CHROM. 8722

Note

Thin-layer chromatography of cinoxacin and some related compounds

RAFIK H. BISHARA and IVAN M. JAKOVLJEVIC

The Lilly Research Laboratories, Indianapolis, Ind. 46206 (U.S.A.)

(Received September 5th, 1975)

Cinoxacin, 1-ethyl-1,4-dihydro-4-oxo[1,3]dioxolo[4,5-g]cinnoline-3-carboxylic acid is a potent antimicrobial drug synthesized at the Lilly Research Laboratories[1,2]. Its *in vitro*[3–6] and *in vivo*[3,7] antimicrobial activity has been reported. Cinoxacin was found to have the same *in vitro* antimicrobial activity as the synthetically prepared nalidixic acid and oxalinic acid[6]. However, its *in vivo* activity against experimental pyelonephritis in rats was superior to the other two previously mentioned synthetic antimicrobial agents[7].

To assure the chromatographic purity of cinoxacin, a thin-layer chromatographic (TLC) system was needed to check the presence of some related compounds. In this paper we report a TLC method that resolves cinoxacin from five related compounds and the utilization of this procedure for the semiquantitation of possible impurities. The relationship between the chromatographic mobility and the chemical structure of the six studied compounds belonging to either the methylenedioxybenzene or the methylenedioxycinnoline ring structure is discussed. The lower limit and means of detecting the related compounds in the raw material of the new drug substance are also included.

EXPERIMENTAL

Chemicals and solutions

All chemicals were analytical-reagent grade and were used without further purification. The following compounds were obtained from in-house sources: 2′-nitro-4′,5′-(methylenedioxy)acetophenone (I), [1,3]dioxolo[4,5-g]cinnolin-4-ol (II); 3-bromo[1,3]dioxolo[4,5-g]cinnolin-4-ol (III), 3-bromo-1-ethyl[1,3]dioxolo[4,5-g]-cinnolin-4(1H)-one (IV), 1-ethyl-1,4-dihydro-4-oxo[1,3]dioxolo[4,5-g]cinnoline-3-carbonitrile (V), 1-ethyl-1,4-dihydro-4-oxo[1,3]dioxolo[4,5-g]cinnoline-3-carboxylic acid (cinoxacin). The structures of these six compounds are shown in Fig. 1.

The solvents used were: sample solvent, chloroform–dimethylsulfoxide (2:1); developing solvent A, ethyl acetate–chloroform (7:3); developing solvent B, acetonitrile–water–ammonium hydroxide (100:15:15).

Equipment

Pre-coated, 0.25-mm thick, silica gel 60 F_{254} plates (20 × 20 cm) from Merck (Darmstadt, G.F.R.) were employed. Further equipment included: rectangular
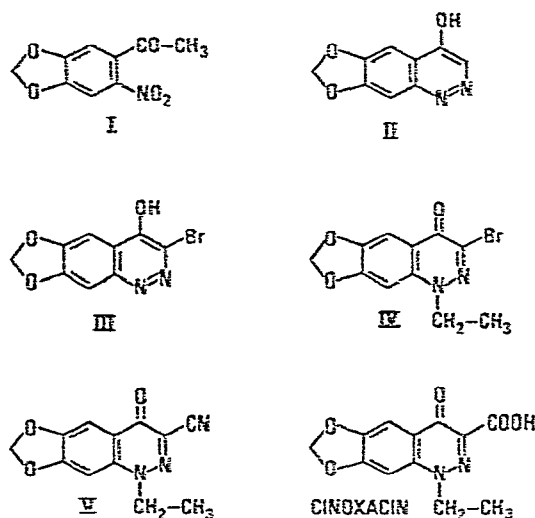
Fig. 1. Structure of cinoxacin and related compounds.

developing glass tanks, $28 \times 22 \times 8$ cm (Brinkmann Instr., Westbury, N.Y., U.S.A.); a chromatographic viewing chamber, "Chromato-Vue", equipped with short- (254 nm) and long-wavelength (366 nm) ultraviolet (UV) lamps (Ultra-Violet Products, San Gabriel, Calif., U.S.A.); an ultrasonic bath (Heat Systems-Ultrasonics, Plainview, N.Y., U.S.A.); glass disposable micropipettes, $50 \mu l$ and $100 \mu l$ (Curtin Matheson, Houston, Texas, U.S.A.); $5-\mu l$ and $10-\mu l$ Microcaps pipettes, Drummond type, distributed by Ace Glass Company (Louisville, Ky., U.S.A.).

Procedure

Each compound was dissolved at a concentration of 1 mg/ml in the sample solvent and placed in a 2-ml plastic-stoppered glass vial. A mixture of all six solutions was also prepared by pipetting 100 μl of each solution into a second 2-ml vial. The points of application (POAs) were marked at 2.5 cm from the bottom edge of the plate. The adsorbent layer was scored across the plate at a distance of 15 cm above the POA. A $5-\mu l$ volume of each compound, equivalent to 5 μg, was spotted on a different lane of the plate. A $30-\mu l$ volume of the mixture solution, equivalent to 5 μg per compound, was also spotted. The sample solvent was evaporated completely using a gentle current of warm air from an air blower. The plate was then introduced into the developing tank containing 100 ml of developing solvent A. The solvent front was allowed to run to the scored line, 15 cm above the POA, approximately 70 min. The plate was dried in a ventilated hood for 15 min and examined under short and long UV light in the Chromato-Vue chamber. The adsorbent layer of the plate was marked 3 cm above the POA (broken line in Fig. 2) and the plate was introduced into a second developing tank containing 100 ml of developing solvent B. The developing time of solvent B to the 3-cm mark was about 6 min. The plate was again dried and viewed as described above. The spots were marked.

RESULTS AND DISCUSSION

Under short UV light (254 nm), all six compounds appeared as purple spots on a yellowish-green fluorescent background due to quenching of the fluorescence of the indicator incorporated in the adsorbent layer. Compound V also showed blue fluorescence. Under long UV light compound I appeared as a non-fluorescent dark blue spot; compound II, V and cinoxacin as blue fluorescent spots; compound III as a yellow fluorescent spot; and compound IV as a faint blue fluorescent spot.

The spotted sample solvent should be completely evaporated before introducing the TLC plate in the developing tank. Residual solvent left on the plate distorts the shape of the resolved spots, (Fig. 2) particularly compound II where a crescent-shaped spot resulted.

Developing solvent A resolved cinoxacin which remained at the POA from its related compounds. However, in order to move the cinoxacin spot from the origin, the plate was re-developed for 3 cm with solvent B. This treatment assured complete resolution and mobility of all six compounds. Fig. 2 shows a chromatogram of the six resolved compounds and the relative position of the solvent front for system A and B.

The R_F values for compounds I–V and cinoxacin were 0.75, 0.23, 0.55, 0.62, 0.47 and 0.05, respectively. The chromatographic mobility of cinoxacin was calculated by dividing the distance that its spot moved, using solvent system B, by 15 (distance
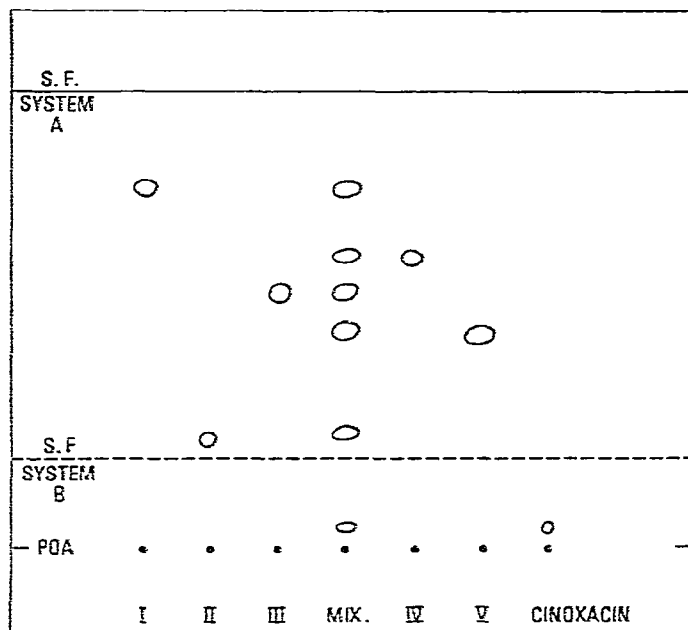

Fig. 2. Thin-layer chromatogram of cinoxacin and related compounds (5 µg per compound) on silica gel 60 F$_{254}$. Solvent system A, ethyl acetate–chloroform (7:3); system B, acetonitrile–water–ammonium hydroxide (100:15:15). Roman numerals of compounds correspond to Fig. 1. POA = point of application; MIX. = mixture of cinoxacin and the five related compounds; S.F. = solvent front.

moved by solvent front of solvent system A). The R_F values are the average of five determinations on separate plates.

Compound I with the highest R_F value hams a ethylenedioxybenzene ring which is smaller in size than the methylenedioxycinnoline ring structure of the other five compounds. This, coupled with the presence of the relatively non-polar nitro and acetyl substituents[8], favored the highest R_F value of compound I. No chromatographic mobility for cinoxacin was observed in solvent A because of the high adsorption affinity of its polar carboxyl group. The polar solvent B was required to move it. Compound II has a hydroxyl group which is slightly less polar than the carboxyl group[8,9] and hence this compound is the next to the slowest-moving cinoxacin. Introduction of the non-polar bromine atom in compound III made it less polar than compound II and correspondingly more mobile. Changing the hydroxyl group in compound III to the less polar keto group[9] in compound IV contributed to increased mobility of the latter compound. A substituted nitrile group showed higher adsorption tendency on silica gel layer than that of a substituted bromine atom[10]. This may be due to the extra carbon atom of the nitrile group as well as the triple bond between the carbon and the nitrogen ($-C \equiv N$), thus the lower R_F value of compound V.

For the semiquantitation of these related compounds in the raw material of cinoxacin, 100 μg of the drug sample (20 μl of a 5 mg/ml solution) and reference standard material were spotted on lanes 1 and 3 of a TLC plate. Mixtures containing 1 and 2 μg of the related compounds were spotted on lanes 2 and 4, respectively. The plate was developed and evaluated as mentioned above. This test allows the visual semiquantitation at the 1 and 2% contamination levels. When the intensities of the impurities were less than 1%, diluted solution was used. Table I summarizes the lower limit and means of detection of the five related compounds in the presence of 100 μg of cinoxacin after separation.

TABLE I

LIMIT AND MEANS OF DETECTION OF SOME RELATED COMPOUNDS IN CINOXACIN BY TLC ON SILICA GEL 60 F_{254}

Developing solvent: ethyl acetate-chloroform (7:3). UV detection by means of Chromato-Vue chamber. Compounds are indicated by Roman numerals as in Fig. 1.

Compound	Lowest detectable quantity (μg)	Wavelength (nm)
I	0.25	254
II	0.05	366
III	0.10	366
IV	0.10	366
V	0.10	366

A phosphorescence was noticed when cinoxacin as the sodium salt was applied to Whatman No. 1 filter paper or to silica gel plate. The spot has to be dried extremely. Cinoxacin in the acid form also exhibits phosphorescence if applied to sodium hydroxide-impregnated paper, or silica gel plate.

The preliminary work, which includes the excitation and phosphorescence

curves at room and liquid nitrogen temperatures as well as decay curve, has been done using the Cold-Finger Phosphoroscope J4-8236 (courtesy of American Instrument Company, Silver Spring, Md., U.S.A.). Details will be published in the near future.

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