1. PROTOCOL FOR THE SCREENING AND CONFIRMATION OF DRUGS IN BLOOD AND URINE BY SPE AND LCMS-QQQ

PURPOSE

The following method describes the qualitative screening and quantitative confirmation of drugs in blood, urine, and other biological samples with analysis by LC/MS-QQQ.

PLAN

A. Equipment:

(1) LC/MS-QQQ with a C18 column

(2) Positive Pressure Manifold

(3) SPE Column – Silica gel - Co polymeric bonded phase with a hydrophobic cation exchange (CSDAU203)

(4) Sample concentrator with UHP Nitrogen

(5) Centrifuge

(6) Vortex mixer / Multi-tube vortex mixer

B. Reagents:

(1) **100 mM Phosphate buffer solution**. Dissolve 1.70 grams of Na_2HPO_4 and 12.14 g $NaH_2PO_4 \cdot H_2O$ in 800 ml of deionized water. Dilute to 1000 ml with deionized water. Mix well. pH should be 5.5-6.0. If necessary, adjust with 100 mM monobasic sodium phosphate (lowers pH) or 100 mM dibasic sodium phosphate (raises pH). Store refrigerated. Stable for six months.

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

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

(4) **100 mM Hydrochloric Acid (HCL)**. To 400 ml of deionized water, add 8.4 ml concentrated HCl. Dilute to 1 L with deionized water. Mix well. Stable for 2 years.

(5) **78:20:2 methylene chloride: isopropanol: ammonium hydroxide Elution Solvent**. Prepare fresh daily. Add ammonium hydroxide to isopropanol, followed by methylene chloride (i.e. per 10 mL of elution solvent add approximately 200 µL ammonium hydroxide). Mix thoroughly, elution solvent should have a turbid appearance when thoroughly mixed.

(6) **Ethyl Acetate**. Prepare a transfer bottle of ACS/HPLC grade ethyl acetate. Label accordingly. Store in glass at room temperature. Stable until consumed.

(7) **Hexane**. Prepare a transfer bottle of ACS/HPLC grade ethyl acetate. Label accordingly. Store in glass at room temperature. Stable until consumed.

(8) **Hexane/Ethyl Acetate (50:50).** Prepare fresh daily.

(9) **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.

(10) **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)

(1) **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):**

Buprenorphine	Butorphanol	Fentanyl
Haloperidol	Norbuprenorphine	Naloxone
Naltrexone	para-Fluorofentanyl	Norfentanyl
Zopiclone	Risperidone	9-Hydroxyrisperidone
Zaleplon	Zolpidem	Ziprasidone
Aripiprazole	Quetiapine	Norquetiapine
6-β Naltrexol	Trazodone	Acetyl Fentanyl
7-Hydroxyquetiapine	Ramelteon	Pregabalin
Butyryl Fentanyl	Gabapentin	Desomorphine
Tramadol	O-Desmethyltramadol	U47700
Mitragynine	7-Hydroxymitragynine	

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

D_4-Buprenorphine	**D_5**-Fentanyl	**D_5**-Norfentanyl
D_5-Naloxone	**D_3**-Norbuprenorphine	**D_4**-Risperidone
D_8-Aripiprazole	**D_7**-Zolpidem	**D_4**-Zaleplon

D. Calibrators and Internal Standards:

(1) **Calibrator Stock Solution (0.1/1.0/10 ng/µl)**. Prepare by adding the appropriate amount of the standards from C1 to achieve the desired concetrations listed below to a 10 ml volumetric flask. Dilute to volume with methanol. Store refrigerated in glass. Stable for 1 year. (*screening method only)

(0.1 ng/µl)	**(1.0 ng/µl)**	**(10 ng/µl)**
Buprenorphine	Norbuprenorphine	Ziprasidone
Butorphanol	Naloxone	Aripiprazole
Fentanyl	Naltrexone	Zolpidem
Haloperidol	Zopiclone	6-β Naltrexol
Norfentanyl	Risperidone	Quetiapine
Acetyl Fentanyl	9-Hydroxyrisperidone	Norquetiapine
Butyryl Fentanyl	Zaleplon	7-Hydroxyquetiapine
*para-Fluorofentanyl	Ramelteon	Trazodone
	*Desomorphine	*Gabapentin
	*Mitragynine	*Pregabalin
	*7-Hydroxymitragynine	*Tramadol
	*U47700	*O-Desmethyltramadol

(2) **Internal Standard (0.1/0.5/2.0 ng/µl)**. Prepare by adding 25 µl of a 100 µg/ml stock of **D_4**-Buprenorphine, **D_5**-Fentanyl, and **D_5**-Norfentanyl; 150 µl of a 100 µg/ml stock of **D_5**-Naloxone, **D_3**-Norbuprenorphine, **D_4**-Zaleplon, and **D_4**-Risperidone; and 500 µl of a 100 µg/ml stock of **D_8**-Aripiprazole and **D_7**-Zolpidem to a 25 ml volumetric flask. Dilute to volume with methanol. (May also prepare alternate stock volume by adjusting volumes accordingly to account for equivalent concentrations of each analyte.) Store refrigerated in glass. Stable for 1 year.

E. Quality Controls: (Store Refrigerated)

(1) **Positive Control Stock Solutions**. Prepare by adding the appropriate amount of the standards from C1 to achieve the desired concetrations listed below to 3 separate 10 ml volumetric flasks. Dilute to volume with methanol. Store refrigerated in glass. Stable for 2 years. (*screening method only) When available a secondary source will be used for preparation of controls.

Low (ng/µl) (0.004/0.016/0.08)	**Med (ng/µl) (0.004/0.16/0.8)**	**Hi (ng/µl) (0.4/1.6/8.0)**
Buprenorphine	Norbuprenorphine	Ziprasidone
Butorphanol	Naloxone	Aripiprazole
Fentanyl	Naltrexone	Zolpidem
Haloperidol	Zopiclone	6-β Naltrexol
Norfentanyl	Risperidone	Quetiapine
Acetyl Fentanyl	9-Hydroxyrisperidone	Norquetiapine
Butyryl Fentanyl	Zaleplon	7-Hydroxyquetiapine
*para-Fluorofentanyl	Ramelteon	Trazodone
	*Desomorphine	*Gabapentin
	*Mitragynine	*Pregabalin
	*7-Hydroxymitragynine	*Tramadol
	*U47700	*O-Desmethyltramadol

(2) **Positive Control (*Screening*)**. Prepare on day of use at concentration of 0.20/2.0/20 ng/ml by adding 50 µl of the low control stock to 1 ml blood.

(3) **Positive Controls (*Confirmation*)**. Prepare on day of use Low (0.20/2.0/20 ng/mL), Med (0.80/8.0/80 ng/ml), and Hi (4.0/40/400 ng/ml) controls by adding 50 µl of each stock solution (see E1 above) to 1 ml of blood. Additional controls shall be prepared, when appropriate, to coincide with any limited sample volumes and/ or dilution of case samples.

(4) Performance Standard (*Optional*): Prepare an unextracted performance standard to check retention times and transition ratios on day of use. For example to prepare at 0.1/1.0/10 ng/µl, make an interim stock by adding 10 µl of the 0.1/1.0/10 ng/µl calibrator stock to an autosampler vial and diluting to 100 µl with methanol. Add 10 µl of the interim stock, 50 µl internal standard, 40 µl methanol and 100 µl H_2O to an autosampler vial, cap and vortex. (Alternatively, can use an extracted calibrator or positive control.)

(5) **Negative 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.

F. Solid Phase Extraction (SPE)

(1) ***Sample Preparation***
Prepare in appropriately labeled culture tubes as follows:

(a) 1 ml* of negative control, positive control(s), case samples, and for confirmation a set of calibrators at 0.1/1.0/10, 0.25/2.5/25, 0.5/5.0/50, 1.0/10/100, 2.5/25/250, and 5.0/50/500 ng/ml from calibrator stock solution above each in 1 ml of blank blood. Add 50 µl* of internal standard.

*In case samples where a limited amount of sample is received use the same fraction of internal standard as the sample, as an example ½ ml of blood and 25 µl of internal standard.

(b) Add 1.5 ml DI water and vortex until thoroughly mixed

(c) Add 1 ml of 100 mM phosphate buffer and vortex until thoroughly mixed

(d) Centrifuge for 5 minutes at 3500 rpm

(2) ***Column Conditioning***
Pass through the column sequentially the following reagents at <1.0 ml/min, or gravity only:

(a) 2 ml of methanol

(b) 2 ml of deionized water

(c) 1 ml of 100 mM phosphate buffer

Take care to prevent sorbent from drying out.

(3) ***Sample Application***
Apply sample to column, being careful to not allow the sediment, if present, which will be in the base of the centrifuge tube to pass. Flow rate should be 1-2 ml/minute.

(4) ***Column Rinse***
Pass through the column sequentially the following reagents, at 1-2 ml/min:

(a) 3 ml of deionized water

(b) 1 ml of 100 mM HCl

(c) Dry column under full pressure for (≥15 inches Hg) for 10 minutes

(d) 1 ml of hexane

(e) 2 ml of 50:50 hexane/ethyl acetate

(f) 250 µl methanol

(5) ***Elution***

(a) Elute by gravity with 500 µl methanol

(b) Dry column under full pressure for (≥15 inches Hg) for 5 minutes

(c) Elute drugs by gravity using 1.5 ml of 78:20:2 methylene chloride: isopropanol: ammonium hydroxide elution solvent

(d) Evaporate eluate in microvials to dryness under nitrogen

(e) Reconstitute with 100 µl of methanol and 100 µl of H_2O, vortex and cap

G. Data Acquisition and Analysis:

(1) Perform Autotune or checktune if not previously done (weekly)

(2) Purge LC pump lines of any bubbles. At equilibrium the pump pressure should reside near 120-130psi. Binary ripple should not exceed 1%. Let LC stabilize before analysis.

(3) Perform needle rinse

(4) The trays for samples are P1 in front and P2 in back. They have rows a-f, columns 1-9. Run a performance standard to check retention time(*optional*). Prepare a sequence beginning with two blanks (to stabilize LC column flow) followed by the calibrators (for confirmations), negative control, positive control(s) and case samples. For a screening batch place controls at the beginning, for a confirmation batch positive controls should be placed throughout the batch, i.e. beginning, mid-run and end of run. LC solvent blanks should precede case samples. For samples requiring a dilution add the appropriate sample multiplier in the sequence table. 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. Retention times and transition ratios can be set by an unextracted standard or calibrator. Retention times should be set prior to running multiple samples.

(5) Analyze using the appropriate method on LC/MS-QQQ

H. Results and acceptability:

(1) ***Screening***

(a) Calibration should be updated at a minimum once per year

(b) Identification of analyte in positive control

(c) Negative control < 25% of cutoff calibrator

(d) Retention time within 5% as stored or set from an unextracted standard or calibrator

(e) Transition ratios within 20% as stored or set from an unextracted standard or calibrator

(f) Quantitation ≥ 0.1/1.0/10 ng/ml

(2) ***Confirmation***

(a) Calibration R≥0.99 and calibrators within 20% of set value

(b) Positive controls within 20% of target concentration

(c) If the above two criteria are not met the analyte may be reported qualitatively

(d) Negative control < 25% of area count of cutoff calibrator

(e) Retention time within 5% as stored or set from an unextracted standard or calibrator

(f) Transition ratios within 20% as stored or set from an unextracted standard or calibrator

(g) Chromatographically acceptable i.e. peak purity ≥90%

(h) Blank prior to sample < 25% area count of cutoff calibrator.

(i) Quantitation ≥ lowest calibrator concentration

(j) Results will be truncated and documented in case notes to two significant figures