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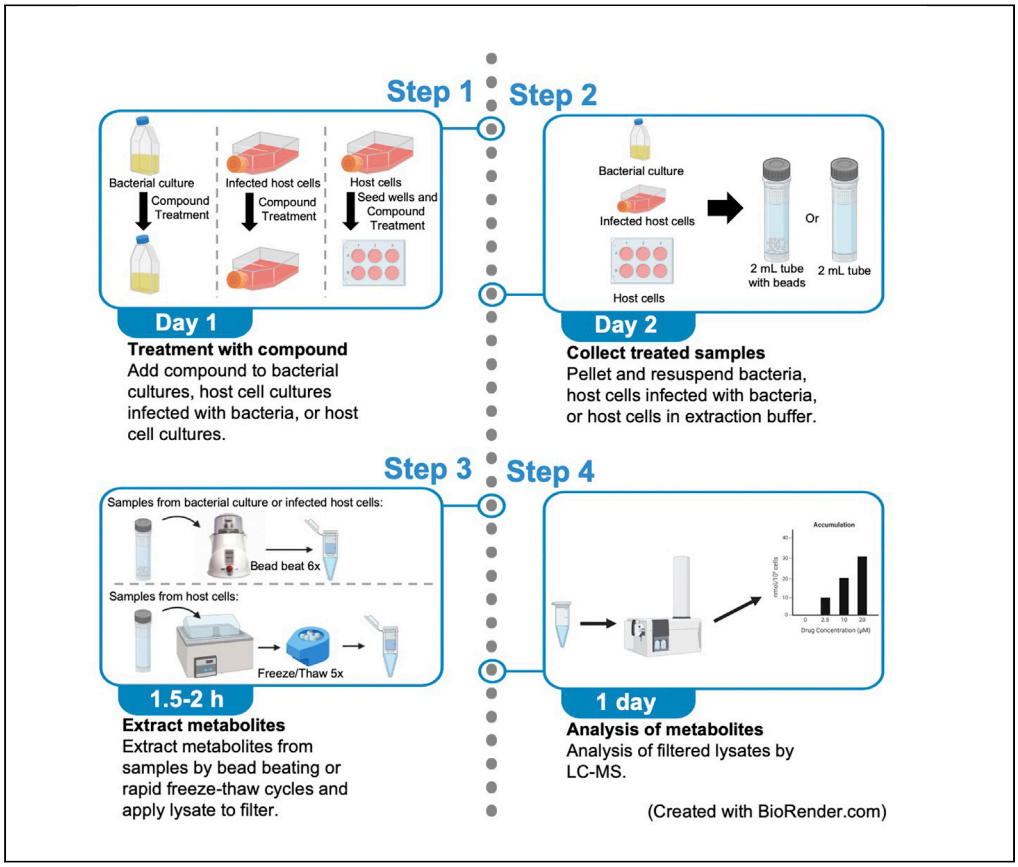
Liquid chromatography-mass spectrometry-based protocol to measure drug accumulation in *Mycobacterium tuberculosis* and its host cell



The extent to which a drug accumulates in *Mycobacterium tuberculosis* (Mtb) and its host cell can affect treatment efficacy. We describe protocols measuring drug accumulation in Mtb, macrophages, and Mtb-infected macrophages. The method leverages drug extraction from the cellular lysate and drug-level quantification by liquid chromatography-mass spectrometry. The general methodology has broad applicability and can quantify drug accumulation in other cell types, while being extended to quantification of drug metabolites formed within the cell under study.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol measures drug accumulation in *Mycobacterium tuberculosis* and its host cell

Protocol relies on drug extraction from the cell lysate followed by LC-MS quantification

Protocol applies to other cell types and may be extended to drug metabolites

These measurements may be used to explain drug efficacy and mechanism

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Liquid chromatography-mass spectrometry-based protocol to measure drug accumulation in *Mycobacterium tuberculosis* and its host cell

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SUMMARY

The extent to which a drug accumulates in *Mycobacterium tuberculosis* (Mtb) and its host cell can affect treatment efficacy. We describe protocols measuring drug accumulation in Mtb, macrophages, and Mtb-infected macrophages. The method leverages drug extraction from the cellular lysate and drug-level quantification by liquid chromatography-mass spectrometry. The general methodology has broad applicability and can quantify drug accumulation in other cell types, while being extended to quantification of drug metabolites formed within the cell under study.

For complete details on the use and execution of this protocol, please refer to Lavin et al. (2021).[1]

BEFORE YOU BEGIN

Institutional permissions

Experiments with live *Mycobacterium tuberculosis* (Mtb) must be performed in a Biosafety Level 3 (BSL-3) facility, with inactivation steps validated and approved by the relevant institution's biosafety office and institutional biosafety committee.

Prepare buffers and media

Timing: 30 min

1. Prepare medium for culturing Mtb, J774 infection medium, and experimental buffers as needed.
2. Mtb culture medium (7H9 OADC + glycerol) is as routinely used in the field.[2]

Start bacterial and cell cultures

Timing: 5–10 d in advance of assay start

3. Start J774 macrophage-like cells from frozen stock and expand to obtain a sufficient number of cells for the assay.

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4. Start Mtb from frozen stock and expand to obtain sufficient bacterial culture at log-phase (OD_{600} ~ 0.6) for the assay.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
M. tuberculosis CDC1551	Lab stock	BEI NR-13649
Chemicals, peptides, and recombinant proteins		
Small molecules under study, where the term "drug" is used to mean either a clinically utilized small molecule treatment or an experimental small molecule under study as a chemical tool/probe, e.g., C6	For C6: Freundlich lab or Molport Inc.	JSF-4116 or Cat# MolPort-016- 629-492
Verapamil hydrochloride	Spectrum Chemicals	Cat# V1072
Sodium pyruvate (100 mM)	Thermo Fisher	Cat# 11360070
L-glutamine (200 mM)	Thermo Fisher	Cat# 25030081
Phosphate-buffered saline, 1× without calcium and magnesium, pH 7.4 ± 0.1	Corning	Cat# 21-040-CV
Bovine serum albumin	Thermo Fisher	Cat# BP1600-1
Gelatin	Sigma	Cat# G7041
Acetonitrile (HPLC grade)	Thermo Fisher	Cat# A998-4
Formic acid (99.0+%, Optima™ LC/MS Grade)	Thermo Fisher	Cat# A117-50
Methanol (Optima™ LC/MS Grade)	Thermo Fisher	Cat# A456-4
Experimental models: Cell lines		
Mouse: J774A.1 cells	ATCC	ATCC TIB-67
Software and algorithms		
MassHunter Qualitative Analysis (Version 8.0)	Agilent	https://www.agilent.com/en/product/software-informatics/mass-spectrometry-software
Other		
DMEM, high glucose	Thermo Fisher	Cat# 11965092
Fetal bovine serum (FBS)	Thermo Fisher	Cat# 10437028
UltraPure DNase/RNase-free distilled water	Thermo Fisher	Cat# 10977023
BD tuberculin syringes 25G × 5/8 inches	BD	Cat# 309626
T25 cell culture flasks	Thermo Scientific	Cat# 156367
T75 cell culture flasks	Thermo Scientific	Cat# 156499
6-well tissue culture plates	Corning Costar	Cat# 3516
15 mL conical tubes	Sarstedt	Cat# 62.554.100
Cell scraper	Sarstedt	Cat# 83.130
0.1 mm zirconia/silica beads	BioSpec Products	Cat# 11079101Z
2 mL screw cap tubes with O-rings	USA Scientific	Cat# 1420-8710
FastPrep-24 bead beater	MP Biomedicals	Cat# 116004500
Microcentrifuge	Eppendorf	Cat# 5425 R
Ultraspec 10 cell density meter	Biochrom US	Cat# 80-2116-30
0.22 μm Spin-X centrifuge tube filters	Corning Costar	Cat# 8160
LC-MS System	Agilent	1260 Infinity II LC 6230 TOF MS
EMD Millipore Chromolith SpeedRod RP-18e column (50 × 4.6 mm)	EMD Millipore Sigma	Cat # 151450

MATERIALS AND EQUIPMENT

J774 infection medium

Reagent	Final concentration	Amount
Fetal bovine serum (FBS), heat-inactivated	10%	50 mL
Sodium pyruvate (100 mM)	1 mM	5 mL
L-glutamine (200 mM)	2 mM	5 mL

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
DMEM, high glucose	N/A	440 mL
Total	**N/A**	**500 mL**

The FBS and L-glutamine stocks are aliquoted in 50 mL and 5 mL volumes respectively and stored at - 20°C until needed. After the J774 infection medium is made, it should be filter-sterilized through a 0.22 μm pore filter and stored at 4°C.

Basal uptake buffer		
Reagent	Final concentration	Amount
Phophate-buffered saline (PBS)	N/A	150 mL
Bovine serum albumin	0.5%	0.75 g
Dextrose	25 mM	0.6756 g
$MgCl_2$ hexahydrate	0.5 mM	150 μL of 0.5 M stock
$CaCl_2$ dihydrate	1 mM	150 μL of 1 M stock
Gelatin	0.1%	0.15 g
Total	**N/A**	**150 mL**

The $MgCl_2$ hexahydrate and $CaCl_2$ dihydrate stock solutions are stored at 20°C–25°C. After the basal uptake buffer is made, it should be filter-sterilized through a 0.22 μm pore filter and stored at 4°C.

Extraction solvent system		
Reagent	Final concentration	Amount
Acetonitrile	40%	20 mL
Methanol	40%	20 mL
UltraPure DNase/RNase-free distilled water	20%	10 mL
Total	**N/A**	**50 mL**

This solvent system is stored at −20°C.

Water-formic acid for LC-MS		
Reagent	Final concentration	Amount
ddH_2O	N/A	999 mL
Formic acid	0.1%	1 mL
Total	**N/A**	**1,000 mL**

Prepare in a glass bottle and store at 20°C–25°C.

Acetonitrile-formic acid for LC-MS		
Reagent	Final concentration	Amount
Acetonitrile	N/A	999 mL
Formic acid	0.1%	1 mL
Total	**N/A**	**1,000 mL**

Prepare in a glass bottle and store at 20°C–25°C.

⚠ CRITICAL: Caution: Acetonitrile, methanol, and formic acid should be handled in a fume hood and appropriate safety gloves and safety goggles should be worn.

Alternatives: This protocol uses a FastPrep-24 bead beater from MP Biomedicals, but any other bead beater with equivalent settings can alternatively be used.

Alternatives: This protocol uses an Agilent 1260 Infinity II liquid chromatography system coupled to an Agilent 6230 time-of-flight mass spectrometer for the liquid chromatography-mass spectrometry (LC-MS) analysis, but any equivalent system can alternatively be used. The protocol also utilizes a reverse-phase EMD Millipore Chromolith SpeedRod RP-18e column (50 × 4.6 mm), but an equivalent column may be implemented alternatively.

Alternatives: This protocol may be modified to study drug accumulation in other cell types. Specific examples from our laboratory include *Staphylococcus aureus*[3] and Vero cells.[4] These are complemented by approaches from other laboratories.[5–8] Furthermore, our methodology may also be leveraged to identify intrabacterial metabolites, representing the biotransformation products of the drug with which the bacterium was incubated.[3,6,9,10]

STEP-BY-SEP METHOD DETAILS

Treatment of bacterial cultures for intrabacterial drug accumulation measurement

Timing: 1–2 h, with a 6 d growth step prior to drug addition and a subsequent 24 h incubation step

Mtb is grown under the desired conditions, before treatment with the drugs/compounds being studied for downstream sample extraction and analysis. Medium conditions of interest may include environmental signals experienced by Mtb during infection, such as high chloride.[1,11]

Note: Samples should be run in triplicate, so each time-point and condition requires three flasks to be set up.

Note: The extraction solvent system should be pre-chilled at −20°C.

1. Start Mtb cultures at OD_{600} = 0.05 in each medium condition to be tested (10 mL in standing T25 flask).
   a. Seed the cultures using log-phase Mtb cultures ($OD_{600} \sim 0.6$).
   b. Incubate in a 37°C, 5% CO_2 incubator for 6 d, until the cultures are at mid-log phase ($OD_{600} \sim 0.6$).
2. When the cultures are ready 6 d later, for t = 0 (no drug treatment) samples, pellet 10 mL log phase Mtb cultures by centrifugation at 3,200 × *g* for 10 min.

Note: The t = 0 samples serve as a control to demonstrate that the untreated samples do not exhibit peak/s in the LC-MS spectra with the same retention time and mass spectrum profile as the parent drug under study.

3. Remove the supernatant and resuspend the Mtb pellet in 1 mL of pre-chilled extraction solvent system.

Note: Be sure to remove all the medium in the flask at this step prior to extraction solvent system addition. Use of a P1000 pipette to remove the last remaining medium is recommended.

4. Transfer each sample to 2 mL screw cap tubes containing 500 µL of 0.1 mm zirconia/silica beads.
5. Store at −80°C at least 18 h before proceeding to drug extraction.
6. To each flask of 10 mL log-phase Mtb culture to be treated, add drug (typically 1000× in DMSO) to afford the desired final concentration. Incubate in a 37°C, 5% CO_2 incubator for 24 h.
7. 24 h post drug addition, pellet the Mtb cultures by centrifugation at 3,200 × *g* for 10 min (t = 24 h samples).
8. Remove the supernatant, resuspend the Mtb pellet in 1 mL of ice-cold PBS, and transfer to a 2 mL screw cap tube.
9. Pellet the Mtb culture by centrifugation at 9,400 × *g* for 5 min at 4°C.
10. Remove the supernatant, and repeat steps 8 and 9 (i.e., for a total of 2 washes).
11. Remove the supernatant and resuspend the Mtb pellet in 1 mL of pre-chilled extraction solvent system.

Note: One can start with a drug concentration above its MIC (e.g., 5× MIC) to ensure quantifiable accumulation is observable, while checking for a lack of detectable cidality within the time frame of the experiment by standard plating methods. Subsequently, the drug concentration can be optimized to capture differential accumulation levels of the drug upon consideration of the different experimental conditions relevant to the study.

Note: Due to the slow-growing nature of Mtb, we chose in this experiment, as well as those with the uninfected and infected J774 cells, to use a drug incubation period of 24 h. This time period can be modified to suit other experimental scenarios.

Note: Be sure to remove all the medium in the tube at this step prior to extraction solvent system addition. Use of a P1000 pipette to remove the last remaining medium is recommended.

12. Transfer the sample to a 2 mL screw cap tube containing 500 μL of 0.1 mm zirconia/silica beads.
13. Store at −80°C at least 18 h before proceeding to drug extraction.

Pause point: The samples can be stored at −80°C for up to a month, unless previous experiments evidence instability of the drug in solution at −80°C.

Infection of J774 cell cultures for combined intrabacterial/intracellular drug accumulation measurement

Timing: 7 d total (schematic of process shown in Figure 1)

J774 macrophage-like cells are seeded and infected with Mtb, before treatment with the drug/compound being studied for sample extraction and downstream analysis.

Note: Samples should be run in triplicate. Thus, each condition requires three T75 flasks to be set up.

14. Seed 2 × 10^7 J774 cells per T75 flask in 12 mL of J774 infection medium. Incubate ∼16–18 h.
15. The next morning, bring the T75 flasks with the J774 cells to the BSL-3.
16. Determine OD_{600} of the Mtb cultures to be used for the infection.

Note: Mtb cultures should be in log-phase (OD_{600} ∼ 0.6).

17. Pellet appropriate amount of Mtb culture by centrifugation at 3,200 × *g* for 10 min.
    a. Resuspend pellet in 1 mL basal uptake buffer.
    b. Pass through a 25G × 5/8″ tuberculin syringe 6–8×.

Note: For determining the amount of Mtb culture that should be pelleted, note that for each T75 flask to be infected, 4 mL of Mtb suspension at OD_{600} = 0.2 will be needed.

18. Add pre-warmed J774 infection medium to the Mtb suspension.
    a. Determine the OD_{600} of the suspension.
    b. Adjust with more pre-warmed J774 infection medium as needed to obtain a suspension at OD_{600} = 0.2.
19. Remove all medium from T75 flasks seeded with J774 cells. Add back 14 mL pre-warmed J774 infection medium.
20. Add 4 mL of Mtb suspension at OD_{600} = 0.2 to each T75 flask to be infected. Carefully rock the flask to ensure the bacterial suspension is well mixed into the medium.
21. Incubate in a 37°C, 5% CO_2 incubator for 2 h.

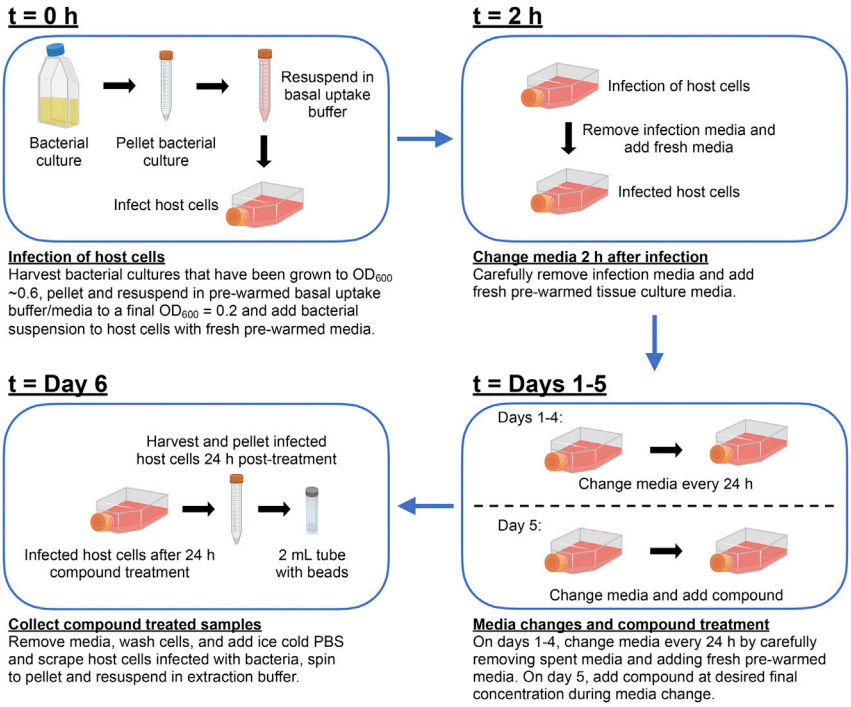

Figure 1. Schematic of infection of J774 cell cultures for combined intrabacterial/intracellular drug accumulation analysis

Created with BioRender.com.

22. After 2 h, remove all medium from flasks, add back 12 mL of pre-warmed J774 infection medium to each flask. Return flasks to the 37°C, 5% CO_2 incubator.
23. For the next 4 d, replace medium daily with 12 mL fresh pre-warmed J774 infection medium.
24. On day 5 post-infection, remove all medium from flasks, and add 12 mL of fresh pre-warmed medium containing desired amount of drug to be tested as appropriate. Incubate in a 37°C, 5% CO_2 incubator for 24 h.
25. 24 h post drug addition, remove all medium from flask and add back 10 mL fresh pre-warmed J774 infection medium (with no drug).
26. Remove medium and repeat the addition of 10 mL fresh pre-warmed J774 infection medium (with no drug) (i.e., 2 washes).
27. Remove all medium and add back 5 mL PBS.

Note: Be sure to remove all the medium in the flask at this step prior to PBS addition. Use of a P1000 pipette to remove the last remaining medium is recommended.

28. Scrape cells off with a cell scraper, and transfer to a 15 mL conical tube for each T75 flask.
29. Pellet the cells by centrifugation at 3,200 × *g* for 10 min at 4°C.

Note: The centrifuge should be pre-chilled to 4°C.

30. Remove the supernatant and resuspend the cell pellet in 1 mL of pre-chilled extraction solvent system.

Note: The extraction solvent system should be pre-chilled at −20°C.

31. Transfer the sample to a 2 mL screw cap tube containing 500 μL of 0.1 mm zirconia/silica beads.

32. Store at −80°C at least 18 h before proceeding to drug extraction.

Pause point: The samples can be stored at −80°C for up to a month, unless previous experiments evidence instability of the drug in solution at −80°C.

Extraction of drug from Mtb-containing samples

Timing: 1.5–2 h

Samples are processed for extraction of drug.

33. Remove samples (from steps 13 and/or 32) from the −80°C freezer and thaw on ice.
34. Lyse bacterial or mixed bacterial/mammalian cell samples by bead beating (6 × 45 s, at 6.5 m/s), placing samples on ice in between each bead beating cycle.
35. After all bead beating cycles are complete, centrifuge samples at 16,600 × *g* for 10 min at 4°C.

Note: The microcentrifuge should be pre-chilled to 4°C.

36. For each sample, transfer 800 µL of supernatant to a 0.22 µm Spin-X centrifuge tube filter.
37. Centrifuge samples at 16,000 × *g*, 10 min, 4°C, or until all the sample is completely filtered.
38. Surface decontaminate sample tubes, using a method approved by the relevant institution's biosafety office and institutional biosafety committee (e.g., by wiping with 1:128 vesphene), and remove from the BSL-3. Store samples at −80°C until LC-MS analysis.

Pause point: The samples can be stored at −80°C for up to a month, unless previous experiments evidence instability of the drug in solution at −80°C.

Treatment of J774 cell cultures for intracellular drug accumulation measurement

Timing: 2–3 h, with a 24 h incubation step

J774 macrophage-like cells are seeded before treatment with the drugs/compounds being studied, for downstream sample extraction and analysis.

Note: Samples should be run in triplicate. Thus, each condition requires three wells to be set up.

39. Seed 1 × 10^6 J774 cells in 3 mL of J774 infection medium per well of a 6-well tissue culture plate. Incubate in a 37°C, 5% CO_2 incubator for 1 h.
40. After the 1 h incubation, add drug (typically 1000× in DMSO) directly to each well at desired final concentration. Incubate in a 37°C, 5% CO_2 incubator for 24 h.
41. 24 h post drug addition, remove medium from the wells and add back 1 mL pre-chilled PBS.
42. Remove PBS and add 1 mL pre-chilled PBS back to wells and repeat once more (i.e., 3 washes total).

Note: Be sure to remove all the medium in the wells at this step prior to PBS addition.

Note: PBS should be pre-chilled at 4°C.

⚠ CRITICAL: Pre-chilled PBS should be added to each well gently by pipetting gently down the side of each well to prevent perturbation of adhered cells prior to scraping.

43. Remove PBS, add 1 mL pre-chilled PBS, and incubate for 5 min at 4°C.

44. Scrape cells off with a cell scraper, and transfer sample to a 2 mL screw cap microcentrifuge tube for each well.
45. Pellet the cells by centrifugation at 400 × *g* for 5 min at 4°C.

Note: The microcentrifuge should be pre-chilled to 4°C.

46. Remove the supernatant and resuspend the cell pellet in 1 mL of pre-chilled extraction solvent system.

Note: The extraction solvent system should be pre-chilled at −20°C.

Pause point: If not proceeding directly to drug extraction, the samples can be stored at −80°C for up to a month, unless previous experiments evidence instability of the drug in solution at −80°C.

Extraction of drug from mammalian cell culture only samples

Timing: ~1 h

Mammalian cell culture only samples are processed for extraction of drug.

47. Freeze the samples from step 46 by placing on an ethanol/dry ice bath for 5 min.

Note: If samples have been frozen previously, proceed directly to step 48.

48. Move the tubes to 20°C–25°C water bath and incubate for 30 s.
49. Vortex the samples for 10 s at maximum speed.
50. Repeat steps 47–49 another four times (i.e., five total freeze/thaw cycles).
51. After all freeze/thaw cycles are complete, centrifuge samples at 16,000 × *g* for 10 min at 4°C.

Note: The microcentrifuge should be pre-chilled to 4°C.

52. For each sample, transfer 800 µL of supernatant to a 0.22 µm Spin-X centrifuge tube filter.
53. Centrifuge samples at 16,000 × *g*, 10 min, 4°C, or until all the sample is completely filtered.
54. Store samples at −80°C until LC-MS analysis.

Pause point: The samples can be stored at −80°C for up to a month, unless previous experiments evidence instability of the drug in solution at −80°C.

LC-MS analysis of extracted samples

Timing: 1 d

Separation, identification and quantification of intrabacterially/intracellularly accumulated drug by time-of-flight LC-MS.

55. Prepare the Agilent 1260 Infinity II liquid chromatography system coupled to an Agilent 6230 time-of-flight mass spectrometer or its equivalent LC-MS system.
    a. The elution is performed by using a water/acetonitrile solvent system, with each solvent containing 0.1% v/v formic acid.
    b. Connect the Chromolith SpeedRod column, or its equivalent, to the LC system.
    c. Set the gradient program as follows (expressed as the percentage of acetonitrile): 0–6.5 min, 10%–100%; 6.5–8 min, 100%; 8–11 min, 10%.

d. Set the flow rate to 0.5 mL/min.

56. Identify the mass ratio (m/z) value for drug and use the value to extract ion peak of drug.
57. For the quantification of the drug, first run authentic standard mixtures containing the drug and verapamil, the internal standard, and confirm that they produce a robust peak on the LC-MS system.
    a. Prepare the calibration curve on the same day to quantify the drug.
    b. Inject 20 μL of the diluted authentic standards with the internal standard (e.g., verapamil) at the following drug concentrations: 0.0125, 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, and 10 μM.
    c. The verapamil concentration is fixed at 500 ng/mL as 1 μL of verapamil (125 μg/mL DMSO stock solution) was added to 250 μL drug authentic standard solution.
    d. Extract the peak areas of the drug and verapamil in the extraction ion chromatograms of the diluted standards.
    e. Calculate the drug to the internal standard (D/I) peak area ratio.
58. Plot the ratio versus drug concentration calibration curve as y = mx, where this represents a linear regression of Drug/Internal standard (D/I, see quantification section below) peak area ratio as a function of drug concentration forced to a y-intercept of 0.

Note: If a non-linear calibration curve results, consult the literature for the appropriate mathematical fit to the data (e.g., https://www.intechopen.com/chapters/58596), while considering statistical tests for outliers that may result in the observed non-linearity. Or, if the non-linearity is due to the signal intensity saturation at higher concentrations, consider dilution of the higher concentrations by 10-fold.

59. Inject the extracted samples (20 μL) into the LC-MS. The verapamil concentration is fixed at 500 ng/mL as 1 μL of verapamil (125 μg/mL DMSO stock solution) was added to 250 μL extracted sample.
60. Calculate concentration of the drug in each sample using the calibration curve.

Note: The solvent gradient offered is fairly general for drug-like small molecules. It may be altered as necessary to properly separate the drug from other molecules in the cellular lysate.

EXPECTED OUTCOMES

This protocol facilitates the downstream identification and quantification of the drug under study within bacterial or mammalian cells. The mass chromatograms of drugs/compounds will be generated by the Agilent Masshunter Qualitative Analysis software, or its equivalent. The drug will be observed as a single peak at the specific retention time. For example, as shown in Figure 2, accumulation of C6 is clearly detected. As C6 concentration is increased, a dose-dependent accumulation in C6 may be observed. Further analyses of the kinetics of drug accumulation may be realized as dictated by the experiment.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data processing

LC-MS data are loaded into the Agilent Masshunter Qualitative Analysis software platform. First, open a sample folder to display a sample chromatogram. Multiple sample chromatograms can be loaded and displayed. Select extract chromatogram to extract peaks corresponding to the drug m/z. The peak area will be automatically integrated for quantification. The peaks can be re-integrated by manually drawing appropriate baselines on individual chromatograms. A representative mass chromatogram of the sample and the peak/peak area of drug accumulation is shown in Figure 2.

Quantification

A drug is identified based on its LC-MS peak with its associated retention time and m/z, while the drug accumulation level is calculated from the peak area in the mass chromatogram. Verapamil

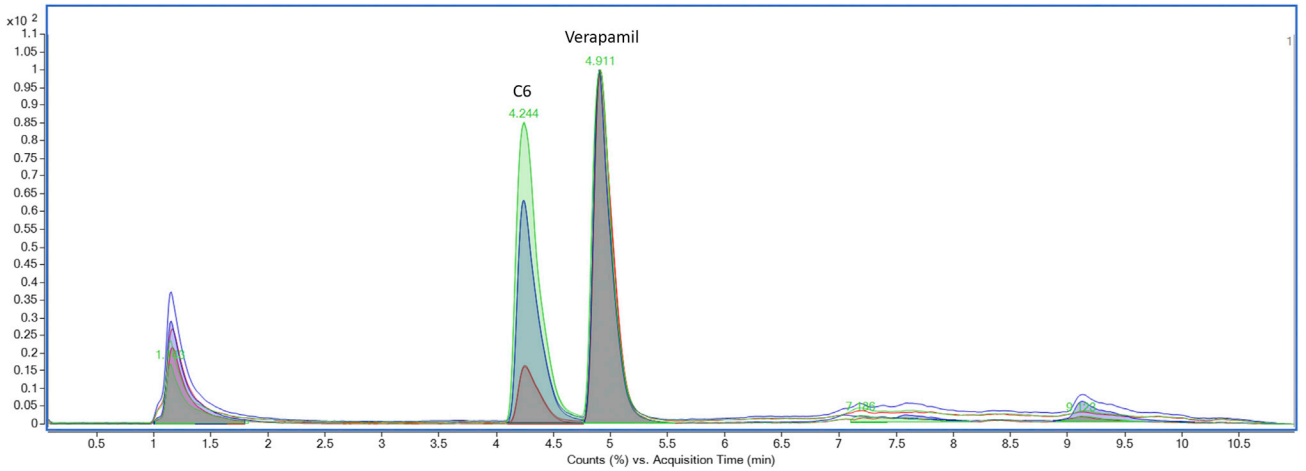

Figure 2. Representative mass chromatogram of C6 accumulation in the presence of verapamil, the internal standard

The retention times for C6 and verapamil are 4.2 min and 4.9 min, respectively. The C6 peak increases in a dose-dependent manner (following the change in the peak color), while the internal standard peak remains constant across doses. Created with MassHunter Qualitative Analysis (Version 8.0).

can be used as an internal standard, although other molecules, such as a stable isotopologue of the drug, may be chosen based on the experiment. These results, the peak areas of the accumulated drug (D), are normalized by the peak area of the internal standard (I) to afford the D/I ratio. The ratio may then be converted to a drug concentration using the slope of the drug calibration curve. The molar concentration of each sample can be further converted to the final yield, moles per number of cells, expressed as mol/1 × 10^6 cells by using the volume and cell count of the culture.

$$\text{Drug accumulation} = \frac{\frac{D}{I}\,\text{ratio}}{\text{Slope of the standard curve}} * \frac{\text{Volume of the culture}}{\text{Cell count of the culture}}$$

For example, treating cells with C6 for 24 h resulted in the accumulation of 25 pmol/1 × 10^6 cells in Mtb, 12 nmol/1 × 10^6 cells in J774, and 20 nmol/1 × 10^6 cells in Mtb infected J774 (scaled by number of J774 cells only). Alternatively, the mols of drug may be scaled by the bacterial cell culture density as measured by OD_{600}.

LIMITATIONS

The protocol is optimized to maintain a high recovery of C6 and its analogs (or compounds with similar physicochemical properties) using a C18 column. Testing other drugs/compounds may require re-optimization of LC-MS conditions and the use of a different column.

If a cellular metabolite or other endogenous analyte(s) has the same m/z and a similar retention time as the drug under study, a background peak will be present, which may need to be subtracted from the drug peak. In this case, when the accumulation level of the drug is not sufficiently high, it may be difficult to distinguish between the cellular metabolite and the drug.

TROUBLESHOOTING

Problem 1

No drug accumulation is detected in step 60.

Potential solution

The failure to observe quantifiable drug accumulation could be caused by incubating cells with too low of a drug concentration, or from insufficient cell lysis. While insufficient cell lysis may be unlikely, this may be checked using a positive control such as C6 (as detailed in this protocol). If the use of the positive control suggests insufficient cell lysis may be the issue, the number of bead beating or

freeze/thaw cycles for cell lysis may be increased. To improve the yield, the drug concentration may be increased. Alternatively, the cell lysate may be evaporated and then dissolved in a smaller volume (< 1 mL) of pre-chilled extraction solvent system to afford a more concentrated sample for LC-MS analysis.

If the drug polarity is very high, separation and detection of a drug peak using a C18 column may be difficult. Check if the authentic standard of the drug of interest is detectable on the LC-MS system and consider using a different type of column if it is not detectable. Alternatively, another LC-MS system affording a lower limit of detection, such as a triple quadrupole system, may be used.

If these alternatives fail to lead to an observation of quantifiable drug within the cell, the conclusion may be that the drug does not accumulate within the cell.

Problem 2

A detectable peak at the retention time of the drug in the no-drug treatment samples is observed in step 59.

Potential solution

Sample subtraction would need to be performed such that the ion count for the peak in the no-drug treatment sample (e.g., an average ion count with respect to the replicates) would need to be subtracted from each of the ion counts quantified in the drug-treated samples.

Problem 3

LC-MS accumulation results are not consistent among sample replicates in step 60.

Potential solution

This problem can be caused by inconsistent internal standard injection or sample extraction variability. Prepare vials containing 250 μL extraction solvent system including 1.0 μL of the internal standard. Then, inject 20 μL from each of the vials into the LC- MS system. Perform LC-MS test runs to check that a consistent peak area (e.g., ±10% deviation from the mean value of the internal standard peak area) among the runs is obtained.

Problem 4

The drug precipitates in steps 6, 24, or 40.

Potential solution

This problem may be addressed by decreasing the drug concentration incubated with the cells, while keeping in mind that one must be able to quantify drug (i.e., Problem 1).

Problem 5

The zirconia/silica beads can occasionally be carried over to the filtration in steps 37 and 53, and can tear the filter.

Potential solution

The contents of the Spin-X tube may be carefully transferred to another tube which may be spun down. The supernatant may be collected and then subjected to steps 37/53 once again.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Joel Freundlich (freundjs@rutgers.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze datasets/code.

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AUTHOR CONTRIBUTIONS

Conceptualization, Y.A., R.C.L., S.T., J.S.F.; Investigation, Y.A., R.C.L., S.T.; Funding acquisition, S.T., J.S.F.; Supervision, S.T., J.S.F.; Writing – original draft, Y.A., R.C.L., S.T., J.S.F.; Writing – review and editing, Y.A., R.C.L., S.T., J.S.F.

DECLARATION OF INTERESTS

S.T. and J.S.F. are listed as inventors on a patent filing related to compound C6, as employees of Tufts University and Rutgers University, respectively.

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