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Note

Thin-layer chromatographic determination of the antitumor agent 3,6-bis-(5-chloro-2-piperidinyl)-2,5-piperazinedione in fermentation broth

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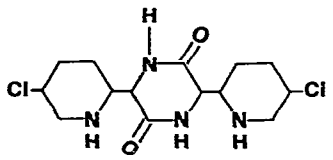
3,6-Bis-(5-chloro-2-piperidinyl)-2,5-piperazinedione dihydrochloride (PD), known as compound 593A, has the structure shown below. It was first reported as an antitumor agent by Gitterman *et al.*[1] and is a product of an actinomycete fermentation; it was detected in fermented broth by the human tumor-egg host system, which was used in work on isolation of the pure crystalline compound[1].



3,6-bis(5-chloro-2-piperidinyl)-2,5-piperazinedione

This agent (PD) was selected by the National Cancer Institute for human clinical trials on the basis of its activity in the mouse leukemia L1210 system[2,3]. Studies in cancer patients have been or are being carried out at several institutions across the U.S.A.[3–7]. Partial responses and improvements have been noted in patients with melanoma, chronic myelogenous leukemia, acute myelogenous leukemia, renal carcinoma, prostate cancer, Hodgkin's disease and non-Hodgkin's lymphomas; other studies on the biological activity of PD have also appeared[8–11].

A procedure for the isolation of PD from fermentation broths has been described[1]. In the course of producing more PD for clinical studies, the Chemotherapy Fermentation Laboratory of the NCI Frederick Cancer Research Center required a rapid quantitative assay for PD in fermentation broths; this paper presents a new procedure, in which one- and two-dimensional thin-layer chromatography (TLC) systems are used for the separation of PD from broth, and describes the use of a spray reagent, sensitive in the nanogram range, for PD quantification.

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METHODS

Apparatus

Standard TLC glass tanks were used for plate development. Quantitative densitometric measurements were made (at 425 nm) on a Schoeffel SK 3000 spectrodensitometer connected to a Hewlett-Packard 3352A laboratory data system through an A/D converter. Elution of PD from the TLC plates was performed with an Eluchrom Automatic Elution System (Camag, New Berlin, Wis., U.S.A.). The IR spectra were recorded on a Perkin-Elmer 180 spectrophotometer having a 6× beam condenser and silver chloride mini-cell windows (Wilks Scientific, Norwalk, Conn., U.S.A.). Mass-spectrometric data were obtained on a JEOL JMS-01SG-2 double-focusing mass spectrometer (operating conditions: source temperature 200°, ionizing current 200 μA, electron energy 70 eV, accelerating voltage, 100 kV).

Reagents

All solvents used were glass-distilled (Burdick and Jackson, Muskegon, Mich., U.S.A.) or of Fischer certified ACS grade. The reagents were of analytical grade. PD was supplied by Merck Sharp and Dohme (Rahway, N.J., U.S.A.) and was used without further purification; TLC of the compound showed only one major spot (R_F 0.4) in a chloroform–methanol (2:1) solvent system on silica gel plates (Brinkman, EM silica gel 60). Drummond micropipettes were used for applying the samples. The *o*-tolidine reagent used was prepared according to Reindel and Hoppe[12].

Preparation of standard

A 1-mg portion of PD was dissolved in 10 ml of deionized water, the pH of the solution was adjusted to 8.6 with 1 *N* sodium hydroxide, and the solution was extracted with ethyl acetate (3 × 4 ml). The combined extracts were dried over anhydrous sodium sulfate and evaporated to dryness under nitrogen, and the residue was dissolved in 1 ml of ethyl acetate.

Preparation of broth extracts

The fermentation broth (50 ml) from the Chemotherapy Fermentation Laboratory was adjusted to pH 2 with 1 *N* hydrochloric acid, then extracted with ethyl acetate (3 × 20 ml), and the extracts were discarded. The pH of the aqueous solution was then adjusted to 8.6 with 1 *N* sodium hydroxide, the solution was extracted with ethyl acetate (3 × 20 ml), and the combined extracts were dried over anhydrous sodium sulfate. The ethyl acetate solution was then evaporated to dryness, and the residue was dissolved in 0.1 ml of ethyl acetate.

TLC development and detection

Standard PD solution and broth extract (2 μl of each) were applied side-by-side on a 20 × 20 cm TLC plate, and development was carried out with butanol–acetic acid–water (13:2:5) (solvent A). After development, the plate was dried at 110° for 10 min, then allowed to cool to room temperature before being exposed to chlorine vapor and sprayed with the *o*-tolidine reagent[13]. Two-dimensional TLC was performed by development with chloroform–methanol (2:1) in the first dimension and with sol-

vent A in the second dimension. The spots were eluted from the plate in situ as described previously[14].

RESULTS AND DISCUSSION

Elution and structural confirmation

Although the presence of PD in the broth was indicated from R_F data, it was necessary to confirm the identity of the spot by spectroscopic techniques. After *in situ* elution[14] of the "PD" spot obtained from the broth, the plates were treated with chlorine and sprayed with *o*-tolidine; densitometric measurements on the eluted region indicated that the elution efficiency was 91%. The mass-spectral and IR data obtained for PD were identical with those reported previously[15].

The first objective of this study was to isolate PD from the fermentation broth in a purity suitable for further analytical studies. This was achieved by extraction of the broth with ethyl acetate at different pH values. Confirmation of the isolated substance as PD was achieved from TLC R_F data, mass spectrometry and IR spectroscopy. After TLC development in solvent A, and visualization, a spot was observed at R_F 0.4, which matched that of the PD standard spot. All major interfering spots in the broth extract were at higher R_F values than the PD standard spot. In order to verify that the spot at R_F 0.4 represented only one compound, another plate was spotted with the broth extract and developed in two dimensions, with chloroform–methanol (2:1) in the first dimension and solvent A in the second dimension. After development, only one spot was obtained from the broth extract, and this exactly matched that obtained for the PD standard. This displaced-spotting technique allows the analyst to compare standard and unknown spots on the same plate under the same conditions.

Another purpose of this study was to develop a quantitative TLC assay for PD. Since PD is colorless and does not fluoresce under UV radiation nor absorb at 254 nm, it was necessary to follow one of the following approaches, choosing the most sensitive and convenient method.

(a) Charring with, or without, sulfuric acid
(b) Reacting with iodine vapor
(c) Spraying with a reagent

The first two techniques were not particularly sensitive, *ca.* 200–300 ng of sample being required for reasonable quantification. Since PD has a secondary-amine group, a logical spray reagent should be ninhydrin, but this, too, lacked the required sensitivity. Previous experience with secondary amines suggested the use of *o*-tolidine reagent after chlorination of the spots[13]. Use of this reagent gave a sensitivity in the range 10–20 ng, which was *ca.* 10 times as sensitive as ninhydrin.

After spraying the plate with *o*-tolidine reagent, the spots appeared dark bluish, and the color intensity was stable for at least 2 h, which made the spots suitable for densitometry. To check the feasibility of quantitative measurements, a calibration graph was prepared; this was linear for 1–15 μg of PD (see Fig. 1).

The amount of PD in the fermentation media was determined by adding 300, 600 and 900 μg of standard PD to three 50-ml samples of fermentation broth; a fourth

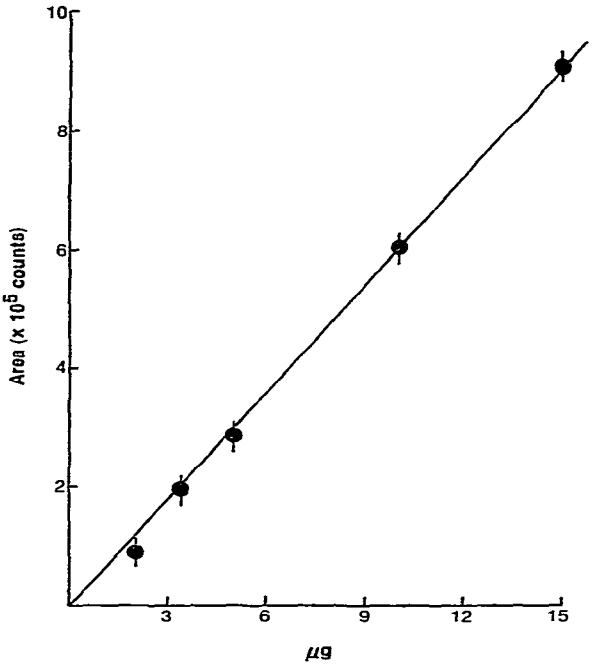

Fig. 1. Densitometric calibration graph of PD after treatment with the *o*-tolidine reagent.

50-ml sample was unspiked. After extraction and drying under nitrogen, each residue was dissolved in 0.5 ml of ethyl acetate, and 2 μl of each solution were spotted and developed in solvent A. From the densitometric measurements (after development, chlorination and spraying with *o*-tolidine), the amount of PD in the fermentation broth was found to be 14 μg/ml.

In summary, this TLC method provides easy separation of PD from other fermentation products. *o*-Tolidine is a sensitive reagent for this antitumor agent (10–20 ng) and provides a product suitable for quantitative analysis by densitometry. This method is currently in routine use for the assay of PD in fermentation media by the Chemotherapy Fermentation Laboratory at this Center.

ACKNOWLEDGEMENTS

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