Qualitative and Quantitative Assay of Trichothecin: a Mycotoxin Produced by Trichothecium roseum

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A method for quantitative determination of trichothecin in crude culture filtrates was presented. The method utilized an agar diffusion bioassay against Candida albicans, a colorimetric test involving a halochromatic reaction with sulfuric acid, and subsequent formation of blue color with methanol, and thin-layer chromatography of trichothecin and its dinitrophenylhydrazine derivative. A positive result in all three systems confirmed the presence of trichothecin. Quantitative results were generally in close agreement.

Trichothecin (T-cin) is a member of a family of mycotoxins known as the 12,13-epoxytrichotheecenes. Members of this family share the property of strongly inhibiting protein synthesis in eucaryotic cells, although at least two different mechanisms of inhibition exist within the group (3,10). T-cin is one of the most potent antifungal members of the group and one of the least effective against mammalian cells (2).

Until recently, demonstration of the trichothecenes required relatively pure samples since these compounds are not fluorescent and do not lend themselves easily to the usual methods of assay. For these reasons, assay procedures applicable to crude samples are badly needed (2). Ueno et al. (12) described a procedure for screening cultures and foodstuffs for Fusarium trichothecenes based on both biological (animal toxicity and inhibition of reticulocyte protein synthesis) and chemical (thin-layer chromatography) properties. No such procedure is currently available for T-cin.

Ikediobi et al. (6) have developed a gas chromatography system for the detection and quantitation of several trichothecenes including T-cin. Although their system has been used to detect T-2 toxin in crude grain extracts, its use with T-cin has so far been confined to mixtures of pure crystalline samples.

Thin-layer chromatographic methods have been described for all of the trichothecenes (2,12). We have tried several of these with crude extracts of Trichothecium roseum cultures but were unable to achieve adequate separation.

The purpose of this communication is to describe procedures currently being used in our laboratory for the detection of T-cin in crude culture extracts.

MATERIALS AND METHODS

T-cin was prepared by cultivation of T. roseum (Pers.) Link ex S. F. Gray (NRRL 1665) by the method of Freeman and Morrison (5), except that Fernbach flasks were used, each containing 750 ml of medium. After 21 days of incubation, the mycelium was removed by filtration, and the filtrate was extracted three times with carbon tetrachloride (200 ml/liter of filtrate). The combined extracts were dried by filtration through anhydrous Na₂SO₄, concentrated in vacuo, and diluted to a final volume of 10.0 ml in a volumetric flask. These crude extracts were used for qualitative and quantitative assays and for preparation of crystalline toxin.

Standard samples of crystalline T-cin were prepared from crude CCl₄ extracts by column chromatography on Al₂O₃ (II) with the benzene-ether stepwise elution method of Achilladelis and Hanson (1). Column fractions were screened by bioassay, and active fractions were pooled and concentrated in vacuo. T-cin was crystallized from CH₃OH-H₂O mixtures and recrystallized from petroleum ether. Its identity was confirmed by nuclear magnetic resonance analysis.

Bioassay. Qualitative and semiquantitative assay was carried out by the agar diffusion method against Candida albicans strain MI-023 obtained from the Center for Disease Control in Atlanta, Ga. Inoculum was prepared in a modified Wickerham medium (13) consisting of Wickerham's trace elements and salts (ammonium sulfate omitted), 1% glucose, 0.5% vitamin-free Casamino Acids (Difco), and 0.01% yeast extract. A 10-ml basal agar layer was covered by a 6-ml seed agar layer containing 24-h cells at a density of 2 x 10⁶ organisms/ml. Nutrient agar (Difco) was used in both layers. A standard volume (0.1 ml) of the extract or dilution to be tested was added to Schleicher and Schuell antibiotic disks (12.7 mm), and the disks were permitted to stand a minimum of 30 min at ambient room temperature to allow evaporation of the solvent. The disks were applied to the plates 30 min after the seed agar layer was poured,
and the plates were incubated at 37 C for 24 h.

**Colorimetric assay.** Colorimetric assay of these extracts was accomplished by a modification of the procedure developed by Poltorak (7). Concentrated H$_2$SO$_4$ (1.5 ml) was added to 0.5 ml of extract (in CCl$_4$ or benzene) to be tested. The tube was then agitated immediately on a Vortex mixer and placed in a boiling-water bath for 4 min. The presence of T-cin or other $\alpha,\beta$-unsaturated ketones is indicated by the development of a red-brown color (7). After cooling for 1 to 2 min, this mixture was added cautiously to 7.5 ml of absolute methanol. Under these conditions, T-cin produces a blue color within a few minutes which is stable for several hours (7). The final solutions were read at 600 nm in a Beckman DB-G spectrophotometer after a minimum of 30 min.

**Thin-layer chromatography.** Silica Gel (Brinkman) at a thickness of about 375 $\mu$m was applied to plates (20 by 20 cm) with a Desaga spreader. Mallincrodt Silicar 7G (250 $\mu$m) produced essentially similar results. The plates were activated at 110 C overnight and stored in a Lab Con Co vacuum desiccator (atmospheric pressure) prior to use. Separation of substances by chromatography can often be enhanced by repeated development in the same solvent system (11). The plates were spotted for either single or double development (12 cm) and were developed by the following solvent systems: (i) benzene-ethyl acetate (95:5), double development; (ii) benzene-acetone (95:5), double development; and (iii) chloroform-ethyl acetate (9:1), single development.

After development, the plates were sprayed with a 1:1 mixture of concentrated H$_2$SO$_4$ and absolute methanol and developed at 110 C for 15 min. T-cin appeared as a brown nonfluorescent spot (long-wave ultraviolet) which eventually began to fade and became surrounded by a white halo.

To enhance the value of thin-layer chromatography in identifying T-cin, 2,4-dinitrophenylhydrazine derivatives were prepared from crude extracts, crystalline T-cin, and acetone. The 2,4-dinitrophenylhydrazine reagent was prepared by the method of Roberts et al. (9). The reagent (1 ml) was mixed with 0.5 ml of extract on a watch glass and stirred occasionally until 2,4-dinitrophenylhydrazones were formed. These were collected by filtration through glass wool in a capillary pipette and washed repeatedly with ethanol. The product was then eluted into a test tube with about 1 ml of chloroform. Dinitrophenylhydrazine derivatives were used as a confirmatory test and were not used for quantitative purposes. The acetone-dinitrophenylhydrazine was used for relative $R_f$ determinations (not shown).

Semiquantitative estimation of T-cin concentrations in crude extracts was done by visual comparison with known concentrations of crystalline T-cin on the same plate.

To compare these procedures for the quantitative analysis of individual samples, a series of eight culture filtrates was prepared and analyzed as described above. In each case, dilutions were prepared with CCl$_4$ whenever required. Analysis of variance was done by standard methods for two factors and one observation per cell (4).

Several strains of *T. roseum* were used to compare these methods of analysis and to determine whether different strains might present unique problems in extraction or analysis. These were obtained from C. W. Hesseltine of the Northern Regional Research Laboratory (NRRL 1665 and 2307), from J. L. Richard of the National Animal Disease Laboratory, Ames, Iowa (MC-156 and MC-176), and from the American Type Culture Collection (ATCC 13411 and 13422). Two of the strains were isolated from natural sources in Oklahoma (FL-1 from soil in Norman and RH-1 from a squash from Elk City). Each strain was cultured and extracted as described above.

**RESULTS**

Although the bioassay was intended initially only for qualitative purposes, it can be used semiquantitatively as well; a linear relationship holds between the log of concentration and the diameter of the zone of inhibition over the concentration range of 50 $\mu$g/ml to 2 mg/ml (Fig. 1). Since only 0.1 ml of extract is used per disk, a definite zone of inhibition is produced if about 2 to 5 $\mu$g of T-cin is present on the disk. The Poltorak colorimetric procedure yields a linear relationship between 0.2 to 3.0 mg/ml (Fig. 2), although certain unknown substances may cause interference in some cases. The presence of interfering $\alpha,\beta$-unsaturated ketones is indicated by a pink or purple color resulting from residual red-colored products of the first reaction.

$R_f$ values for T-cin, T-cin–dinitrophenylhydrazine, and acetone-dinitrophenylhydrazine are presented in Table 1. The minimum amount of T-cin or T-cin–dinitrophenylhydrazine which could be easily detected was 6 to 10 $\mu$g/spot. These solvent systems afford good separation of T-cin and an unknown contaminating sub-

![Graph](image-url)  
**Fig. 1. Linear relationship between the diameter of the zone of inhibition of *C. albicans* and the log of the T-cin concentration ($y = 14.0x + 7.0; r = 0.990$).**
thin-layer chromatography is the least sensitive of the methods. A major disadvantage of the bioassay is its lack of specificity, since many naturally occurring substances inhibit C. albicans. Its convenience, simplicity, and economy make it a useful screening technique.

The specificity of the colorimetric test is currently not known. According to Poltorak (7), it is dependent upon a halochromatic reaction between concentrated H₂SO₄ and the α,β-unsaturated keto group of T-cin. While we have not studied this point, we have tried quinhydrone (a mixture of quinone and hydroquinone) with H₂SO₄ under the conditions of the Poltorak test. Only a faint yellow color developed at a concentration of 1 mg/ml. Therefore, the red-brown color resulting from halochromation of T-cin is not a general phenomenon of α,β-unsaturated ketones.

It would be helpful to know the structural requirements for this reaction and the subsequent reaction, i.e., the formation of the blue color with methanol. The Poltorak reaction is somewhat limited by its uncertain specificity and by the possibility of interference resulting from the occasional occurrence of turbid solu-

**Table 1. Characteristic Rₜ values**

| Solvent system          | Substance tested | T-cin | T-cin-DNP | Acetone-DNP |
|-------------------------|------------------|-------|-----------|-------------|
| Benzene-etac* (95:5),   |                  | 0.14  | 0.22      | 0.67        |
| double development      |                  |       |           |             |
| Benzene acetone (95:5), |                  | 0.35  | 0.48      | 0.76        |
| double development      |                  |       |           |             |
| Chloroform-etac (9:1),  |                  | 0.44  | 0.58      | 0.74        |
| single development      |                  |       |           |             |

* DNP, Dinitrophenyhydrzone.
* Etac, Ethyl acetate.

stance which overlapped T-cin in several other solvent systems previously reported (Fig. 3).

Table 2 presents the results of a quantitative comparison of a series of eight culture filtrates. Although agreement was not complete (e.g., RH-1 and MC-156), the results of the three assay methods were in general agreement. Analysis of variance of these data (Table 3) shows that the different strains used were significantly different in their production of T-cin, but that there was no statistically significant difference in results obtained by the three assay methods.

**DISCUSSION**

The minimum detectable levels in the bioassay, Poltorak colorimetric reaction, and thin-layer chromatography are 50 µg/ml, 200 µg/ml, and about 6 µg, respectively. Under the conditions employed, the bioassay is the most sensitive of the three methods. Because of the limited loading capacity inherent in analytical thin-layer chromatography, working samples must contain about 0.5 to 1.0 mg/ml. Therefore

**Table 2. Comparative analysis of crude extracts**

| Strain   | Assay method |
|----------|--------------|
|          | Bioassay*    | Colorimetric* | TLC* |
| 1665     | 16.2         | 14.2          | 13.3 |
| 2307     | 16.6         | 16.1          | 16.7 |
| FL-1     | 5.5          | 5.8           | 5.0  |
| RH-1     | 11.6         | 6.8           | 11.4 |
| MC-156   | 0.3          | 1.5           | 0.3  |
| MC-176   | 1.9          | 2.3           | 2.0  |
| 13411    | 1.3          | 1.5           | 1.2  |
| 13422    | 2.3          | 2.9           | 2.0  |

*Expressed as milligrams of T-cin per milliliter of extract.

*Average values: bioassay, 7.0; colorimetric, 6.4; and thin-layer chromatography (TLC), 6.5.

**Table 3. Analysis of variance**

| Source of variation | DF* | SS* | MS* |
|---------------------|-----|-----|-----|
| Strain              | 7   | 822.09 | 117.44* |
| Method of assay     | 2   | 1.51  | 0.76* |
| Error               | 14  | 19.69 | 1.41  |

*Analysis of data in Table 2.
*DF, Degrees of freedom.
*SS, Sum of squares.
*MS, Mean square.
*Significant at the 5% level of probability.
/Insignificant.

![Graph](image.png)

**Fig. 2. Linear relationship between absorbance of reaction products and T-cin concentration (y = 0.4x - 0.038; r = 0.981).**
grams. Also it allows charring of organic compounds present and gives a recognizable reaction with T-cin.

Richard et al. (8) previously reported that T. roseum NRRL 2307 does not produce T-cin. However, our results indicate yields comparable to those of NRRL 1665. Perhaps the subculture used in their work had lost its ability to produce the compound. We were able to detect T-cin in all of the extracts reported herein, although the yields were often quite low. It is interesting that we have tried only two fresh isolates from Oklahoma, and that both produced the toxin under these conditions in yields comparable to those originally reported by Freeman and Morrison (5).

Although each of the assay procedures used has certain inherent disadvantages, their use in combination allows qualitative determination of T-cin without purification, and quantitation by these methods yields results which are generally in close agreement.

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