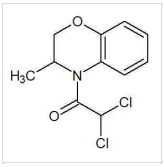

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Standard Operating Procedure (SOP) for the Analysis of benoxacor in subcellular fraction incubations by gas chromatography mass spectrometry/electron capture detector

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1 Works for me dx.doi.org/10.17504/protocols.io.bb52iq8e

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ABSTRACT

This standard operating procedure uses liquid-liquid extraction for the determination of benoxacor in incubations with liver microsomes, liver cytosol, and other samples using a gas chromatography-mass spectrometry (GC-MS) and gas chromatography with electron capture detection (GC-ECD).

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Simonsen D, Cwiertny DM, Lehmler HJ. Benoxacor is enantioselectively metabolized by rat liver subcellular fractions. Chem Biol Interact. 2020 Oct 1;330:109247. doi: 10.1016/j.cbi.2020.109247. PMID: 32866466; PMCID: PMC7556730.

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KEYWORDS

Agrochemicals, safener, benoxacor, microsomes, cytosol, metabolism, enantioselective, chiral, gas chromatrography, liquid-liquid extraction

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MATERIALS TEXT

Accessories and supplies

Crimp-top GC-vial (Fisher, 03-391-6)
Eppendorf centrifuge 5810 R (Eppendorf)
Furnace (Thermolyne F30400 Furnace)
Disposable culture tube, size 12*125 mm (Fisher, 1495935A)
Pasteur pipettes (Fisher, 22-183632)
Pipette sizes: 20-200 µL, 100-1000 µL
Pipette tips 100-1000 µL
Screw caps, thread 14-415 mm (Fisher, 14-930-15E)
Vortex mixer (Fisher)

Chemicals

Benoxacor (Sigma-Aldrich; 46001) CAS number = 98730-04-02
Ethyl acetate, pesticide grade (Fisher, E191-4)
Magnesium sulfate (Sigma-Aldrich, 208094-500G)

Instruments

Agilent Technologies 6890 gas chromatograph equpped with a 5975 MSD, a Gerstel MPS Autosampler, and an Agilent SLB-5MS column (30 m length, 250 µm inner diameter, 0.25 µm film thickness)

Agilent Technologies 7890A gas chromatograph equipped with a 63Ni-micro electron capture detector (µECD), an Agilient 7693 Autosampler, and an Agilent Chiralsil-Dex CB capilary column (25 m length, 250 µm inner diameter, 0.25 µm film thickness)

Standard Solutions

Benoxacor 2.5 mM in DMSO
Trifluralin 20 µg/mL in ethyl acetate as surrogate recovery standard
Acenaphthene 20 µg/mL in ethyl acetate as internal standard

Preparation and cleaning of glassware

After each use glassware for incubations are washed with water and then triple rinsed with acetone, dichloromethane, and hexane. All extraction glassware is disposed of after use.

SAFETY WARNINGS

Benoxacor SDS.pdf

Trifluralin SDS.pdf

Acenaphthene SDS.pdf

DISCLAIMER:

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BEFORE STARTING

Benoxacor will readily degrade in the inlet of a gas chromatograph. The inlet temperature of the gas chromatograph needs to be kept relatively low and the glass liner (e.g., inlet liner, splitless type, Supelco, 2046605) should not contain glass wool

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Sample preparation

1

Label disposable culture tubes and GC vials. Tubes and vials that need to be labeled include quality assurance/quality control (QA/QC) samples (i.e., buffer, cofactor with no benoxacor, inactivated subcellular fractions, subcellular fractions without c-substrate, and DMSO spiked buffer with cofactor), experimental samples and the Reference Standard.

Important: Use unique sample numbers that include your initials and identifiers for the experiment (e.g., notebook number) and the sample. The goal is to avoid identical sample numbers across experiments.

1.1

At least one tube for method blanks (buffer only).

1.2

At least one tube per sample blank (matrix samples from species/sex analyzed).

1.3

Three tubes for Ongoing Precision and Recovery (OPR) samples (matrix samples spiked with known amounts of the target analyte(s), i.e., benoxacor).

1.4

Tubes for all samples (depends on number of samples to be analyzed; it is recommended that 20% of the samples are QA/QC samples; increase the number of blank samples accordingly).

1.5

One tube for the Reference Standard (will include known amounts of all analytes, including the surrogate recovery standards and internal standard)

(Note: Solutions of all analytes are added to the tube with the Reference Standard at the same time when the samples are spiked)

Protein degradation

2

Immediately after incubation is completed place samples in furnace for 10 minutes at 110 ^{o}C

(Note: Leaving samples in furnace longer to 10 minutes will reduce the recovery of the analytes)

2.1

Cool samples to room temperature before continuing extraction.

2.2

Spike samples with 25 µL of 20 µg/mL trifluralin dissolved in ethyl acetate

Extraction

3

Add 2 mL pesticide grade ethyl acetate to all samples

(Note: Do not add extra ethyl acetate to reference)

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4

Put samples on tube rotator for 1 minute at 40 rpm to mix buffer and ethyl acetate.

(Note:) Make sure caps are tight so they do not leak

5

Transfer the samples to a centrifuge and centrifuge at 3,000 rpm (1811 x g) for 5 minutes to facilitate the phase separation

5.1

Transfer 2 mL of ethyl acetate into a clean culture tube

5.2

Repeat steps 3-5.1

(Note: Combine the 2 mL of ethyl acetate to the previous 2 mL ethyl acetate generated from the first extraction)

6

Add 2 mL of 1% KCl to the 4 mL of ethyl acetate to remove residual proteins.

6.1

Put samples on tube rotator to mix the ethyl acetate and 1% KCl

6.2

Transfer the samples to a centrifuge and centrifuge at 3,000 rpm (1811 x g) for 5 minutes to facilitate the phase separation

6.3

Transfer the 4 mL ethyl acetate to a clean culture tube containing 0.25 mg of magnesium sulfate

7

Briefly vortex each tube.

7.1

Transfer the samples to a centrifuge and centrifuge at 3,000 rpm (1811 x g) for 5 minutes to facilitate precipitation of magnesium sulfate

7.2

Carefully transfer the ethyl acetate into a clean tube while attempting to not transfer any magnesium sulfate

(Note: if small amounts of magnesium sulfate does get transferred it can be removed in subsequent steps)

8

Evaporate each tube under nitrogen to approximately 500 µL

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9 Spike each tube with 25 µL with 20 µg/mL acenaphthene

10 Transfer samples into a GC vial and dilute to 1 mL

GC/MS Analysis

11 Run samples on GC/MS followed by GC/ECD.

12 GC/MS Parameters

Samples were injected by splitless injection at 280 ^{O}C. We used a Supelco SLB-5MS column (30 m length, 0.25 mm inner diameter, 0.25 µm film thickness) with a column helium flow rate of 1 mL/min. The oven parameters were programmed as follows: Initial temperature, 50 ^{O}C hold for 1 min, 15 ^{O}C/min to 240 ^{O}C, hold for 13 min, 15 ^{O}C/min to 300 ^{O}C, hold for 10 min. The temperatures of the MS source and the MS quad were 230 ^{O}C and 150 ^{O}C, respectively.

A	B	C	D
Compound	Avg. Rention time	Quant	Qual
Acenaphthene	11.35	153	154
Trifluralin	12.74	264	306
Benoxacor	15.57	120	259

SIM Mode Monitored Ions

13 GC-ECD Parameters

A ^{63}Ni-µECD detector was used to monitor enantioselective metabolism. We used an Agilent CP-Chrialsil Dex CB (CB) column (25 m length, 0.25 mm inner diameter, 0.25 µm film thickness) to determine the enantiomeric fraction of benoxacor using the following temperature program: 50 ^{O}C, hold for 1 min, 10 ^{O}C/min to 145 ^{O}C, hold for 50 min, 15 ^{O}C/min to 200 ^{O}C, hold for ^{63}Ni-µECD detector 12 min. The injector temperature was set at 250 ^{O}C, and a constant helium flow rate of 2 mL/min was used.

14 **Example Quantification of Benoxacor.xlsx**

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