METHOD EVALUATION CONFIRMATION FOR DRUGS OF ABUSE BY TURBOFLOW LCMS

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REASON FOR EVALUATION

The first stage of a routine drug screen is to test by immunoassay for different classes of drugs. This broad spectrum screen will detect a range of classes of drugs, but will not specifically identify them. A second, more specific method is used to confirm the presence of the drug, and also to classify the opiates or amfetamine class substances present. The existing method is based on an online SPE extraction method that has been in use for over a year in the Toxicology Unit, but it has shortfalls, including poor sensitivity of morphine-glucuronide, a major analyte, and being qualitative only. The Turboflow method is more sensitive and reproducible for morphine glucuronide, amongst other analytes. This will increase confidence in the results, reduce the turnaround times, increase the range of drugs that we can confirm and eventually increase the repertoire of the laboratory.

PROCEDURE DETAILS

975 µL urine and 25 µL internal standard are added to a 2mL HPLC vial. 100 µL is injected directly onto the Turboflow system. Turboflow technology is based on a mixed anion exchange/reversed phase column which also operates in a size exclusion mode. Proteins and salts are washed off the column at high flow rates, leaving analytes bound to the solid phase. These are then eluted with a pH shift onto a 100x2.1mm 5µm PFP Gold HPLC column running a gradient of acetonitrile and water with 0.1% formic acid. The eluant is then introduced into the ion trap mass spectrometer. While the gradient runs, the Turboflow column is cleaned and regenerated ready for the subsequent sample.

In each cycle of the analysis, a full scan is obtained to identify the parent masses present. These masses are compared to a mass list, and if a defined amount of a specific mass is present within a specified time window, a MS2 scan is triggered. This MS2 scan will produce a product ion scan of the drug, which can be compared to a standard library to confirm the presence of the drug. Following this scan, the five most intense ions in the full scan are fragmented in the ion trap to produce MS2 scans. If any of these scans produces an ion that corresponds to a loss of a glucuronide moiety (176 mass units) or sulphate group (80 mass units), an MS3 scan is performed on the daughter ion. All scans may be compared to the MS spectral library to identify the drugs and metabolites present. The final scan event in the sequence is a defined MS2 scan which is designed to identify specific and/or low intensity ions which might otherwise be missed.

The analytical run is split into 2 time segments. In Segment 1 (0 – 4 minutes), the final scan event looks specifically for morphine glucuronide. In segment 2 (4 minutes – end of run), scan event 7 looks for 6-monoacetylmorphine, and scan even 8 looks for 6-acetylcodeine. Further segments may be added in the future if there are specific or low intensity ions that need to be reliably identified.

Any drug detected in a scan will then be placed on a reject list, meaning that it will be excluded from subsequent MS2 scans for 2.5 seconds. This is more than twice the duration of a scan sequence, so that a minimum of 3 overlapping peaks may be simultaneously detected.

On completion of the analysis, the MS2 spectra are compared with library spectra and the configuration file, and if they give an acceptable match and the retention time is similar, the drug is determined to be present.

For more information on TurboFlow technology, see reference:

Chassaing C, Robinson S., Turbulent Flow Chromatograhpy: an Evolving Solution for Bioanalysis. *Chrom Today*. 2009: 2; 20-24

SCOPE

This evaluation covers the analytes currently detected by on-line SPE and Ion Trap LC-MS, listed below. Many of these drugs and metabolites will be only occasionally identified, and it is important that a positive control is periodically analysed to ensure that the retention times have not drifted outside the identification window.

Qualitative	
Morphine	Dihydrocodeine glucuronide
Morphine Glucuronide	Codeine
Codeine Glucuronide	Methadone
6-Monoacetylmorphine	EDDP
Dihydrocodeine	Amfetamine
Benzoylecgonine	Metamfetamine
Acetylcodeine	Hydroxypapaverine glucuronide
Ketamine	Dihydroxypapaverine glucuronide
MDMA (Ecstasy)	Pholcodine
Hydroxypapaverine	Nortriptyline
Dihydroxypapaverine	Noscapine

SAMPLE REQUIREMENTS

This method has been validated for urine samples, for which 1mL of sample is needed. Smaller volumes can be used, although the quantity of internal standard may need to be adjusted.

RISK ASSESSMENTS

WORK ACTIVITY RISK ASSESSMENT

A risk assessment for the LCMS, the HPLC and the complete procedure has already been performed. [HR-CB-TOX-LCQFLEET], [HR-CB-TOX-JASCO], [HR-CB-TOX-UDS]

COSHH RISK ASSESSMENT

Substance	Handling requirements	Risk Rating
Acetonitrile	Gloves, goggles and lab coat must be worn when handling acetonitrile. Acetonitrile is flammable and only working quantities of solvent should be stored on the LCMS.	L

	Solvent must be prepared in the fume cupboard	
Formic Acid	Formic acid is corrosive and gloves, goggles and lab coat must be worn when preparing the HPLC solvents.	L
Ammonia solution		L
Ammonium Formate	Irritant to eyes. Goggles must be worn when handling ammonium formate or aqueous HPLC eluant	L
Calibrators & QC	Treat as infectious samples. Gloves must be worn when handling these materials	L
Helium Cylinder	Helium is non-hazardous when used as directed. The helium cylinder is heavy and should be considered a manual handling risk.	L

ASSESSMENT OF STAFF EXPOSURE:
RISK RATING: Low.

COMPARISON OF RESULTS

Patient sample results from the proposed method were compared with results from samples analysed by the existing online SPE LCMS method.

All results are qualitative by SPE extraction and so cannot be processed using a Deming regression. Direct comparison of patient results shows the following:

	Morphine Gluc	Codeine	Methadone	Benzoylecgonine	Amfetamine
True POS	80	23	71	39	1
True NEG	20	77	29	61	99
False POS	1	11	9	1	1
False NEG	3	0	1	5	0
SENSITIVITY	96.39 %	100 %	98.61 %	88.64 %	50 %
SPECIFICITY	95.24 %	87.50 %	76.32 %	98.39 %	99 %

The results of the SPE method are taken as "TRUE" and Turboflow LCMS results are compared to these.

$$\text{Sensitivity} = \frac{\text{True Positives}}{(\text{True POS} + \text{False NEG})}.$$

$$\text{Specificity} = \frac{\text{True Negatives}}{(\text{True NEG} + \text{False POS})}.$$

The anomalous results have been investigated, with the following results.

The high number of false positives for codeine and methadone are caused by the fact that the SPE method was not very sensitive for these analytes. Therefore whilst it

shows as a false positive, in fact they were all genuine positives that the SPE method did not detect.

The few false negatives that have been found were likely caused by the analytes being present at such low concentrations that they were below the cutoff and would not have been reported as positives even with the SPE method.

INTRA ASSAY PRECISION

Intra assay precision was measured for three of the most commonly seen analytes in a C4 QC. Intra assay precision was acceptable, see below:

Sample	Codeine	Morphine Glucuronide	Benzoylecgonine
1	3177	719	1199
2	2683	674	985
3	2615	580	1236
4	3177	612	999
5	3426	553	1226
6	2793	587	1017
7	2627	556	1027
8	2410	638	745
Mean	**2863.50**	**614.88**	**1054.25**
SD	**353.32**	**58.76**	**164.36**
% CV	**12.34**	**9.56**	**15.59**

INTER ASSAY PRECISION

Inter assay precision was measured for three of the most commonly seen analytes in a C4 QC. Inter assay precision was acceptable, see below:

Sample	Codeine	Morphine Glucuronide	Benzoylecgonine
1	1175	399	423
2	1199	501	305
3	1169	606	410
4	1237	550	298
5	1358	390	353
6	1575	517	312
7	1138	474	331
8	1127	604	349
Mean	**1247.25**	**505.13**	**347.63**
SD	**151.12**	**82.47**	**46.95**
% CV	**12.12**	**16.33**	**13.50**

LINEARITY

MS2 data can not be used to assess concentration, as above a threshold the trap admits fewer ions. This mechanism (automatic gain control or AGC) is intended to maintain mass resolution, but reduces the counts for concentrated analytes.

INTERFERENCE STUDIES

The combination of chromatography and spectrum matching should remove most interferences. Where possible, spectra have been produced using a collision energy optimised to retain the parent ion at a lower intensity than the most intense daughter ion. MS3 spectra are also used to increase the specificity of detected analytes.
One of the most significant sources of interference in LCMS is ion suppression.

DETECTION AND QUANTITATION LIMITS

External quality control material is used as a cutoff for this assay, see table below for concentration of analytes in cutoff. The method is qualitative only therefore it is not possible to state a limit of detection. The cutoff is analysed at the beginning and the end of each batch, if a patient sample is present at an intensity higher than the cutoff then it can be reported as positive, otherwise it is to be reported as negative even if a small amount is present.

Analyte	**Concentration (ng/mL)**
3,4-Methylenedioxymethamfetamine (MDMA)	94
d-Amfetamine	500
d-Methamfetamine	500
Benzoylecgonine	150
Methadone	300
Codeine	938
Morphine-3-β-D-glucuronide	938

CARRY OVER/CARRY UNDER

Patient samples with low and high values were analysed in the following order: LLLHHHLLL. The mean of the three results was calculated and the individual differences from the mean calculated. Where carry over is present, the first result for the second set of the low samples will be higher than for those following, and higher than the mean for the first set.
Where carry under is present, the first result for the high samples will be lower than those of the following high samples.
Benzoylecgonine

	Result	Mean	% Difference
L	123	142.33	-13.58
L	168	142.33	18.04
L	136	142.33	-4.45
H	1226	1043.33	17.51
H	905	1043.33	-13.26
H	999	1043.33	-4.25
L	78	93.00	-16.13

L	99	93.00	6.45
L	102	93.00	9.68

Amfetamine

	Result	Mean	% Difference
L	219	233.00	-6.01
L	237	233.00	1.72
L	243	233.00	4.29
H	751	713.67	5.23
H	676	713.67	-5.28
H	714	713.67	0.05
L	295	307.67	-4.12
L	349	307.67	13.43
L	279	307.67	-9.32

Carry over and carry under data were acceptable.

REAGENT/SAMPLE STABILITY

HPLC solvents appear to be stable for at least 1 month, and workload should ensure that solvent throughput is faster than this. QC samples are supplied liquid stable, and each open bottle should last 30 days, or until the use by date on the pack.
Calibration and internal standards should be made up fresh at least once / month, and stock solutions should be stable for at least 6 months when stored in the fridge.
Samples are stable for these analytes for at least 2 weeks when kept at 4ºC and for several months when frozen.

QUALITY CONTROL

Three quality control samples from Bio-Rad will be used in this assay; C2, C3 (low opiate) and C4. Each QC contains different concentrations of a range of analytes that we are interested in, so that we have low and high QCs for the major analytes. A negative QC from Bio-Rad is also to be included with each batch.

REFERENCE RANGE

There is no reference range with this test. Results are reported as either Positive or Negative.

STAFF TRAINING

Staff using the LCMS should be familiar with the principles of drug testing and will understand the differences between screening and confirmation assays. There is no further training required beyond existing LC-MS operation training and an overview of the principles of Turboflow. Beyond this, it is expected that training will be through routine rotational training.

COSTS

Bottom up costs suggest that the cost of a batch of 20 samples will be approximately £5.23. The current workload is around 20-30 samples/day. This is based on a new HPLC column and SPE cartridge being required every month. These are conservative figures, and an HPLC Column will probably last significantly longer, up to approximately 1000 samples.
The current bottom up cost of opiate confirmations is approximately £5.10, suggesting that no change in pricing is required.
A full drug screen, including confirmation, currently costs £20.

COMPUTER CHANGES

The existing test code (OPIC) will be kept, and no changes will be required.

IDENTIFICATION OF OTHER DRUGS

The ion trap mass spectrometer can be used to identify unknown drugs in patient samples. A full formal evaluation may not be appropriate for all drugs, but it is important that an evaluation is performed to ensure correct sample identification. It is intended that the following guidelines are used when identifying other drugs.

A full evaluation must be done for drugs that are to be reported in all patients (eg cannabis, benzodiazepines). For less common drugs, one of the following cases must apply:

- If a drug is easily obtainable commercially, this is analysed as a patient sample and a library spectrum and retention time are recorded.
- If a drug or metabolite is not easily obtainable, but there is a sample that is known positive for this drug (eg control, NEQAS sample, patient known to be taking drug), the drug should be identified from a clear peak in an extracted ion chromatogram (XIC), together with an MS2 spectrum that is a good match with a stored library spectrum.
- Where there is no known positive sample as a comparison, an identification may be made with a clear XIC peak (S/N >10) **and** a very good library match (SI >600 & RSI >700) **and** a >90% probability that this is the correct identification (from the library search window). There should also be some other supporting evidence, such as suspicion of use, an MS3 spectrum matching a theoretical or published spectrum or a similar retention time to a related compound. It is NOT intended that this procedure should be used for routine "unknown drug screening".

NEW/ALTERED PROCEDURE ACCEPTANCE FORM
Please ensure the procedure acceptance form is fully completed and signed.

Procedure Title: Opiate / Amfetamine Confirmation

Brief description of procedure:
Urine samples are extracted by online SPE and drugs separated by HPLC. Detection is by ion-trap MS with spectral library matching of drugs.

Is this procedure:
☐ a new assay? ☐ new equipment? ☑ a modified procedure?

Evaluation Checklist

Tick the boxes when the following have been completed or are not applicable (N/A):

Yes	N/A	
☑	☐	Work activity risk assessment
☑	☐	COSHH risk assessments
☑	☐	Comparison of results
☑	☐	Intra assay imprecision
☑	☐	Inter assay imprecision
☐	☑	Linearity
☑	☐	Interference studies (list): Ion suppression, mebeverine and quinine
☑	☐	Sensitivity
☑	☐	Carry over
☑	☐	Reagent stability
☑	☐	Sample stability
☑	☐	External Quality Assurance samples (name scheme): Heathcontrol Drugs of Abuse scheme

Tick the boxes below when the following have been completed:

☐Yes	☑ N/A	Reference range established
☑		Procedure/ instructions written, tested, accepted
☑		Staff trained
☑Yes	☐ N/A	Participation in External Quality Assurance scheme arranged (name): Heathcontrol Drugs of Abuse Scheme
☑		Computer changes completed
☑		Changes made to other laboratory documentation (list): SOP Instrument SOP Training Logs

Note any comments related to the new procedure (which are not covered above) below:

New Procedure Acceptance

Procedure Title: Opiate and Amphetamine confirmations by LCMS

Name of person introducing new procedure:
Michelle Birch
Grade :
Senior Toxicologist
Date:

Signature:……

Date Ratified at Executive meeting:

Proposed start date for new procedure:

Letter sent to clinicians? ☑ Yes ☐ Not required