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Note

Rapid quantitative assay for 3,6-bis-(5-chloro-2-piperidyl)-2,5-piperazinedione in fermentation broth using high-performance liquid chromatography

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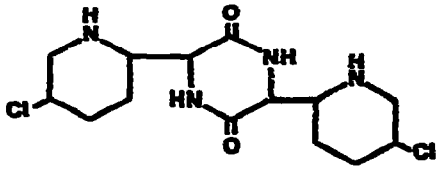
The compound 3,6-bis-(5-chloro-2-piperidyl)-2,5-piperazinedione (PZD), a product of actinomycete fermentation[1], was first reported as an antitumor agent by Gitterman *et al.* Its structure is given below. It has been shown to be highly active in the L1210 murine lymphocytic leukemia system[2], and in phase II clinical trials has demonstrated definite antitumor activity in patients with Hodgkin's disease[3].



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

The Chemotherapy Fermentation Program of the NCI Frederick Cancer Research Center is involved in the production of more PZD for clinical studies. Until recently this laboratory relied on a procedure reported by Issaq *et al.*[4] for routine assay of PZD fermentation development runs. This procedure involves a cumbersome ethyl acetate extraction of the broth, thin-layer chromatographic (TLC) development, detection by exposure to chlorine gas and spraying with the carcinogenic reagent *o*-tolidine (3,3-dimethylbenzidine)[5,6]; quantitation is then achieved by TLC densitometric reading. We wish to report a much more rapid assay procedure using high-performance liquid chromatography (HPLC). This new method is now in use in our laboratory for in-house evaluation of PZD fermentation broth samples and involves a simple cleanup by column chromatography followed by HPLC assay and quantitation. The method is sensitive for broth samples with titers down to *ca.* 2 μg/ml of PZD.

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EXPERIMENTAL

Apparatus

Liquid chromatography was carried out on a Waters Assoc. ALC/GPC 200 series instrument equipped with a Model 6000A solvent pump, U6K septumless injector, and a Schoeffel SF770 Spectroflow monitor set at 215 nm (0.04 a.u.f.s.). A Waters reversed-phase C_{18} μBondapak column (30 cm × 3.9 mm I.D.) was used at ambient temperature with a flow-rate of 2.0 ml/min and a nominal pressure of 2000 p.s.i. Standards were weighed on a Cahn 21 electro-balance. Column chromatography was performed using Amberlite XAD-2 disposable columns (4 × 1 cm I.D.) obtained from Applied Science Lab., State College, Pa., U.S.A. Reversed-phase TLC plates were from Whatman (Clifton, N.J., U.S.A.), 0.2 mm thickness with fluorescent indicator, and silica gel plates were from Merck (Darmstadt, G.F.R.) silica gel 60, 0.25 mm without indicator.

Reagents

Unless stated otherwise, all chemicals used were ACS reagent grade. Water used in preparing the HPLC solvent system was deionized and double glass-distilled. Methanol used for the HPLC solvent system was also glass-distilled (Burdick & Jackson, Muskegon, Mich., U.S.A.). The HPLC solvent system consisted of 0.02 *M* phosphate buffer (pH 7.8)–methanol (65:35, v/v) and was filtered through a 0.45-μm Millipore filter prior to use. PZD·2HCl was recovered from a production fermentation and found by chromatography and spectroscopy to be identical with an authentic PZD·2HCl sample supplied by Merck, Sharp & Dohme (Rahway, N.J., U.S.A.).

Standards

Standards were prepared by dissolving PZD free base (prepared from the dihydrochloride) in methanol at the following concentrations: 0.02 mg/ml; 0.04 mg/ml; 0.08 mg/ml; 0.12 mg/ml; and 0.16 mg/ml. Standard solutions were filtered through a 0.45-μm Millipore filter prior to use.

Assay and isolation of PZD from fermentation broth

A 320-ml aliquot of filtered PZD fermentation broth (pH 8.2–8.5) was divided into four portions of 80 ml each. Three of these portions were spiked with PZD·2HCl to give final broth titers of 8.3, 16.6, and 24.9 μg/ml, expressed as the free base. The fourth portion was unspiked. Each of the 80-ml portions was then further divided into four 20-ml aliquots which were arranged in four sets of four. Two of these sets were extracted according to the method of Issaq *et al.*[4], except that the residue was reconstituted with 5.0 ml of a mixture of 50% 0.1 *N* HCl in acetone and filtered through a 0.45-μm Millipore filter.

The other two sets were treated in the following manner: A disposable XAD-2 column was prepared by washing with 10 ml of methanol and 50 ml of water. The 20-ml broth aliquot was then passed through the prepared column. The broth eluate was discarded and the resin bed washed with 10 ml of water; the water wash was also discarded. The PZD was then eluted off the column with the 50% 0.1 *N* HCl–acetone mixture. The first 5.0 ml of eluent was collected in a 5-ml volumetric flask and the solution filtered through a 0.45-μm Millipore filter. A 25-μl aliquot of each sample

solution was then injected into the HPLC (chart speed of 0.2 in./min). The five standard solutions were also injected, 25 μl each. All injections were done in duplicate to obtain an average peak height for each sample and each standard.

The samples from the XAD-2 column and extraction procedures were then reinjected a number of times, and the PZD peak from each procedure was collected. The HPLC eluent was adjusted to pH 5.5 and the methanol evaporated off. The remaining aqueous layer was readjusted to pH 8.6 and extracted with ethyl acetate. The ethyl acetate was dried over anhydrous sodium sulfate and evaporated to dryness, and the residue was reconstituted in methanol. This solution was then used to spot TLC plates in the following systems:

(I) methanol; reversed-phase C_{18} plate
(II) chloroform–methanol (2:1); silica gel plate
(III) *n*-butanol–acetic acid–water (65:10:25); silica gel plate.

The plates were eluted and spots detected by means of the chlorine–*o*-tolidine reagent.

RESULTS AND DISCUSSION

Peak heights obtained for each sample were plotted *versus* the amount of PZD added to the spiked broth for both the XAD-2 column and the extracted sample sets, each point representing an average value for the duplicate samples. The curves obtained for the two procedures were fitted to the best straight line, using the method of least squares, and extrapolated to the abcissa to obtain the amount of PZD

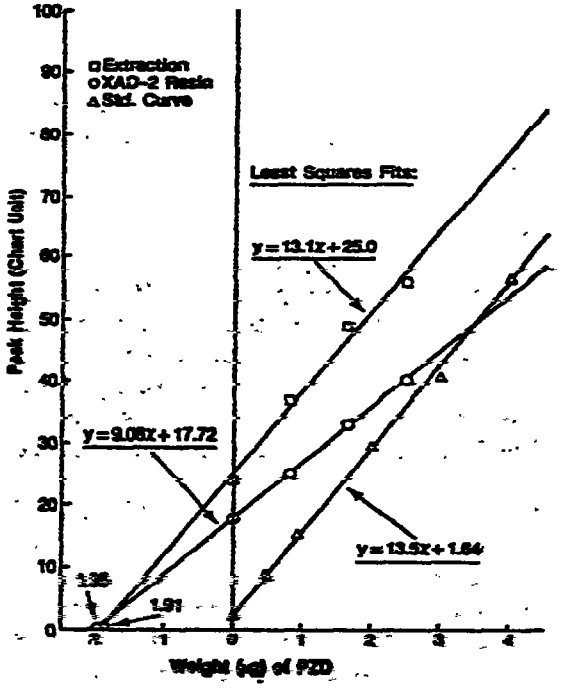

Fig. 1. PZD assay curves.

originally present in the unspiked broth (method of multiple standard addition). The curves from the two different methods of sample cleanup and the standard curve are shown in Fig. 1. The resolution of the PZD peak in the HPLC profile is shown for a XAD-2 column clean-up sample in Fig. 2. HPLC results obtained by standard addition and by reading the unspiked samples from the standard curve are displayed in Table I. The isolated HPLC peaks from all the samples prepared by XAD-2 clean-up or ethyl acetate extraction showed essentially a single spot with the same R_F as authentic PZD in three different TLC systems, and the co-spotted samples gave an enhanced PZD spot.

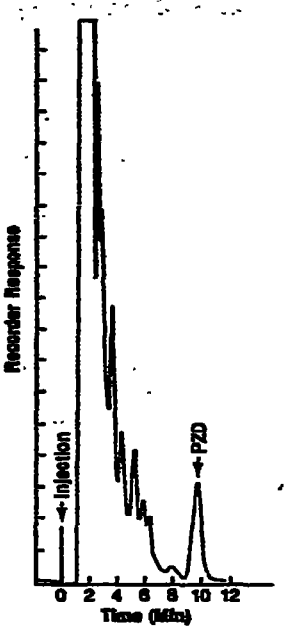

Fig. 2. HPLC chromatogram of PZD fermentation broth after XAD-2 column clean-up. Conditions as described in Experimental section.

The time required for a single PZD assay is substantially reduced by using the XAD-2/HPLC procedure. On a routine basis only the external standard method is used as the basis for reporting titer values. It was observed that results obtained using the XAD-2 columns were consistent from day to day and from sample to sample. It was also found that the resin columns would not work at all when the initial methanol washing step was omitted.

TABLE I

PZD TITER BY XAD-2 PROCEDURE *VERSUS* EXTRACTION

Procedure	*PZD titer (μg/ml)*	
	Standard addition	*Standard curve*
XAD-2 Column	19.5	20.2
Extraction	19.1	17.1

In conclusion, the assay procedure reported here is fast, easy, convenient and safe, and in all of these respects it offers an improvement over the previously reported method of quantitative assay.

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