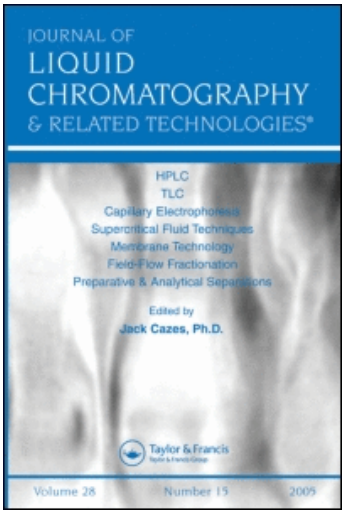

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HPLC WITH ELECTROCHEMICAL AND FLUORESCENCE DETECTION OF THE OPA/2-METHYL-2-PROPANETHIOL DERIVATIVE OF FUMONISIN B_1

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ABSTRACT

The o-phthalaldehyde (OPA) derivative of fumonisin B_1 was prepared in the presence of 2-methyl-2-propanethiol (tert-butyl thiol). A hydrodynamic voltammogram for the derivative indicated that the optimum voltage for maximum electrochemical response was +0.7 V. The electrochemical response of the OPA/tert-butyl derivative was unstable. However, the fluorescence response was found to be stable for over an hour after an initial 30 minute reaction time. The minimum detectable limit (MDL) of the OPA/tert-butyl derivative using fluorescence detection was 30 ng/ml as compared to 250 ng/ml for electrochemical detection.

INTRODUCTION

Fumonisins are mycotoxins produced by the fungus Fusarium moniliforme, which are common fungal pathogens of corn (1). Two metabolites from Fusarium moniliforme cultures, identified as fumonisins B_1 (FB_1) and B_2 (FB_2), have cancer-promoting activity (2). Intravenous injection of FB_1 in horses induces

the neurotoxic disease leukoencephalomalacia (3,4). Fumonisin B_1 has also been implicated in inducing pulmonary edema in swine (5) and causing hepatocarcinogenic and hepatotoxic effects in rats (6).

The molecular structure of fumonisin B_1 contains a primary amine moiety (FIGURE 1) which can be utilized to form highly sensitive fluorescent derivatives. A sensitive method commonly used for quantitating fumonisin B_1 is that of Shephard et al. (7), which uses HPLC with fluorescence detection after derivatization with OPA in the presence of the reducing agent 2-mercaptoethanol. To remove interfering compounds when forming the OPA derivative, cleanup is necessary before derivatization and analysis by HPLC. The cleanup involves solvent extraction of the original sample followed by passing the extract through a strong anion exchange (SAX) column prior to derivatization and HPLC analysis. The OPA derivative with 2-mercaptoethanol is unstable, making it necessary to time the reaction of OPA with the sample to obtain reproducible results.

Allison et al. (8) obtained more stable derivatives of primary amines (amino acids) by reaction with OPA in the presence of tert-butyl thiol instead of 2-mercaptoethanol. This OPA/tert-butyl derivative was stable up to 4 hours; the background fluorescence was overcome by using electrochemical detection.

We have compared the sensitivities of fluorescence and electrochemical detection for the OPA/tert-butyl derivative of fumonisin FB_1 and looked at the possibility of improving an existing analytical method for trace amounts of fumonisin B_1 by determining the derivative's stability.

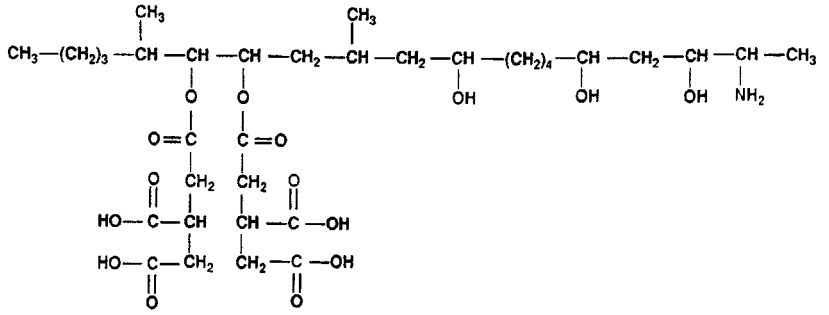

FIGURE 1. Structure of Fumonisin B_1.

EXPERIMENTAL

Instrumentation

The high performance liquid chromatography (HPLC) system consisted of a Waters Model M 6000A pump (Waters Chromatography Division, Milford, MA, USA) and an Altex Model 100A pump (Beckman Instruments, Fullerton, CA USA). A guard column (Upchurch Scientific, Catolog # C-135B, Dark Harbor, WA USA) with a 2 μm frit and containing 100 mg of C_{18} column packing was placed between the injector and the column. The column was a 250 X 4.6 mm 5 μm Supelco reversed-phase C_{18} column (Supelco, Bellefonte, PA USA). A Shimadzu Model RF-535 fluorescence detector was used with a Shimadzu Model C-R3A integrator (Shimadzu Scientific struments, Columbia, MD USA). A BAS LC-4B amperometric electrochemical detector (Bioanalytical Systems, West Lafayette, IN USA) with a dual glassy carbon electrode (GCE) and a Ag/AgCl reference electrode was used with a Model 3390 Hewlett-Packard integrator (Hewlett-Packard Company, Avondale, PA USA). A TG-5M(BAS)0.005 in. thick red gasket separated the stainless steel auxiliary electrode from the glassy carbon electrodes. The first electrode was maintained

at +0.4 V to remove background noise. The voltage of the second electrode or working electrode was varied from +0.5 to +0.9 V. The electrochemical detector was operated at 100 nA.

Reagents

The 100 mM borate buffer was prepared by dissolving 38.137 g of sodium tetraborate decahydrate (Fluka Chemical Company, 99% purity, Ronkonkoma, NY USA) in 1 L of deionized H_2O and adjusting the pH to 9.5 with dilute HCl.

The o-phthalaldehyde/tert butyl thiol (OPA/tert-butyl) reagent was prepared by first dissolving 27 mg of OPA (Sigma Chemical Company, 99% purity, St. Louis, MO USA)in 2 ml of methanol. The solution was transferred to a 10 ml serum bottle and then 20 μl of tert-butyl thiol (Aldrich Chemical Company, Milwaukee, WI USA) and 4.5 ml of borate buffer was added. This OPA-thiol reagent was stable for 3 days when stored in a sealed serum vial at 4°C.

The fumonisin B_1 standard (FB_1) (CSIR, 99% purity, Pretoria, South Africa) was used as received in a 10 mg vial. A stock standard of FB_1 was prepared by quantitatively transferring the FB_1 from the vial to a 100 ml volumetric flask using acetonitrile/water (50:50 v/v) to give a final concentration of 100 μg/ml. The stock standard is stable for 6 months when stored at 4°C. Working standards were made up from this stock standard in acetonitrile/water (50:50 v/v).

Precolumn Derivatization and HPLC

For the precolumn derivatization, 40 μl of the FB_1 standard and 40 μl of the OPA/tert-butyl reagent were reacted from 2 to 120 min at room temperature (27°C). A 50 μl aliquot of the reacted solution was injected into the HPLC.

The mobile phase used was acetonitrile/pH 7 KH_2PO_4 (45:55 v/v) with 1 mM of Na_2EDTA. The flow rate was 1.5 ml/min.

For optimum response, the fluorescence detector had the excitation and emission wavelengths set at 345 nm and 435 nm, respectively, with both the attenuation and gain at 10.

RESULTS AND DISCUSSION

The literature indicated that one of the most sensitive methods available for the analysis of fumonisins was HPLC analysis with fluorescence detection after derivatization with OPA in the presence of 2-mercaptoethanol. However, several problems exist with this method, including an unstable fluorescent derivative and a high fluorescence background even after solvent extraction of samples and additional cleanup with solid phase extraction (SPE) on a strong anion exchange (SAX) column. The OPA/tert-butyl derivative of fumonisin B_1 was investigated with the objective of obtaining a more stable fluorescent derivative. Also, electrochemical detection was evaluated to determine if high sensitivity could be obtained for fumonisin B_1 and hence, eliminate the high background fluorescence seen when fluorescence detection is utilized.

The mobile phase used for isocratic HPLC was acetonitrile/ pH 7 KH_2PO_4 with 1 mM Na_2EDTA. Several ratios of acetonitrile/ pH 7 KH_2PO_4 were tried with a final ratio of (45:55 v/v) selected since this could be used successfully to resolve the fumonisin B_1 peak using fluorescence detection.

Various quantities and ratios of standard FB_1 and OPA/tert-butyl reagent were tried in forming the derivative of fumonisin B_1, with equal amounts of each (40 μl) giving the optimum results.

The electrochemical detector took up to a day to equilibrate enough to give a stable baseline. Even though a great effort was taken to de-gas the mobile phase by filtering daily through 0.45 μm filters and sparging with nitrogen to keep out oxygen, the electrochemical detector was not very easy to operate. However, after equilibration, good HPLC chromatograms for FB_1 could be obtained with electrochemical detection (FIGURE 2).

A hydrodynamic voltammogram, comparing the detector response with applied voltage, showed the optimum voltage to give maximum response for the electrochemical detector to be +0.7 V.

The fluorescence detector on the other hand was quick to stabilize and easy to operate and consequently good chromatograms could be obtained after only a 10 to 15 min warm-up period (FIGURE 3). The retention times corresponding to fluorescence and electrochemical detection was 13.46 and 13.55 min, respectively. The peak for the FB_1 derivative was determined by comparing chromatograms of standard FB_1 at different concentrations to derivatization of blanks containing no FB_1. This made it possible to distinguish between the FB_1 derivative peak and the artifact peaks in the chromatograms.

Sensitivity studies were done on the OPA/tert-butyl derivative of FB_1, using both the electrochemical and fluorescence detectors. As the level of the OPA/tert-butyl derivative dropped below 1 μg/ml, longer periods of time were necessary for the electrochemical detector to equilibrate and stabilize, whereas this was not a problem with the fluorescence detector. The fluorescence detector could detect approximately 30 ng/ml of FB_1 compared to only 250 ng/ml for the electro-

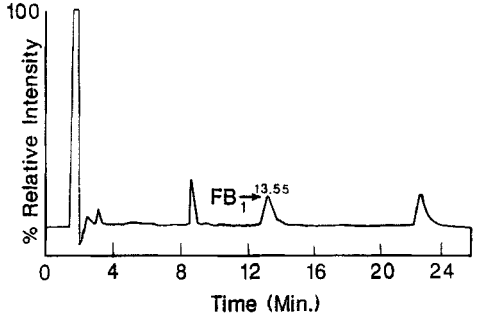

FIGURE 2. HPLC of the OPA/tert-butyl derivative of a 1 μg/ml fumonisin B_1 standard using electrochemical detection.

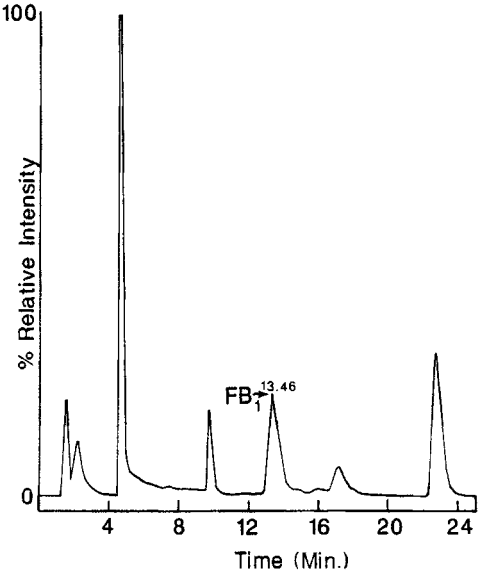

FIGURE 3. HPLC of the OPA/tert-butyl derivative of a 1 μg/ml fumonisin B_1 standard using fluorescence detection.

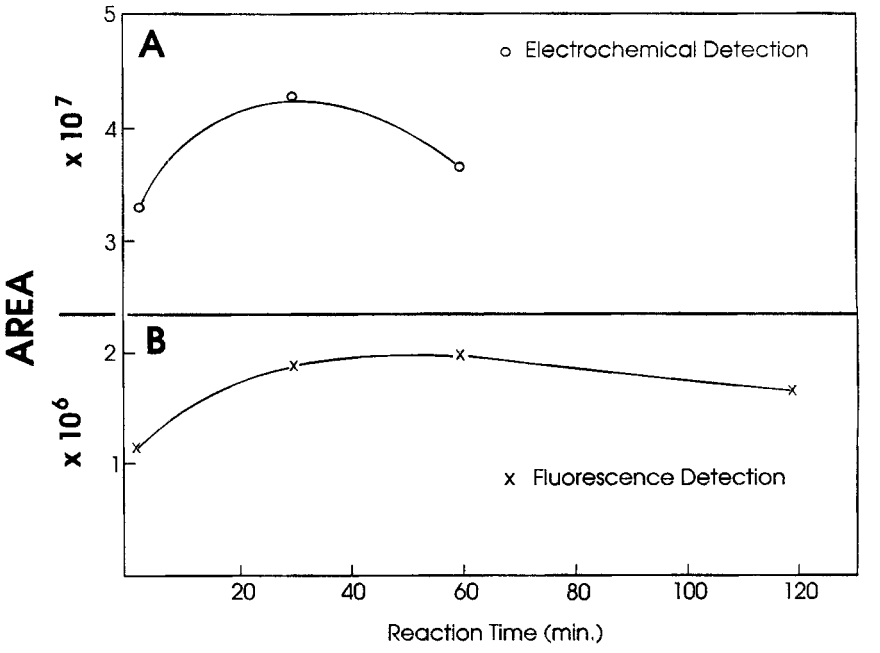

FIGURE 4. (A) Detector response vs reaction time of the OPA/tert-butyl derivative of fumonisin B_1 using electrochemical detection. (B) Detector response vs reaction time of the OPA/tert-butyl derivative of fumonisin B_1 using fluorescence detection.

chemical detector. Detector sensitivity was determined as twice background.

The electrochemical response for the OPA/tert-butyl derivative of FB_1 was not stable (FIGURE 4A).

The fluorescence response for the OPA/tert-butyl derivative was stable for at least an hour after an initial reaction time of 30 min (FIGURE IVB). Increasing the reaction time for the OPA/tert-butyl derivative from 2 min to 30 min resulted in a 63% increase in fluorescence response (FIGURE 4B).

CONCLUSION

Our study indicates that fluorescence detection is more sensitive than electrochemical detection for the OPA/tert-butyl derivative of FB_1. Also, a more stable derivative is formed when FB_1 is reacted with OPA in the presence of 2-methyl-2-propanethiol instead of 2-mercaptoethanol.

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