Replacement of Benzene as a Solvent for Aflatoxin Standards

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ABSTRACT

Cyclohexane, heptane and toluene were evaluated for possible replacement of benzene in the preparation of aflatoxin standard solutions. The results of a 6-month study showed that these solvents can be substituted for benzene, provided they are not unduly exposed to light. The superiority of benzene as a solvent for aflatoxin standards was clearly demonstrated in a test in which these solvent systems containing 5 μg B_1/mL, were exposed to daily lighting in the laboratory for 3 months. Complete photodegradation of aflatoxin B_1 occurred in the cyclohexane, heptane and toluene systems whereas only a 15% B_1 decrease occurred in the benzene system.

INTRODUCTION

At the 1980 meeting of the AOCS Aflatoxin Subcommittee in New York City, investigation was recommended of the possible replacement of benzene as a solvent for aflatoxin standards. The request was made for 2 reasons: (a) the carcinogenic potential of benzene requires strict controls to comply with OSHA regulations, and (b) the costs incurred to meet these regulations, as well as increased insurance premiums, are substantial.

Three solvents were selected for this study: cyclohexane, heptane and toluene. The results of a 6-month study in which aflatoxin B_1 standard solutions were prepared using these solvents and compared with that of benzene is reported.

EXPERIMENTAL PROCEDURES

Materials

Aflatoxin B_1 was obtained from Supelco, Inc., Bellefonte, PA. Reagent grades of benzene, cyclohexane, heptane and toluene were used. Thin layer chromatographic (TLC) strips, 5 × 20 cm, layered with silica gel on aluminum support from EM Laboratories Inc. were obtained from Scientific Products, Columbia, MD. Clear-shell vials were of 7-mL capacity with screw-type stoppers lined with Teflon. Beckman double beam (Model 24) and single beam (Model DU) spectrophotometers were used to measure absorbance.

Preparation of Aflatoxin B_1 Standard Solution

A standard solution of aflatoxin B_1 (100 μg/mL) was made by weighing and dissolving crystalline B_1 in *n*-propanol. Aliquots of 5 mL were transferred to four 100-mL volumetric flasks with a pipet, and each made to volume with benzene, cyclohexane, heptane and toluene. These standard solutions thus contained 5 μg B_1 and 5% of *n*-propanol/mL. Blank solutions were also prepared by pipeting 5 mL *n*-propanol into four 100-mL volumetric flasks and making them to volume with benzene, cyclohexane, heptane and toluene.

Absorption Maxima

The 5 μg B_1/mL standard solutions were scanned against their prepared blank solutions using the double beam spectrophotometer. The B_1 standard solutions were also measured with the single beam spectrophotometer. Spectrophotometric measurements were made with both the ultraviolet (UV) and tungsten light sources. The wavelength of maximal absorption of each of the standard solutions was recorded.

Stability Study

Duplicate aliquots of each B_1 standard solutions (5 μg/mL) and blank solutions were pipetted into 7-mL clear-shell vials. One set of standards was stored in the dark at room temperature. At monthly intervals, the absorbance of the standard solutions was measured. After measurement, the solutions were returned to their vials and stored. The second set of standards was weighed and the vials placed in a test tube rack which had open sides. The rack was placed on top of a bench shelf for 3 months. At the end of 3 months, the vials were reweighed to determine solvent loss and the absorbance of the solutions was measured.

TLC Development

The standard solutions from the stability study were chromatographed on 5 × 10 cm strips (5 × 20 cm strips cut in half) layered with silica gel. Five μL from each of the standards to be stored in the dark were spotted and developed with diethyl ether/methanol/water (96:3:1) for 15 min. At the end of the 6-month period, 5 μL of the same solutions were spotted on a second TLC strip and developed. A third TLC strip was used to spot 5 μL of the standard solutions placed on the bench top. These solutions were spotted at the end of the 3-month test, developed and a composite picture of the 3 TLC strips was taken (Fig. 1).

RESULTS AND DISCUSSION

Previous studies on stability of aflatoxin standard solutions have indicated that the greatest source of error in maintain-

TABLE I

Comparison of Solvent Properties

	OSHA maximal exposure/8 hr[a] (ppm)	Boiling point (C)	Vapor pressure (mm)	UV cutoff (nm)
Benzene	10	80.1	81	278
Cyclohexane	300	81.4	77	200
Heptane	500	98.4	39	200
Toluene	200	110.6	24	284

[a]1975 OSHA concentration limits for gases (maximal allowable exposure).

TABLE II

Molar Absorptivities of Aflatoxin B_1 in Solvents

	Wavelength maxima (nm)	Molar absorptivity (e)
Benzene	345	19500
Cyclohexane	350	20500
Heptane	345	20300
Toluene	350	19100

TABLE III

Comparison of Aflatoxin B_1 Stability in Solvents (5 μg/mL)

Solvent	Time (months)[a] (absorbance 350 nm)[b]							abs loss (%)
	0	1	2	3	4	5	6	
Benzene	0.313	0.297	0.287	0.293	0.287	0.280	0.285	9.0
Cyclohexane	0.334	0.310	0.294	0.287	0.290	0.287	0.280	16.2
Heptane	0.320	0.313	0.305	0.315	0.303	0.290	0.290	9.4
Toluene	0.310	0.302	0.298	0.284	0.290	0.300	0.297	10.3

[a]Standard B_1 solutions stored in the dark.

[b]Absorbance measured with Beckman spectrophotometer DU using UV lamp.

ing laboratory standard solutions has been the increase in concentration due to evaporation of solvent. This error also has been singled out by EPA from studies conducted on pesticide standard solutions. The boiling point or vapor pressure of a solvent, therefore, becomes a major consideration in the preparation of standard solutions. Another important factor which must now be considered is the tolerance level set by OSHA for exposure to solvent vapors in a laboratory or plant.

Thus, in addition to the normal considerations of, e.g., cost and availability, the group of solvents selected to test and compare with benzene are shown in Table I. The physical properties of these solvents show that, from the standpoint of minimizing errors due to evaporation, toluene would be the solvent of choice followed by heptane, cyclohexane and benzene. From the standpoint of industrial health safety, heptane would be preferred, followed by cyclohexane, toluene and benzene. The obvious choice of solvent for aflatoxin standards from this table would be heptane with a high exposure tolerance level of 500 ppm and a boiling point of 98.4 C. Evaluation of aflatoxin B_1 absorptivity is shown in Table II. Wavelength maxima of B_1 in this group was found to be within a very narrow range of 5 nm (345-350 nm). The molar absorptivity of B_1 in these solvents was also found to be within a small range, 19,100-20,500 (toluene/cyclohexane), with benzene (19,500) and heptane (20,300) falling in between. From the standpoint of absorptivity, benzene could be replaced by any of the solvents in this group.

Further evaluation was conducted by comparing the stability of aflatoxin B_1 in the solvent systems. The results of the stability study in which standard solutions (5 μg/mL) were stored in the dark at room temperature for 6 months are shown in Table III. Measurements of the solutions taken at monthly intervals show a decrease in absorbance for all solvent systems. The greatest decrease was that of cyclohexane (16.2%), followed by toluene (10.3%), heptane (9.4%) and benzene (9.0%). The stability of B_1 in heptane or toluene was thus found to be near equal to that of benzene when stored in the dark and at room temperature.

The most significant difference in the study was found in the bench life of the B_1 standard solutions. In this test, the standard solutions were exposed to the daily lighting in the laboratory for 3 months, after which their absorbance was measured. The results are shown in Table IV. Only the aflatoxin B_1 solution in benzene withstood the test. All other standards showed a drastic decrease in aflatoxin B_1 absorbance. The greatest decrease was that of the cyclohexane system which showed a loss of 82.6% followed by toluene, 76.8%, heptane, 65.6%, and benzene, which showed a loss of 16.3%. The stability of B_1 in benzene is impressive considering that a 9% absorbance loss was noted with storage in the dark compared to a 16% loss

TABLE IV

Comparison of Stability of Aflatoxin B_1 in Solvent Exposed to Light in Laboratory

Solvent	Time (months) (absorbance 350 nm)		Abs loss (%)
	0	3	
Benzene	0.313	0.262	16.3
Cyclohexane	0.334	0.058	82.6
Heptane	0.320	0.110	65.6
Toluene	0.310	0.072	76.8

with exposure to light. A more graphic result of the stability study is shown in Figure 1. This figure shows a composite picture of the B_1 standard solutions developed on TLC strips of silica gel at the beginning and at the end of the stability study. The standard solutions were spotted in solvent arranged in alphabetical order and from left to right; benzene/cyclohexane/heptane/toluene. An equivalent of 25 ng B_1 in 5 μL of solution was spotted. The group of 4 fluorescent spots on the left TLC strip of the picture are those of the standards at 0 time of dark storage. The group of 4 spots in the middle TLC strip is of the standard solu-

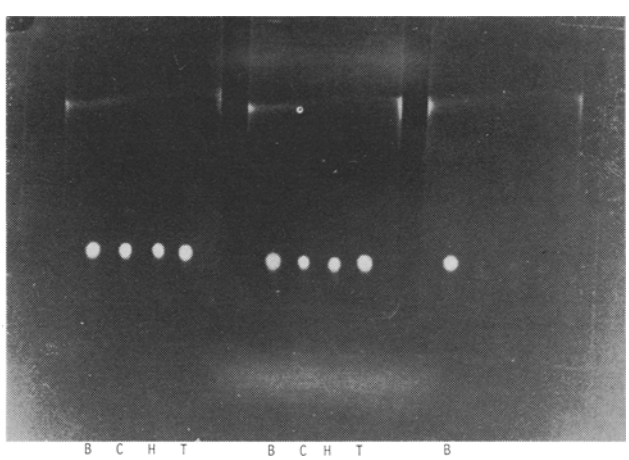

FIG. 1. Composite picture of aflatoxin B_1 standard solutions (5 μg/mL) spotted on TLC strips. Strips represent solutions at 3 storage levels. Each spot contains 5 μL of standard solution: b = benzene, c = cyclohexane, h = heptane, t = toluene. First TLC strips on left with 4 spots are standards after 0 time of storage. Second or middle TLC strip with 4 spots are standard after 6 months storage in the dark and at ambient temperature. Third, TLC strip on right with lone spot is that of standards after 3 months storage in open laboratory light and at ambient temperaure. Development was made with diethyl ether/methanol/water (96:3:1) for 10 min. Detection was made with long-wave UV light.

tions after 6 months' storage in the dark. One can notice a slight decrease in fluorescent intensity compared to those on the left. Also notable in both groups is that the cyclohexane and heptane B_1 spots are tighter than the benzene or toluene spots. The third TLC strip, on the right with the lone spot, is that of standards after 3 months of exposure to normal lighting in the laboratory. The lone spot is that of the benzene system. Complete photodegradation of B_1 occurred in the cyclohexane, heptane and toluene systems, even though the absorbance of these solutions indicated a level of B_1 greater than 1 μg/mL. The superiority of benzene as a solvent for aflatoxin is clearly evident from this exposure test to light.

Replacement of benzene by heptane or toluene in preparing aflatoxin standards appears feasible, provided that exposure to light is minimized. Of the 2 solvents, heptane would be a better choice, as it has a higher vapor exposure safety level. Laboratories not equipped to comply with OSHA regulations for benzene vapors could thus substitute heptane for benzene. Laboratories that have adequate controls to meet OSHA regulations for benzene vapors could continue to use this solvent for aflatoxin standards.

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Enzyme-Linked Immunosorbent Assay for T-2 Toxin

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ABSTRACT

An enzyme-linked immunosorbent assay (ELISA) was developed for the rapid quantitation of T-2 toxin, a tricothecene mycotoxin produced by members of the genus *Fusarium*. T-2 toxin was first converted to the T-2 hemisuccinate (T-2 HS) and then conjugated by the water-soluble carbodiimide method to either bovine serum albumin for use as an immunogen or to horseradish peroxidase for use as an enzyme marker. T-2 antiserum was air-dried onto polystyrene microtissue culture plates and the ELISA conducted by simultaneously incubating standards of T-2 toxin and the T-2 HS-peroxidase conjugate. Competition curves were prepared by determining total bound enzyme. The ELISA took about 2 hr to complete and allowed minimal detection of T-2 at levels of 2.5 pg/assay. Average recoveries from samples of wheat flour spiked with T-2 toxin in the 1.0-30 ppb range were 95 ± 25% and those for corn meal spiked in the 5.0-30 ppb range were 98 ± 19%. The results suggested the ELISA is a simple and convenient alternative for the screening of T-2 toxin in food and feeds.

INTRODUCTION

T-2 toxin and other related tricothecene mycotoxins are secondary metabolites produced by the fungal genera *Fusarium*, *Trichoderma*, *Myrothecium* and *Stachybotrys* (1). T-2 toxin has been associated with certain mycotoxicoses (1,2), including alimentary toxic aleukia (3), and has recently been suggested to be carcinogenic (4). Because T-2 toxin and the fungi that produce it are found in a number of agricultural commodities (5,6), it presents a potential risk to human and animal health. While a number of analytical methods for T-2 toxin have been developed, including biological assays, thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and gas liquid chromatography-mass spectrometry (GC-MS) (7,8), these either lack sensitivity or specificity or require expensive instrumentation, thus precluding their use in the routine screening of foods and feeds. Recently, this problem was overcome by the development of a radioimmunoassay (RIA) for T-2 toxin (9). The RIA is sensitive and specific for T-2 toxin and can be used in the assay of corn, wheat, urine and serum (10,11).

In spite of its many advantages, the RIA still presents problems related to the disposal and storage of radioactive reagents, the necessity for a scintillation counter, and the number of samples that can be conveniently analyzed. A simpler alternative, the enzyme-linked immunosorbent assay (ELISA), has been applied to the assay of aflatoxin B_1, aflatoxin M_1, and ochratoxin A in our laboratory (12-14). In this report, we describe the development of a solid phase-microtest plate ELISA for T-2 toxin and its application to the assay of artifically contaminated wheat and corn.

EXPERIMENTAL

Materials

Horseradish peroxidase (type VI), bovine serum albumin (BSA), RIA grade, Tween 20, 1-ethyl-3,3-dimethylaminopropyl-carbodiimide (EDPC), 2,2'-azino-di-3-ethyl-benzthiazoline-6-sulfonate (ABTS) and hydrogen peroxide were purchased from Sigma Chemical Co. (St. Louis, MO). Complete and incomplete Freund adjuvants were purchased from Difco Laboratories (Detroit, MI). T-2 and T-2 hemisuccinate (T-2HS) were prepared according to previously published methods (9,15). Standard preparations of T-2 were kindly supplied by E.B. Smalley of the University of Wisconsin. All other chemicals were of reagent-grade quality or better. Sep-Pak™ C_{18} cartridges were obtained from Waters Associates (Milford, MA). Albino rabbits were purchased from Klubertanz's Rabbit Farm (Edgerton, WI) and tested to be *Pasteurella* negative before use. Corn meal and wheat flour were purchased from a local grocery store.

Production of Hyperimmune Serum

Antisera specific for T-2 was prepared by multiple site injections of albino rabbits with a T-2 HS-BSA immunogen in the manner described by Chu et al. (9). The resulting hyperimmune serum was purified by the ammonium sulfate method of Herbert et al. (16) and titer was determined by RIA (9).

Preparation of T-2 HS-Peroxidase Conjugate

The method for T-2 HS-peroxidase preparation was essentially that used for aflatoxin B_1 oxime-peroxidase (12).