 cm, 250 μm thickness, with yellow-green fluorescent indicator under 254 nm UV light). These plates were coated both with silica gel (17 cm × 20 cm) and C_{18} reversed-phase layer (3 cm × 20 cm). The plates may be prewashed with methanol–acetone (1:1) for best results. The concentration (w/w) for the gels were 0.025% and 0.01% and for the creams 0.05% and 0.1% (Retin-A-Gel® and Retin-A-Cream®, Ortho Pharmaceutical Corp., Raritan, NJ, U.S.A.).

Sample solutions were spotted onto the TLC plates with either a micropipet (2–5 μl sizes, Drummond Microcaps, Fisher Scientific, Plainfield, NJ, U.S.A.) for cream samples or a microsyringe (50 μl size, Hamilton, Reno, NV, U.S.A.) for gel samples. TLC developing chambers were wrapped in aluminium foil and lined with filter paper, saturated with the developing solvent, and equilibrated for at least 10 min before plate development. All the work was done in reduced daylight and/or fluorescent light or under yellow light. The standards and samples were prepared in low actinic volumetric flasks or translucent volumetric flasks wrapped in aluminium foil. In general, all manipulations related to the handling of retinoic acid analogues should be done expeditiously, minimizing light exposure. This is essential to prevent isomerization during sample preparation. A methanolic solution of butylated hydroxytoluene (BHT) was employed to retard oxidation of the retinoic acids.

Solutions

The BHT–methanol solution was prepared by dissolving 5.0 g of butylated hydroxytoluene in 1 l of methanol. The developing solvent (System A) for the silica gel mode consisted of diethyl ether–cyclohexane–acetone–glacial acetic acid (40:60:2:1). The developing solvent (System B) for the reversed phase mode consisted of ethanol–distilled water (80:20). Sample spots were visualized using a spray reagent consisting of concentrated sulfuric acid–ethanol (8:92).

Preparation of standards

Standard stock solutions of tretinoin and 13-*cis*-RA were prepared at a concentration of 0.2 mg/ml, dissolved in BHT–methanol. A dilution (1:25) of the 13-*cis*-RA stock solution was prepared using BHT–methanol. This solution was designated as the standard test solution (concentration of 13-*cis*-RA about 8 μg/ml).

Preparation of samples

After discarding the first 1-2 g of the gel (or cream) from a tube, about 3 g of either 0.025% or 0.01% gel or about 4 g of 0.05% and 2 g of 0.1% cream were accurately weighed into respective 25-ml low actinic volumetric flasks. The matrix was dispersed with about 15 ml of BHT–methanol in a Vortex Genie mixer (Scientific Industries) for 2 min and diluted to volume with BHT–methanol. The cream extracts were filtered using Whatman No. 541 filter paper. The gels required no filtration.

Assay procedure

Gel samples. The gel sample solution was applied to the TLC plate in the following manner. A 50-μl aliquot (equivalent to about 1.5 μg of tretinoin) for the 0.025% gel and 100 μl (equivalent to about 1.2 μg of tretinoin) for the 0.01% gel were spotted onto the preadsorbent layer of an HPTLC plate. The sample spots were best applied as vertical strips, no more than 5 mm wide, on the preadsorbent layer, between the boundary of the two layers and the developing solvent depth and at least 1.5 cm away from the edge of the plate. Adjacent to the sample strips on the preadsorbent layer were spotted the 13-*cis*-RA standard test solutions (2, 3 and 4 μl, equivalent to about 16, 24 and 32 ng of 13-*cis*-RA) for comparative reference. All the spots were dried using a stream of nitrogen. The plate was developed in solvent system A for a distance of 9 cm (about 10 min). After development, the plate was dried using warm air (hair-dryer, about 2 min). The spots were visualized under long-wavelength UV light (365 nm) both before and after a light spraying with the sulfuric acid–ethanol mixture (after the spraying, the plate was heated at 110°C for 5 min which produces pink fluorescent spots for all-*trans*- and 13-*cis*-retinoic acids against a dark background).

Cream samples. The cream sample solution (25 μl, equivalent to about 2 μg of tretinoin) was spotted onto the bottom corner of the C_{18}-strip of the Multi-K CS5 TLC plate, about 1.5 cm away from the side and bottom edges. The sample solution was spotted in small increments (about 3 μl) and dried with a stream of nitrogen between applications. The final spot was no larger than about 5 mm in diameter. A 20-μl aliquot of tretinoin standard stock solution (equivalent to about 4 μg of tretinoin) was spotted on the upper corner (opposite to the sample application, spot A in Fig. 1), on the same C_{18}-strip to monitor stability of tretinoin during the analysis. Before development, the plate was sprayed lightly with ethanol while masking the C_{18}-strip, coating only the silica gel layer. The objective of this was to deactivate the silica gel surface sufficiently to assure uniform migration of the solvent front across the plate. The solvent was allowed to migrate a distance of 13–14 cm (about 90 min) in solvent system B (80% ethanol in water). The plate was then thoroughly dried either in an unheated vacuum oven or vacuum desiccator (both covered with aluminum foil to protect from light) for about 2 h. Alternatively, a stream of unheated air may be applied which normally requires 15–30 min.

Just before development in solvent system A, an additional 20-μl aliquot of the tretinoin standard stock solution (equivalent to about 4 μg of tretinoin) was spotted adjacent to the standard spot A (spot B in Fig. 1b). Next to it were spotted 10 μl of the 13-*cis*-RA standard test solution (equivalent to about 80 ng of 13-*cis*-RA) (spot C in Fig. 1b). These fresh standards are intended to monitor potential isomerization of tretinoin to 13-*cis*-RA during the time span the plate is developed

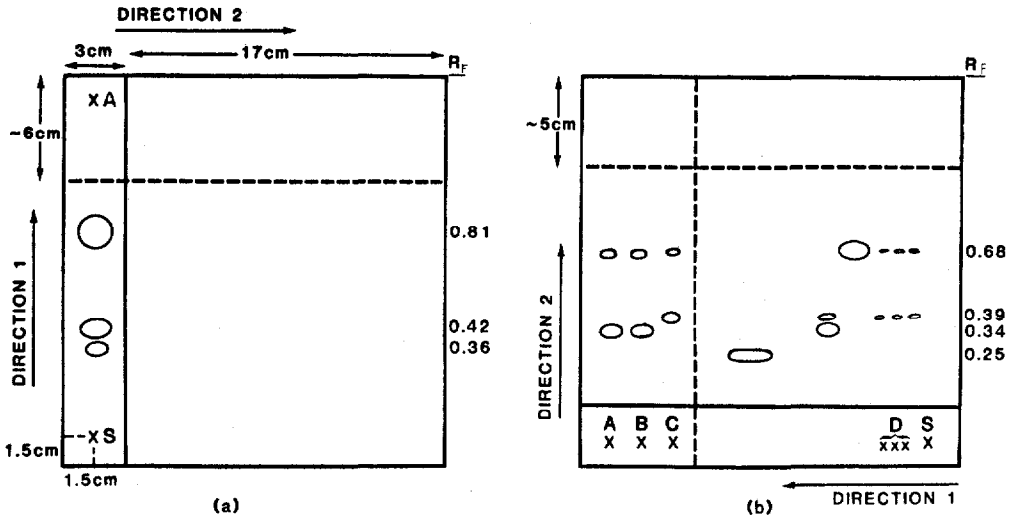

Fig. 1. Typical chromatograms of the cream sample on the Multi-K CS5 TLC plate after development in solvent system B (80% ethanol in water) (a) and of the cream sample and standards, tretinoin and 13-*cis*-RA, after development in solvent system A (diethyl ether–cylohexane–acetone–glacial acetic acid, 40:60:2:1) (b). The spotting areas are designated as follows: S = Sample; A and B = tretinoin standard stock solution at the beginning of analysis and just before development in direction 2, respectively; C and D = 13-*cis*-RA standard test solutions. The broken lines represent solvent fronts. The R_F values in Part a are 0.81 for polar excipients, 0.42 for tretinoin and 13-*cis*-RA and 0.36 for BHT. In Part b, the R_F values are 0.25 for polar expicients, 0.34 for tretinoin, 0.39 for 13-*cis*-RA and 0.68 for BHT.

in solvent system B and subjected to the subsequent drying period. A 10-μl aliquot of 13-*cis*-RA being equivalent to about 80 ng, represents about 2% isomerization of tretinoin in the 20 μl sample aliquot. If the precautions listed in the experimental section were followed, no isomerization at the 2% level was observed during our analysis. Additional aliquots of 13-*cis*-RA standard test solution (5, 6 and 7 μl, equivalent to about 40, 48 and 56 ng) were spotted in an area of the C_{18}-strip between the original spotting area for the sample and the BHT spot developed in direction 1 (spotting area D in Fig. 1). The dried plate was rotated 90° and developed in solvent system A for a distance of 15 cm (about 1 h). The plate was dried using warm air (hair dryer) for about 5 min and the spots were visualized under long-wavelength UV light (365 nm) in the same way described for the gel samples. However, BHT and the polar excipients were best visualized under short-wavelength UV light (254 nm) before spraying.

RESULTS AND DISCUSSION

The R_F values for tretinoin and 13-*cis*-RA on the HPTLC plates were determined using the distance from the boundary of the silica gel and preadsorbent layer to the solvent front. For the Multi-K CS5 plates the distance from the boundary between the two phases to the solvent front was employed. For the gel samples on the HPTLC plate, R_F values were about 0.27 for tretinoin, 0.31 for 13-*cis*-RA and 0.61 for BHT. No interfering gel expicients were observed, and the detection limit was about 20 ng for both isomers. If any 13-*cis*-RA were present in any samples, it

could be detected at a level of 1.2%. This means that if the 16 ng standard spot of 13-*cis*-RA compared in size and intensity to the 13-*cis*-RA spot in the sample, the isomerization of tretinoin to 13-*cis*-RA would be about 1.2% (w/w). Fig. 1 shows the typical chromatograms of the cream sample on the Multi-K CS5 TLC plate after development in solvent system B (Fig. 1a) and of the cream sample and standards after development in solvent system A (Fig. 1b). A photograph of the chromatogram corresponding to Fig. 1b is shown in Fig. 2. In the reversed-phase mode (development 1), tretinoin and 13-*cis*-RA migrate together with a R_F value of about 0.42. The spot below the retinoic acids is due to BHT ($R_F \approx 0.36$) and the more mobile spot ($R_F \approx 0.81$) consists of polar excipients from the cream matrix. In the silica gel mode (development 2), tretinoin ($R_F \approx 0.34$) is resolved from 13-*cis*-RA ($R_F \approx 0.39$). In this mode, BHT ($R_F \approx 0.68$) migrates farthest and the polar excipients are observed at $R_F \approx 0.25$. The reversed-phase mode clean-up is necessary to allow silica gel chromatography where cream excipients interfere with the resolution of the retinoic acids. This is evidenced by the migration of both retinoids along with some excipients as a narrow band with an R_F value slightly greater than that of 13-*cis*-RA standard. If any 13-*cis*-RA isomer were present in the cream samples, it could be detected at the 2% level. This means that if the 40 ng standard spot of 13-*cis*-RA compared in size and intensity to the 13-*cis*-RA spot in the sample, the isomerization of tretinoin to 13-*cis*-RA would be about 2% (w/w). For both gel and cream samples, if the 13-*cis*-RA spots were larger than the detection limits, they could be compared with other 13-*cis*-RA standard spots to estimate the concentrations.

The methods are suitable for a rapid, qualitative and semi-quantitative determination of tretinoin in gel and cream products. The approximation of the isomer-

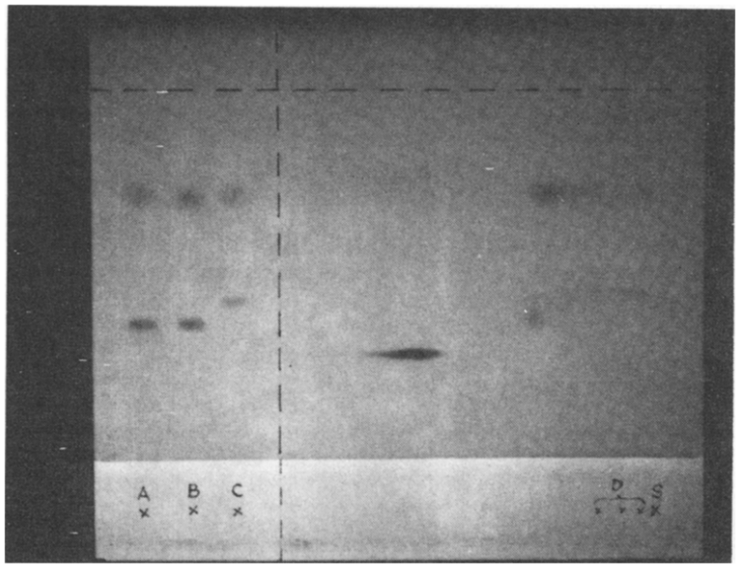

Fig. 2. A photograph of the chromatogram depicted in Fig. 1b as viewed under short-wavelength UV light (254 nm), which is best for visualization of the polar excipients and BHT. However, tretinoin and 13-*cis*-RA become better visualized under long-wavelength UV light (365 nm) after spraying with 8% sulfuric acid in ethanol. See Fig. 1b for identification of spots.

ization to 13-*cis*-RA can be made by inclusion of standards of 13-*cis*-RA at appropriate concentrations.

ACKNOWLEDGEMENTS

The author acknowledges the support of Eugene McGonigle, Philip Lane and Ms. Arlene Holman for technical assistance.

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