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Procedure for Detection of Aflatoxin B1 and M1 in Urine by High Performance Liquid Chromatography with Fluorescence Detection.

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DISCLAIMER

Reference to any commercial materials, equipment, or process does not in any way constitute approval, endorsement, or recommendation by the Food and Drug Administration.

ABSTRACT

The purpose of this SOP is to describe how to determine the presence of aflatoxin B1 (AFB1) and aflatoxin M1 (AFM1) in urine with pre-column derivatization by high performance liquid chromatography (HPLC) with fluorescence detector.

Quantitation range: 0.5-15 ng/g (ppb). Note, sensitivity of the method greatly depends on sensitivity of the fluorescent detector. The method requires 2mL of urine. Quantitation is based on matrix-matched calibration curve. Analytes (AfB1 and AfM1) are extracted, derivatized, cleaned up using Octadecyl (C18) material, injected into HPLC, chromatographed on C18 column and quantitated using Fluorescence detector

Validation data (in-house and via collaborative studies such as Blinded Method Tests) are available in the following two publications:

https://pubmed.ncbi.nlm.nih.gov/28985321/

https://pubmed.ncbi.nlm.nih.gov/36912688/

ATTACHMENTS

Target-SOP-AfB1M1-in-Urine-Iowa_2017-9-7.pdf

MANUSCRIPT CITATION:
1. Intra-laboratory Development and Evaluation of a Quantitative Method for Measurement of Aflatoxins B1, M1 and Q1 in Animal Urine by High Performance Liquid Chromatography with Fluorescence Detection
Xiangwei Du [1], Dwayne E Schrunk [1], Dahai Shao [1], Paula M Imerman [1], Chong Wang [1], Steve M Ensley [1], Wilson K Rumbeiha [1] J Anal Tox. 2017 Oct 1;41(8):698-707. doi: 10.1093/jat/bkx059. https://pubmed.ncbi.nlm.nih.gov/28985321/

2. **Extensive Evaluation of a Method for Quantitative Measurement of Aflatoxins B1 and M1 in Animal Urine Using High-Performance Liquid Chromatography with Fluorescence Detection**
Xiangwei Du [1], Dwayne E Schrunk [2], Paula M Imerman [2], John Tahara [3], Andriy Tkachenko [4], Jake Guag [4], Renate Reimschuessel [4], Wilson K Rumbeiha [5] J AOAC Int . 2023 May 3;106(3):645-651. doi: 10.1093/jaoacint/qsad034. https://pubmed.ncbi.nlm.nih.gov/36912688/

GUIDELINES

1. The correlation coefficient R2 value for the standard curve must be >0.99. The back calculated deviation for each points
must be within 20%.
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2. The method for detection of AFB1 and AFM1 was validated in glassware. 7 mL borosilicate glass scintillation vials are also suitable for this step.

3. The method for detection of AFB1 and AFM1 was validated in glassware. 50 mL screw cap glass tube is also suitable for this step.

4. Each lab should perform a practice run to check the optimal incubation time and HPLC run time at three levels (low at 0.8 ppb, medium at 5 ppb, and high at 10 ppb). After incubation, inject samples at 20 h, 24 h, 28 h, and 32 h to check signal drift for each aflatoxin. The optimal incubation time is the time when minimal signal drift is observed. A signal drift of ± 15% is acceptable.

5. The HPLC run time is optimal as long as the last eluting peak has returned to the baseline (2-4 minutes) before the run has ended. The validated run time is 33 minutes, if needed the run time can be extended in 5-10 minute increments until the run time has been optimized.

Protocol status: Working
We use this protocol and it's working

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MATERIALS

Equipment/Materials

- Balances. Capable of weighing minimum: 0.01 g (Mettler Toledo or equivalent).
- Centrifuges. Bench top centrifuge (IEC Centra-GP8 or equivalent).
- Mixers and shakers. Single tube vortex mixer (VWR or equivalent), Fisher Multi-tube Vortexer (Thermo Scientific or equivalent), Rotor rack shaker (LabQuake, Thermo Scientific or equivalent).
- Pipettes. Variable pipettes to cover ranges of 1-10 µL, 10-100 µL, 100-1000 µL, and 1-10 mL. (Rainin or equivalent).
- Digital Heating bath (GeneMate or equivalent).
- A Waters HPLC or equivalent equipped with a Waters 2695 separation module including a vacuum degasser, a quaternary pump, an automatic sample injection system, a Waters 2475 Multi-wavelength fluorescence detector or equivalent, and the Empower software or equivalent to control the instrument, data acquisition, and data analysis is used for separation and quantification of aflatoxins (or equivalent).
- HPLC column. Biphenyl reversed phase column (Kinetex Biphenyl, 2.6 µm, 100 Å, 100 mm × 4.6 mm, Phenomenex, Torrance, CA, USA, part #: 00D-4622-E0). A guard column (Agilent, Polaris C18-A, MetaGuard, 5 µm, 2.0 mm, part #: A2000MG2) is used.
- Clean-up immuno-affinity column AFLAPREP, R-Biopharm AG (Washington, MO, USA). Part #: P07.
- 7 mL borosilicate glass scintillation vials, Fisher Scientific (Waltham, MA, USA). Part #: 03-337-26.
- 15 mL Falcon conical centrifuge tubes, Fisher Scientific (Waltham, MA, USA). Part #: 14-959-49B.
- Syringes, 30 mL, Monoject, Kendall. Part #: 1180600555.
- 50 mL conical-bottom centrifuge tubes, VMR (Radnor, PA, USA). Part #: 89039-662.
- 55 mL screw cap glass tubes, Fisher Scientific (Waltham, MA, USA). Part #: 14-933D or 50 mL conical-bottom centrifuge tubes, VMR (Radnor, PA, USA). Part #: 89039-662.

Reagents/Controls

- Methanol, HPLC grade, Fisher Scientific (Waltham, MA, USA). Part #: A452-4.
- Acetonitrile (ACN), HPLC grade, Fisher Scientific (Waltham, MA, USA). Part #: A298-4.
- Trifluoroacetic acid (TFA), bioanalysis grade, Acros Organics, Fisher Scientific (Waltham, MA, USA). Code: 293811000.
- Glacial acetic acid (HOAc), ACS reagent grade, Fisher Scientific (Waltham, MA, USA). Part #: A38c-212.
- Hydrochloric acid (HCl), certified ACS grade, Fisher Scientific (Waltham, MA, USA). Part #: A144-212.
- Sodium chloride (NaCl), certified ACS grade, Fisher Scientific (Waltham, MA, USA). Part #: S271-3.
- Potassium chloride (KCl), certified ACS grade, Scientific (Waltham, MA, USA). Part #: P217-3.
- Sodium phosphate dibasic anhydrous (Na2HPO4), certified ACS grade, Fisher Scientific (Waltham, MA, USA). Part #: S374-3.
- Potassium phosphate monobasic (KH2PO4), certified ACS grade, Fisher
- Scientific (Waltham, MA, USA). Part #: P285-3.
- All aqueous solutions are prepared in 18.2 MΩ·cm water by Aries High Purity Water System (Aries Filter Network, USA).
- The reference standard of aflatoxins was purchased from Sigma-Aldrich (St-Louis, MO, USA). AFB1 part #: A-6636; AFM1 part #: A-6428.

SAFETY WARNINGS

SAFETY CONSIDERATIONS

- See [9.1399, current version] Policy for Personal Protective Equipment and Safe Laboratory Practices.
- See Applicable SDS.
- Considerations specific for this procedure:AFB1 and AFM1 are toxic and carcinogenic and hence should be handled with extreme care.

BEFORE START INSTRUCTIONS

Checks Made Prior to Beginning Test Procedure

- We run standard and make sure the retention time drift for each analyte should be within 15%.

Preparation of Standard Stock Solutions and Reagents

1 Methanol/water 80/20 (v/v) is prepared by adding 80 mL deionized water to 320 mL methanol. This amount is good for about 75-80 samples.

2 The standard stock solutions of each aflatoxin is prepared by dissolving the pre-weighed standard in methanol (AFB1) or chloroform (AFM1) and stored in -20 °C when not in use.

Note

The reference standard of aflatoxins was purchased from Sigma-Aldrich (St-Louis, MO, USA). AFB1 part #: A-6636; AFM1 part #: A-6428.

2.1 Example: A 5 µg AFM1 standard is dissolved in 5 mL of chloroform to make a [M] 1 ug/mL stock standard.

2.2 Example: A 5 mg AFB1 standard is dissolved in 5 mL of methanol to make a [M] 1 mg/mL stock standard.

2.3 A working mixed standard solution (250 ng/mL for each aflatoxin) is prepared. Quantitatively transfer 1 mL of the AFM1 stock standard and 1 µL of the AFB1 stock standard to a 7 mL scintillation glass vial. Concentrate to dryness by nitrogen effusion and dilute in 4 mL methanol. It is stored at -20 °C when not in use and is good for one year.

2.4 A working mixed standard solution (25 ng/mL) is prepared by dilution of the 250 ng/mL mixed standard solution using methanol. It is prepared on the day of use. Transfer 0.5 mL of the 250 ng/mL mixed standard to a 7 mL vial, add 4.5 mL of methanol and vortex to mix.

3 1 M HCl solution is prepared by adding 8.3 mL of concentrated HCl and diluting it in a volumetric flask to 100 mL.

4 1x Phosphate-buffered saline (PBS) solution is prepared by adding Sample NaCl, 0.20 g KCl, 1.44 g Na2HPO4, and 0.24 g KH2PO4 in 800 mL deionized water and adjust the pH to 7.4 with 1 M HCl, then diluted to 1.0 L with deionized water. This amount is good for about 25-28 samples.

5 Derivatization reagent 35/10/5 (v/v) water/TFA/glacial acetic acid: Mix 10 mL TFA with 5 mL glacial acetic acid and 35 mL L deionized water. The mixture is mounted on Roto rack for rotate-mixing for 00:30:00, then stored in dark or aluminum-foil-wrapped bottles. This amount is good for about 100-120 samples. We suggest this is good for 3 months 30m

Sample preparation

6 Urine samples were pooled into a beaker and stirred with a stir bar for 00:10:00. After homogenizati 20m the urine was transferred into 50 mL centrifuge tubes and centrifuged at 3800 rpm for 00:10:00 to remove precipitates.

7 The centrifuged urine sample was transferred into brown plastic bottles and stored at -80 oC before use.

Test Procedure

8 **Preparation of calibration curve in urine matrix**

8.1 Thaw samples stored in -80 °C freezer in a water bath and centrifuge the blank urine samples i 10m
plastic centrifuge tubes at 3800 rpm for 00:10:00

8.2 Seven 2 mL control urine samples are weighed into 15 mL Falcon plastic polypropylene conical tubes (See Guideline 2). A series of volumes (4 µL, 8 µL, 16 µL, 40 µL, 80 µL and 120 µL) of the 250 ng/mL working standard solution of AFB1 and AFM1 is added to the 2.0 mL control urine to give a series of fortified concentration of 0.5, 1, 2, 5, 10, 15 ng/mL.

8.3 The fortified samples are mixed thoroughly by vortexing for 00:00:10 at maximum speed 10s
and subject to the following steps 7-9.

9 **Extraction**

9.1 Add 4 mL 80/20 (v/v) methanol/water to the samples. Vortex at 2500 speed for 5m
00:05:00 using the Fisher Multi-tube Vortexer.

9.2 Pipet 2 mL solution into a 50 mL plastic conical-bottom centrifuge tube (See Appendix 3).

9.3 Dilute the 2 mL solution with 14 mL 1X PBS solution, vortex for 00:00:05 5s

10 Clean up

10.1 Immuno-affinity columns (stored at 5 C) are mounted on the vacuum container with the other end of the column connected with 30 mL syringes through adaptors before use. Ensure that the column has not dried out and contains buffer above the gel when loading the 16.0 mL PBS solution. (It is important to note that the antibody included in the immuno-affinity column can be denatured by extreme temperature or pH change).

10.2 Pass solutions through the columns at a steady slow rate of 0.25 - 0.5 mL/min under vacuum or by gravity (This has to be followed exactly). A slow, steady flow rate is essential for the capture of the aflatoxins by the antibody.

10.3 Wash columns by passing 20 mL 1x PBS solution, at a flow rate of approximately 1 drop/sec (3 mL/min). Pass air through the column to remove residual liquid.

10.4 Elute the analytes from the column using 1.0 mL of methanol and collect the eluents in new tubes.

10.5 Pass 1.0 mL deionized water through the column.

10.6 Collect in the same tubes to give a 2 mL total volume. A backflush is needed to remove residual liquid.

10.7 The eluates collected in last two steps are transferred to 7 mL glass scintillation vial. The tubes are washed with 2 x 0.5 mL methanol. The solutions are concentrated to dryness under gentle nitrogen stream at ambient temperature.

11 **Derivatization**

11.1 The residue obtained in the previous step are reconstituted in 400 µL 35/10/5 (v/v/v) water/TFA/glacial acetic acid, vortexed for 00:00:10 at maximum speed, then heated at 65 °C in heating block for 00:15:00 15m 10s

11.2 The solutions obtained from this step are incubated at least for 20:00:00 (This incubation time needs to be optimized under each lab's conditions, see Appendix 5.3) at room temperature before HPLC analysis. 20h

HPLC Conditions

12 The optimized excitation and emission wavelengths for the fluorescence detector are 360 and 440 nm, respectively.

13 The mobile phase consisting of water (A) and acetonitrile (B) is pumped at a flow rate of 1 mL/min. A gradient elution is used to give the optimized separation. The details of the gradient program are as follows: 1 min isocratic step at 100% A; 1 min linear gradient (1-2 min) to 84% A and 16% B; an isocratic step from 2 to 16 min at 84% A and 16% B; a 1 min linear gradient (16-17 min) to 80% A and 20% B; an isocratic step

from 17 to 27 min at 80% A and 20% B; 1 min linear gradient (27-28 min) to 100% A; and a final isocratic step at 100% A to the end (28-33 min)

14 An injection volume of 20 µL is used.

15 Retention time: A total running time of 00:33:00 is normally used. 33m

Result Interpretation

16 The calibration curve is established by plotting the fluorescence intensity (in peak area, fluorescence unit) versus the injected mass of standard (in ng) by linear regression. The concentration of the unknown sample is calculated based on this standard curve.

Reporting Results

17 See [9.3444, current version] Procedure for entering results in ISULIMS.