

Pyxant Labs Inc.
STM2360.03

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1.0 PRINCIPLE AND SCOPE

Fluazinam is extracted from sediment using methanol and a digital sonifier. The extracts are then diluted 1:10 with UHP water. Analysis is performed by LC/MS/MS utilizing TurboIonSpray in the positive ionization mode. This assay uses 10 g sub-samples of sediment. Sediment should be stored at approximately -20°C.

2.0 SAFETY

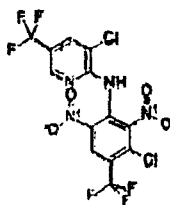
Analysts must be acquainted with the potential hazards of the reagents and solvents employed in the laboratory. Such information may be obtained from the MSDS, literature, and other documents such as the laboratory chemical hygiene plan. Disposal of chemicals and reagents must comply with all federal, state, and local regulations.

Methanol is a flammable reagent and formic acid is corrosive.

3.0 COMPOUNDS

The fluazinam (IKF-1216) reference standard is stored frozen ($\leq -20^{\circ}\text{C}$), protected from light.

4.0 CHEMICAL STRUCTURE



Fluazinam

5.0 REAGENTS, CHEMICALS AND SUPPLIES

- Acetonitrile (HPLC Grade)
- Acetone (HPLC Grade)
- Methanol (HPLC Grade)
- Formic Acid
- Ultra High Purity Water or HPLC Grade Water
- Column: Synergi Polar-RP 4 μm , 50 \times 2.0 mm
- Agilent 1.5 mL Capped Glass Autosampler Vials

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- Glass Qorpak Tubes 27.5 × 95 mm with Teflon cap
- 100 mL glass mixing cylinders

6.0 EQUIPMENT

- Centrifuge Beckman Coulter Allegra X-12R (or equivalent)
- API 5000 Mass Spectrometer
- Symbiosis HPLC system
- Branson 450 digital sonifier with cup horn
- Calibrated adjustable pipettes
- Class A volumetric flasks, various sizes
- Class A volumetric pipettes, various sizes
- Analytical balance, 20 mg – 200 g
- Top loading balance, 1 g – 400 g

7.0 PREPARATION OF SOLUTIONS

UHP water (18-megaohm-cm or equivalent) or HPLC water is used wherever water is required. All solutions are mixed well.

10/90/0.02 Acetonitrile/Water/Formic Acid (v/v): Add 100 mL of acetonitrile to a 1000-mL class A volumetric flask and dilute to volume with water. Add 200 µL of formic acid and mix well.

0.1% Formic Acid in Water: Add 1 mL of formic acid to 1 L of UHP water. Mix well.

0.1% Formic Acid in Acetonitrile: Add 1 mL of formic acid to 1 L of acetonitrile. Mix well.

8.0 STABILITY

Per SOP 1.4005¹, all reagents, except 0.1% formic acid in water, have six months stability at ambient temperatures. An expiration of two weeks is given to the 0.1% formic acid in water.

9.0 PREPARATION OF ANALYTICAL SOLUTIONS

Analytical standard stock solutions are stored at < -10°C, in glass containers protected from light and equilibrated to ambient temperature before use. The solutions are remade every three months. The following charts indicate suggested standard solutions and concentrations.

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Stock and Intermediate Solutions:

| Solution Name | Preparation | Nominal Concentration |
|----------------------|--|------------------------------|
| A1 | *Weigh approximately 50 mg of fluazinam and dissolve in 50 mL acetone (correct for purity) | 1000 µg/mL |

*Use an analytical balance capable of weighing to 0.1 mg
Use glass Class A pipettes and volumetric flasks

Fortification Solutions:

Fortification and calibration solutions are stored at < -10°C, in glass containers protected from light, and equilibrated to ambient temperature before use. The solutions are remade every three months. The following charts indicate suggested standard solutions and concentrations.

| QC Sample | Preparation | Nominal Concentration |
|------------------|--|------------------------------|
| B1 | Add 0.1 mL of A1 to a 100-mL class A volumetric flask and dilute to volume with acetonitrile | 1.00 µg/mL |
| B2 | Add 5 mL of B1 to a 50-mL class A volumetric flask and dilute to volume with acetonitrile | 100 ng/mL |

Use glass Class A pipettes and volumetric flasks

Note: Other dilution aliquots can be used as long as the nominal concentrations are maintained.

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Calibration Solutions:

| Solution Name | Preparation | Nominal Concentration |
|----------------------|--|------------------------------|
| C1 | Add 5 mL of B2 and dilute to 50 mL with 10:90:0.02 acetonitrile/water/formic acid | 10.0 ng/mL |
| C2 | Add 5 mL of C1 and dilute to 25 mL with 10:90:0.02 acetonitrile/water/formic acid | 2.00 ng/mL |
| C3 | Add 1 mL of C1 and dilute to 10 mL with 10:90:0.02 acetonitrile/water/formic acid | 1.00 ng/mL |
| C4 | Add 5 mL of C1 and dilute to 100 mL with 10:90:0.02 acetonitrile/water/formic acid | 0.500 ng/mL |
| C5 | Add 1 mL of C2 and dilute to 10 mL with 10:90:0.02 acetonitrile/water/formic acid | 0.200 ng/mL |
| C6 | Add 1 mL of C3 and dilute to 10 mL with 10:90:0.02 acetonitrile/water/formic acid | 0.100 ng/mL |
| C7 | Add 1 mL of C4 and dilute to 10 mL with 10:90:0.02 acetonitrile/water/formic acid | 0.0500 ng/mL |

Use glass class A pipettes and volumetric flasks

Note: Other dilution aliquots can be used as long as the nominal concentrations are maintained.

10.0 EXTRACTION PROCEDURE

This analysis must be performed as quickly as possible. Do not fortify all QC samples at once or a significant loss of analyte is observed. During validation, two samples were fortified and taken through the preparation prior to fortifying the next two samples.

1. Weigh 10 grams \pm 0.5 g of sediment into a glass 40 mL Qorpak tube.
2. Fortify any required laboratory QC samples at this time.
3. Add 30 mL of methanol to the samples and QC samples.
4. Shake by hand for a few seconds to disperse and wet the sample.
5. Subject samples to pulse sonication for 10 minutes using the Branson 450 digital sonifiers w/ cup horn, pulsed signal at 50 seconds on and 10 seconds off at 80% of capacity.
6. Centrifuge the samples at 2000 rpm for five minutes.

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7. Take 0.100 mL of extract and aliquot it into an autosampler vial containing 900 µL of UHP water for a 1:10 dilution. Other dilution schemes may be used as necessary. Mix by vortexing prior to analysis.

11.0 ANALYSIS PROCEDURE

Typical HPLC Conditions

HPLC System: Symbiosis Pharma
Column: Synergi Polar-RP (4 µm, 50 × 2.0 mm)
Guard Column: Phenomenex C18 (optional)
Column Temperature: Ambient
Injection Volume: 35 µL
Mobile Phase: Solvent A: 0.1% formic acid in water
Solvent B: 0.1% formic acid in acetonitrile
Flow Rate: 0.500 mL/minute

| Gradient: | Time (min) | % A | % B |
|-----------|------------|-----|-----|
| | 0:00 | 90 | 10 |
| | 1:02 | 90 | 10 |
| | 5:02 | 0 | 100 |
| | 5:07 | 90 | 10 |
| | 7:02 | 90 | 10 |

Typical Mass Spectrometer Conditions

Mass Spectrometer: Applied Biosystems. API 5000
Interface: TurboIonSpray, 550°C
Polarity: Positive
Scan Type: MRM
Resolution: Q1: Low, Q3: Unit
Dwell Time: 100 ms, depending on peak width and signal-to-noise ratio observed

| Compound | Transition (amu) | DP |
|-----------------|------------------|-----|
| Fluazinam Quant | 465.2→373.0 | 110 |
| Fluazinam Conf | 465.2→338.0 | 110 |

12.0 TYPICAL ANALYTICAL SEQUENCE

A validation batch's analytical sequence contains at a minimum a reagent blank, two untreated control (UTC) samples, five UTC samples fortified at the limit of quantitation

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(1.OQ), 0.01 µg/g, and five UTC samples fortified at 10×LOQ, 0.1 µg/g. The seven calibration standards are interspersed throughout the run.

13.0 SYSTEM SUITABILITY

The LC/MS system is considered ready for analysis when the signal-to-noise ratio, using peak-to-peak, of the low standard is greater than or equal to 5:1.

14.0 COLUMN PROCEDURES

Synergi Polar-RP columns, when not in use, should be stored in 100% acetonitrile.

15.0 QUANTITATION METHOD

A linear 1/x regression is used.

16.0 CALCULATIONS

Calibration standards can be embedded throughout the analytical batch. Instrument control and data collection are accomplished using the Analyst® 1.4.2 software program. The data are analyzed and the raw nanogram amounts calculated using the Analyst® 1.4.2 software program.

A calibration curve calculated by linear regression with 1/x weighting was determined to be a best fit of the data during method validation. From this curve, the concentration of analyte in the sample was calculated using the equation

$$y = mx + b \quad (1)$$

where

- y = the analyte peak area
- x = the concentration of the analyte in ng/g

The coefficients, m and b, were calculated automatically by the validated Analyst® 1.4.2 software program. The software also corrects for the dilution factor or the amount by which the initial extract was diluted to yield a concentration within the calibrated range.

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The standard concentration in ng/g can be calculated as follows:

$$AC \text{ (ppb)} = \frac{x_i \times V_f}{W} \quad (2)$$

where

- AC = Analyte concentration found
- x_i = Standard concentration (ng/mL)
- V_f = Final volume of extract (30 mL)
- W = Nominal sample weight (10 g)

17.0 ACCEPTANCE CRITERIA

Individual recoveries should be between 70-120%. Calibration curves should exhibit a correlation coefficient of 0.995 or better.

18.0 REFERENCES

1. "Preparation and Labeling of Reagents and Solutions." Pyxant Labs Inc. SOP 1.4005. Rev 13. February 12, 2010.