1. PROTOCOL FOR THE QUALITATIVE CONFIRMATION OF DRUGS BY LC/MS-QQQ

PURPOSE

The following method describes the qualitative confirmation of over 110 drugs and metabolites in blood, serum, plasma and urine by LC/MS-QQQ.

PLAN

A. Equipment:

(1) Ultivo LC/MS-QQQ with a Poroshell Phenyl Hexyl column (3x50mm)

(2) Sample concentrator with UHP Nitrogen

(3) Centrifuge

(4) 12x75 mm culture tubes

(5) 2 ml autosampler vials

(6) Vortexer/Multi-Vortexer

B. Reagents:

(1) **Deionized Water (DI Water)** Label. Stable until consumed.

(2) **Methanol.** Prepare a transfer bottle of ACS/HPLC grade methanol. Label accordingly. Store in glass at room temperature. Stable until consumed.

(3) **Ammonium formate buffer 0.01%**. Prepared by adding 0.36 g of ammonium formate and 100 µl of formic acid to 1.0 L deionized water. Stable until consumed.

(4) **Formic Acid (0.01%) in methanol**. Prepared by adding 100 µl of formic acid to 1.0 L methanol. Stable until consumed.

C. Standards: (Store refrigerated or frozen per manufacturer's recommendation).

(1) Purchase individual 100 µg/ml stock standards of the following (in methanol, acetonitrile, or other solvent depending on what is available from the manufacturers):

d3-Amitriptyline	*d3-Desipramine*	*d8-Norquetiapine*
d8-Aripiprazole	*d3-Diphenhydramine*	*d3-Promethazine*
d3-Buprenorphine	*d3-Doxepin*	*d4-Risperidone*
d3-Norbuprenorphine	*d5-Fentanyl*	*d6-Trazodone*
d10-Carbamazepine	*d5-Norfentanyl*	*d3-Trimipramine*
d7-Carisoprodol	*d10-Gabapentin*	*d4-Zaleplon*
d3-Clomipramine	*d3-LSD*	*d7-Zolpidem*
d3-Codeine	*d7-Meprobamate*	*d4-norZopiclone*
d3-Cyclobenzaprine	*d5-Naloxone*	

(2) Prepare or purchase individual 1.0 mg/ml or 100 μg/ml stock standards of the following (in methanol, acetonitrile, or other solvent depending on what is available from the manufacturers):

4-OH Xylazine	Chlorpromazine	Isotonitazene	Norchlorcyclizine	Ramelteon
6-β Naltrexol	Cinnarizine	Ketamine	Norcitalopram	Risperidone
7-OH-Quetiapine	Citalopram	Lacosamide	Norclomipramine	Scopolamine
9-OH-Risperidone	Clomipramine	Lamotrigine	Norcyclobenzaprine	Sertraline
Acetyl-fentanyl	Clonidine	Levetiracetam	Nordoxepin	Strychnine
Amitriptyline	Clozapine	Lidocaine	Norfentanyl	Suvorexant
Amoxapine	Cyclobenzaprine	Loxapine	Norfluoxetine	Tetrahydrozoline
Aripiprazole	Desipramine	LSD	Norketamine	Thioridazine
Atomoxetine	Dextromethorphan	m-CPP	Norquetiapine	Tizanidine
Atropine	Diphenhydramine	Meclizine	Nortrimipramine	Topiramate
Benztropine	Donepezil	Mephedrone	Nortriptyline	Trazodone
Brompheniramine	Doxepin	Meprobamate	Norzopiclone	Trimipramine
Bufotenine	Doxylamine	Mescaline	Olanzapine	Triprolidine
Buprenorphine	Duloxetine	Metaxalone	O-Norvenlafaxine	U47700
Bupropion	Etomidate	Methaqualone	Oxcarbazepine	Venlafaxine
Buspirone	Eutylone	Methocarbamol	para-Fluorofentanyl	Verapamil
Butorphanol	Fentanyl	Methotrimeprazine	Paroxetine	Vigabatrin
Butyryl fentanyl	Fluoxetine	Methylphenidate	Pheniramine	Xylazine
Carbamazepine	Gabapentin	Metonitazene	Phenytoin	Zaleplon
Carbinoxamine	Guaifenesin	Mirtazapine	Pregabalin	Ziprasidone
Carisoprodol	Haloperidol	Naloxone	Promethazine	Zolpidem
Cetirizine	Hydroxybupropion	Naltrexone	Psilocin	Zolpidem-Ph4-COOH
Chlorcyclizine	Hydroxyzine	NNDMT	Quetiapine	Zopiclone
Chlorpheniramine	Imipramine	Norbuprenorphine	Quinine	

D. Calibrators for BAM confirmation:

(1) **BAM Calibrator Stock Solution (0.5/5.0/25 ng/μl)**. Prepare by adding the appropriate amount of the standards from C.(2) to achieve the desired concentrations listed below to a 10 ml volumetric flask. Dilute to volume with methanol. Store frozen in glass. Stable for 2 years. *Note: Psilocin, Zopiclone and Norzopiclone should be grouped separately in acetonitrile due to instability observed in methanol (referred to as ACN stock in D.(2)). Compare to historic calibration before proceeding to next steps.*

(0.5 ng/µl)	(5.0 ng/µl)			(25 ng/µl)
4-OH Xylazine	6-B Naltrexol	Doxylamine	Nordoxepin	Carbamazepine
9-OH-Risperidone	7-OH-Quetiapine	Duloxetine	Norfluoxetine	Carisoprodol
Acetyl-fentanyl	Amitriptyline	Etomidate	Norketamine	Gabapentin
Atropine	Amoxapine	Eutylone	Norquetiapine	Lacosamide
Buprenorphine	Aripiprazole	Fluoxetine	Nortrimipramine	Levetiracetam
Butorphanol	Atomoxetine	Guaifenesin	Nortriptyline	Meprobamate
Butyryl fentanyl	Benztropine	Hydroxybupropion	Olanzapine	Metaxalone
Clonidine	Brompheniramine	Hydroxyzine	O-Norvenlafaxine	Methocarbamol
Fentanyl	Bufotenine	Imipramine	Paroxetine	Oxcarbazepine
Haloperidol	Bupropion	Ketamine	Pheniramine	Phenytoin
Isotonitazene	Buspirone	Lamotrigine	Promethazine	Pregabalin
LSD	Carbinoxamine	Lidocaine	Psilocin	Topiramate
Metonitazene	Cetirizine	Loxapine	Quetiapine	Vigabatrin
Naloxone	Chlorcyclizine	m-CPP	Quinine	
Naltrexone	Chlorpheniramine	Meclizine	Scopolamine	
Norbuprenorphine	Chlorpromazine	Mephedrone	Sertraline	
Norfentanyl	Cinnarizine	Mescaline	Suvorexant	
Norzopiclone	Citalopram	Methaqualone	Tetrahydrozoline	
para-Fluorofentanyl	Clomipramine	Methotrimeprazine	Thioridazine	
Ramelteon	Clozapine	Methylphenidate	Trazodone	
Risperidone	Cyclobenzaprine	Mirtazapine	Trimipramine	
Strychnine	Desipramine	NNDMT	Venlafaxine	
Tizanidine	Dextromethorphan	Norchlorcyclizine	Verapamil	
Triprolidine	Diphenhydramine	Norcitalopram	Ziprasidone	
U47700	Donepezil	Norclomipramine	Zolpidem	
Xylazine	Doxepin	Norcyclobenzaprine	Zolpidem-Ph4-COOH	
Zaleplon				
Zopiclone				

(2) **BAM Qualitative Confirmation Method Calibrator/QC Levels:** Prepare 5.0 ml calibrator stocks as needed per matrix below. Prepare levels 4, 5, & 6 first, and then levels 1,2, & 3 per matrix below. For simplicity the target concentrations are for the 10 ng/ml cutoff analytes (i.e. diphenhydramine). *Also prepare separate Level 1-6 ACN stocks with the appropriate drugs. Store refrigerated. Stable for 2 years.

	Level	Target conc	BAM 5 ng/µl (Cal stock *D.(1)*)	BAM cal	Methanol
	1	5 ng/ml		500 µl of #4	4.5 mL
CUTOFF	**2**	10 ng/ml		500 µl of #5	4.5 mL
	3	20 ng/ml		400 µl of #6	4.6 mL
	4	50 ng/ml	100 µl		4.9 mL
	5	100 ng/ml	200 µl		4.8 mL
	6	250 ng/ml	500 µl		4.5 mL

E. Internal Standards:

(1) **BAM Internal Standard (0.02/0.2/1.0 ng/µl)**. Prepare interim for 0.02 ng/µl by adding 50 µl of 100 µg/ml stocks of **D_3**-Buprenorphine, **D_3**-Norbuprenorphine, **D_5**-Fentanyl, **D_5**-Norfentanyl, **D_5**-Naloxone, **D_3**-LSD, **D_4**-Risperidone, **D_4**-Zaleplon, and **D_4**-Norzopiclone. Dilute to 1.0 ml with methanol. Add 100 µl of interim stock, 50 µl each of 100 µg/ml stocks of **D_3**-Amitriptyline, **D_8**-Aripiprazole, **D_6**-Trazodone, **D_3**-Clomipramine, **D_3**-Codeine, **D_3**-Cyclobenzaprine, **D_3**-Desipramine, **D_3**-Diphenhydramine, **D_3**-Doxepin, **D_3**-Promethazine, **D_3**-Trimipramine, **D_7**-Zolpidem, and **D_8**-Norquetiapine; 250 µl each of 100 µg/ml stocks of **D_{10}**-Carbamazepine, **D_{10}**-Gabapentin, **D_7**-Carisoprodol, **D_7**-Meprobamate. Dilute to 25 ml volume with methanol. Store refrigerated in glass. Stable for 2 years.

F. Quality Controls:

(1) **Positive Controls.** For qualitative confirmation of drugs on the BAMconf method matrix-based calibration Levels 1 thru 6 will be utilized (0.5/5.0/25, 1.0/10/50, 2.0/20/100, 5.0/50/250, 10/100/500, and 25/250/1250 ng/ml) as calibrators/controls.

(2) **Performance Standard (*optional*)**. Prepare an unextracted performance standard to check retention times and transition ratios on day of use. For example add 50 µl of the Level 5 calibrator stock (and 50 µl of the Level 5 ACN stock) to autosampler vial with 50 µl of internal standard, dry under nitrogen to approximately 50 µl, then add 500 µl of water, vortex.

(3) **Negative Blood Control**. Blank blood prepared in house consisting of 50% saline, 50% packed red blood cells, and 5g sodium fluoride/1g potassium oxalate (per 500 ml prepared blood) will be used as negative control.

(4) **Negative Urine Control**. Urine produced in house will be used as negative control.

G. Sample Preparation (blood or urine)

(1) Prepare a set of calibrators using 50 µl of the Level 1-6 calibrator stock solutions (and 50 µl corresponding ACN stock solutions) respectively in 100 µl of blank blood or urine (matrix match with samples in batch). Prepare negative control (matrix match with samples in batch), and blood/urine case samples using 100 µl each into labeled culture tubes.

(a) Add 50 µl of internal standard

(b) Add methanol needed to arrive at 500 µl total methanol volume* and vortex until thoroughly mixed. *Note: The total volume of methanol should be 500 µl, so if adding the stock cal and internal standard, then only 400 µl of additional methanol is needed (exclude ACN volume).

(c) Centrifuge for 5 minutes at 3500 rpm

(d) Transfer 200 µl of the supernatant to an autosampler vial and dry under nitrogen until approx. 50 µl remaining

(e) Add 500 µl of water, cap and vortex

H. Data Acquisition and Analysis:

(1) Perform Autotune if not previously done (weekly). Attach tuning solution (stored in refrigerator), ensure approximately 20mL volume in container prior to attaching. Load the Pretune.m method, this will ensure the correct temperatures and flows are set. At the top of the screen hover over the QQQ box and turn it on. Wait until the box is green, approximately 20 min. At the bottom left of the screen, under method editor, find and click on “Autotune”; click the key icon (Request tune control) and then select autotune icon, do not select checktune. The tune should take about 20 minutes. If the tune fails, try again and/or sonicate the nebulizer. Electronically save all Autotunes.

(2) Purge the LC pump lines of any bubbles, primarily check the methanol gradient line. This can be done with the pretune.m method loaded as the gradient is 50:50 water:methanol. At equilibrium the pump pressure should reside near 80-100psi. Binary ripple should not exceed 1%. Let LC stabilize before analysis.

(3) Perform needle flush/rinse. At the top left of the screen, find sampler box. Right-click and select needle wash, type in 20 seconds and make sure there is enough rinse solution (methanol) in the wash container.

(4) Prepare a sequence beginning with two blanks (to stabilize LC column flow), followed by Level 1-6 calibrators/positive controls, negative control(s), and case samples. Case samples will be preceded by blanks, preceding urine samples the injected blank will be 50% DI H_2O and 50% methanol. Reinject Level 5 at end of batch to bracket samples and monitor performance throughout the batch. Load samples onto autosampler according to sequence and have it verified by another analyst before or after analysis but prior to unloading.

(5) The transitions are fixed and derived from Method Optimizer. If needed the retention times and transition ratios can be set by an unextracted standard or calibrator. Retention times should be checked and/or set prior to running multiple samples.

(6) Analyze using BAMconf.m on either Ultivo LC/MS-QQQ instruments.

I. Confirmation Results and Acceptability:

(1) Calibration fit is set to quadratic with 1/x weighing

(2) A sample is positive if ≥ cutoff (Level 2) or lowest valid calibrator/control level that is ≥ Level 2 (i.e. validity of controls determined by retention time criteria, transition ratio criteria, concentration within 20% of target)

(3) Retention time within 0.1 min (i.e. -0.05 to +0.05 min) as stored or set from an unextracted standard or calibrator

(4) Chromatographically acceptable i.e. peak purity ≥90%, for primary transition

(5) Transition ratios for target analytes and assigned internal standards are within tolerances (based on table below) as stored or set from an unextracted standard or calibrator

Relative Intensity (qualifier to primary transition)	Tolerances
Greater than 50%	± 20%
20-50%	
10-20%	
Less than 10%	± 30%

(6) Blank prior to sample < 25% area count of cutoff calibrator (Level 2), with the exception of high-level concentrations in a sample (i.e. > calibration range) potential carryover area count in blank needs to be < 5% as compared to the area count in the case sample

(7) Negative control < 25% of area count of cutoff calibrator (Level 2)

(8) Diluted samples may be reported if the raw result is < cutoff (Level 2) but ≥ Level 1 and all criteria for Level 1 and sample are met.

J. Method Limitations:

(1) Zopiclone, Norzopiclone, and Psilocin are prone to degradation in the calibrator stock and extract.

(2) Carryover is possible primarily with urine samples due to high concentrations. In addition to monitoring solvent blanks, be cognizant of drug/analyte presence in samples analyzed after high concentration samples. Re-analyze at end of batch if carryover is suspected and sample result > cutoff value for same analyte.

(3) Vigabatrin will not be reported in urine or blood samples due to significant ion suppression. Psilocin will not be reported in blood samples due to degradation in blood (further research needed). Bufotenine will not be reported in urine or blood samples due to potential for endogenous levels (further research needed).

(4) Dextromethorphan will be reported as Methorphan.

(5) See TOX-SOP-66 for potential drug->metabolite breakdown/conversions

(6) Since this a qualitative confirmation with 6 levels of calibrators, it is acceptable to drop one calibrator per analyte with approval from Technical Lead.

(7) Trace amounts of certain analytes may not be considered positive in the presence of high concentrations of structurally similar analytes for example:

(a) Amitriptyline and Cyclobenzaprine

(b) Nortriptyline and Norcyclobenzaprine

(c) Chlorpheniramine and Brompheniramine

(d) Imipramine and Clomipramine

(8) Exceptions to Cutoff:

BLOOD	ng/mL	URINE	ng/mL
Buprenorphine	5.0	Buprenorphine	5.0
Butorphanol	5.0	Butorphanol	2.0
Chlorpromazine	20	Hydroxybupropion	20
Clonidine	2.0	Naloxone	2.0
Norbuprenorphine	5.0	Norbuprenorphine	5.0
Norfluoxetine	100	Norfluoxetine	100
Norzopiclone	2.0	Tizanidine	5.0
Olanzapine	50	Zaleplon	5.0
Tizanidine	10		
Zaleplon	2.0		