Proof of Concept for Automated SPE/HPLC/MS/MS Methods to Replace Traditional Immunoassay with MS Confirmation of Driving Under the Influence Samples

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Introduction

Immunoassay for screening followed by solid phase extraction (SPE) coupled with GC/MS or LC/MS/MS is well established for identification and confirmation/quantification of drugs and/or poisons from complex biological matrices submitted to forensic laboratories. However, reduced budgets and staffing necessitate improved operational efficiency. This poster details our initial comparison of operational efficiency using in-line automated SPE HPLC/MS/MS, versus traditional methods, for the analysis of urine samples submitted in Driving Under the Influence of Drugs (DUI-D) cases.

Background

Today, many forensic labs face difficulties related to budget cuts, reduced staffing, the need to effectively utilize instrument time and resources, and a need to increase the productivity of the remaining scientists. Instrument Top Sample Preparation (ITSP) coupled to liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) provides a possible solution to improve productivity and reduce the cost of analysis within the Forensic Toxicology laboratory. The ITSP system provides integrated online sample preparation which is controlled via the mass spectrometer software and utilizes disposable extraction cartridges. Urine samples from DUI-D cases were analyzed with ITSP/LC/MS/MS for comparison with results from immunoassay followed by standard solid phase extraction (SPE) and gas chromatography/mass spectrometry (GC/MS) or LC/MS/MS. All results provided in this study are from actual case samples.

Upon initial receipt, samples were screened for amphetamine/ methamphetamine, benzodiazepines, cocaine metabolite (benzoylecgonine), opiates, and THC metabolite (THCA) using Abbott Diagnostics fluorescence polarization immunoassay (FPIA). Previously validated confirmation methods using GC/MS or LC/MS/MS were utilized on samples which were positive on screening for one or more of the previously listed drug classes or had a history of drugs suspected, provided by the submitting agency, which fell outside of the normal five panel immunoassay screening. Aliquots of confirmed positive samples were supplied to OpAns for testing utilizing the ITSP system. Confirmed positives covered all classes of drugs listed previously and accounted for over fifty different analytes of interest.

Each sample submitted by SLED to OpAns for analysis by ITSP/ HPLC/ MS/MS was analyzed by two separate assays: one assay for THCA and barbiturates, the other assay for the remaining compounds of interest (>50 analytes). With the exception of glucuronide cleavage and centrifugation, each assay is fully automated and is performed in less than 10 minutes.

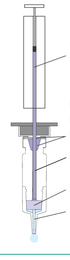
Apparatus

Autosampler: CTC Analytics PAL System HPLC auto sampler or Gerstel MPS with ITSP hardware kit

HPLC: Agilent Model 1200 SL with Binary Pump

MS: Agilent Model 6430 QQQ

ITSP Design



Analytical syringe replaces standard column reservoir found in SPE and Filter media formats

Needle Penetrates Septum and Creates Seal So That When Plunger Is Depressed, Sample is Forced Through Media. Septum Also Grips Needle to Allow Instrument to Pick Up ITSP Cartridge for Movement.

Small Inner Diameter of ITSP Needle Guide Reduces Inner Volume While Assisting in Maintaining a Vertical Perpendicular Position

SPE or Sample Filtration Media

Sample Can Be Eluted into Collection Plate

Basic Extract Sample Preparation

ITSP SPE methods are very similar to other SPE methods with adjustments made for reduced sample and solvent volumes and the use of positive pressure. Samples to be analyzed for basic drugs were assembled for the PAL by combining 25 µL of internal standard, 25 µL of β-Glucuronidase in pH 4.5 buffer and 200 µL urine. The plates were sealed and allowed to incubate at 60ºC for 30 minutes with gentle mixing. The plate was centrifuged for 5 minutes at approximately 2000g.

1. Wash ITSP SPE cartridge with 100 µL of Solvent 1.
2. Condition ITSP SPE cartridge with 100 µL of water.
3. Load 200 µL of sample on the ITSP SPE cartridge.
4. Wash the ITSP cartridge with 100 µL of water.
5. Move ITSP cartridge over collection vial and Elute with 100 µL of Solvent 1. Well contains 200 µL of 100 mM Ammonium Acetate in water.
6. Elute with 100 µL of Solvent 2 into the same vial.
7. Mix by aspirate/dispense.
8. Inject for LC/MS/MS analysis.
9. Peak areas were determined using Agilent MassHunter software.

Solvent 1 – 4:3:3:0.2 v/v THF:Methanol:Water:Ammonium Hydroxide

Solvent 2 – 5% Ammonium Hydroxide in water

Analysis Conditions (Basic Analytes)

ITSP Cartridges: UCT SSDBX (MicroLiter 07-UDBX10-20A)

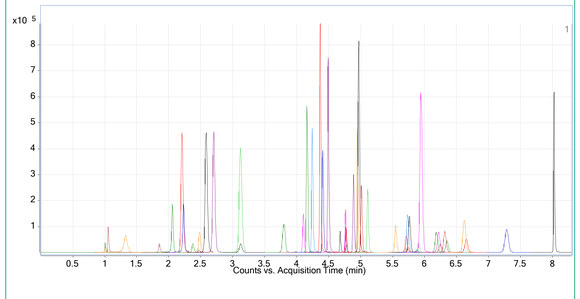
LC Conditions:

Solvent A: Water with 0.1% (v/v) Formic Acid
Solvent B: Methanol with 0.1% (v/v) Formic Acid
Column: 50 x 3mm i.d., Poroshell 120 EC-C18, 2.7 µm (Agilent)
Injection Vol.: 10 µL
Column Temperature: 30ºC
Flowrate: 0.8 mL/min
Gradient:

Time (min)	0.0	0.10	1.00	3.00	4.20	7.00	7.75	8.25	8.30
%B	3	3	15	20	50	55	100	100	3

MS Conditions:

Instrument: Agilent 6430 Triple Quadrupole
Ionization Mode: Electrospray @ 350ºC
Polarity: Positive
Transitions: available upon request



Analytes of interest include

RT	Compound				
0.97	Morphine	3.10	7-Amino Clonazepam	5.05	α-OH Midazolam
1.00	Noroxycodone	3.73	Benzoylecgonine	5.09	Buprenorphine
1.00	Oxymorphone	3.96	Norfentanyl	5.55	Nitrazepam
1.25	Hydromorphone	4.12	7-Amino Flunitrazepam	5.68	Propoxyphene
1.27	Norcodeine	4.14	Tramadol	5.69	Clonazepam
1.83	Dihydrocodeine	4.19	Cocaine	5.72	α-OH Triazolam
1.84	Codeine	4.22	Methylphenidate	5.74	Flunitrazepam
1.86	Norhydrocodone	4.35	Tapentadol	5.81	Norpropoxyphene
2.03	Oxycodone	4.39	Meperidine	5.87	Methadone
2.15	Amphetamine	4.47	Normeperidine	5.89	α-OH Alprazolam
2.20	Hydrocodone	4.66	PCP	6.15	Carisoprodol
2.31	Methamphetamine	4.75	Fentenyl	6.20	Alprazolam
2.41	MDA	4.75	Norbuprenorphine	6.29	Oxazepam
2.43	6-MAM	4.76	Meprobamate	6.31	Lorazepam
2.52	MDMA	4.79	Chlordiazepoxide	6.58	Temazepam
2.67	O-Desmethyltramadol	4.88	Midazolam	6.73	Nordiazeapam
3.04	MDEA	4.92	EDDP	7.33	Diazepam
		4.95	Flurazepam	8.03	Prazepam

Analysis Conditions (Acidic Analytes)

ITSP Cartridges: UCT CSDAU 10 mg (MicroLiter 07-UDAU10-20A)

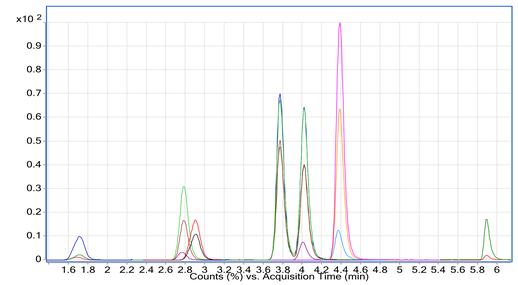
LC Conditions:

Solvent A: Water with 5 mM ammonium acetate and 0.05% (v/v) Ammonium Hydroxide
Solvent B: Methanol with 0.05% Ammonium Hydroxide
Column: 50 x 2.1 mm i.d., XTerra MS C18, 3.5 µm (Waters)
Injection Vol.: 10 µL
Column Temperature: 30ºC
Flowrate: 0.5 mL/min
Gradient:

Time (min)	0.0	0.50	4.00	6.50	7.00	7.50
%B	5	5	45	100	100	5

MS Conditions:

Instrument: Agilent 6430 Triple Quadrupole
Ionization Mode: Electrospray @ 350ºC
Polarity: Negative
Transitions: available upon request



Analytes of interest include

RT	Compound				
1.7	Phenobarbital	2.8	Butabarbital	4.3	Secobarbital
2.7	Butalbital	3.7	Amobarbital	5.9	11-carboxy-THC
		3.9	Pentobarbital		

Discussion

The ITSP methods used in this poster were originally developed for application to the clinical field of pain management. These methods have proven to be sufficiently robust to process forensic urine samples without modification. One hundred six (106) samples were submitted for testing using ITSP coupled to LC/MS/MS.

Review of the data summary reveals the following:

Barbiturates: All samples previously confirmed by SLED as containing barbiturates were confirmed by OpAns.

THC metabolite: Twelve additional cases were found to contain THC-COOH when evaluated by OpAns. All twelve samples screened negative by FPIA at the established cut off of 100 ng/mL. The additional positive cases can be attributed to the lower ITSP Limit of Quantification of 10 ng/mL for THC-COOH.

Amphetamines: Four additional samples were found to contain amphetamine or methamphetamine when evaluated by OpAns. All four samples screened negative by FPIA at the established cut off of 1000 ng/mL. The additional positive cases can be attributed to the lower ITSP Limit of Quantification of 50 ng/mL.

Benzodiazepines: Six additional samples were found to contain one or more benzodiazepines when evaluated by OpAns. All six samples screened negative by FPIA at the established cut off of 200 ng/mL. Additional benzodiazepines were confirmed by OpAns in nine cases. There were 23 cases where OpAns detected benzodiazepines at <50ng/mL but SLED confirmed them to be >10ng/mL

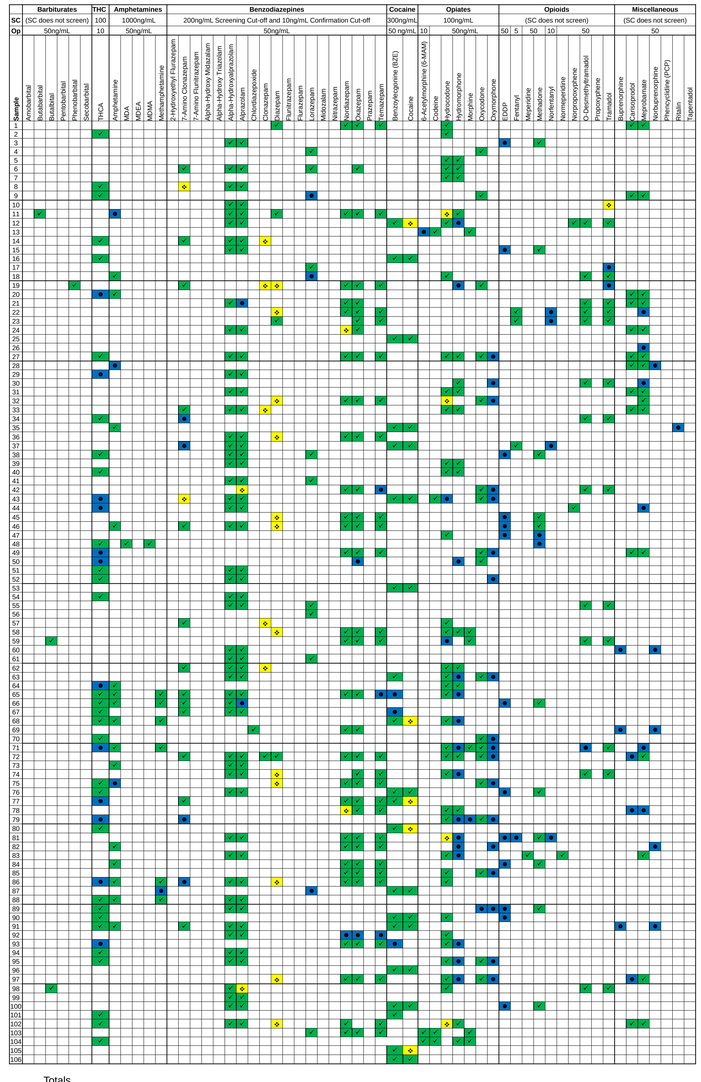
Cocaine/cocaine metabolite: All samples previously confirmed by SLED as containing cocaine or benzoylecgonine were found to contain cocaine and benzoylecgonine upon analysis by OpAns. In a few instances, the concentration of cocaine had decreased so that it was less than the OpAns 50ng/mL cut-off. Three additional samples were found to contain benzoylecgonine when evaluated by OpAns. All three samples screened negative by FPIA at the established cut off of 300 ng/mL.

Opiates: Three additional samples were found to contain one or more opiates when evaluated by OpAns. All three samples screened negative by FPIA at the established cut off of 100 ng/mL. Oxymorphone was confirmed in a total of 17 cases. Oxymorphone is not currently a target analyte of SLED's normal opiate panel. Sample 13 was found to contain 6-monoacetylmorphine when evaluated by OpAns. This sample was originally reported to contain codeine and morphine. The detection of 6-monoacetylmorphine by OpAns may be attributed to the 10 ng/mL LOQ established for the ITSP method.

Opioids/Miscellaneous: Currently, SLED does not perform routine screens for any of the drugs listed in these categories. General acid, base, neutral extractions followed by GC/MS and LC/MS/MS analysis are preformed if a history of suspected drugs is provided and normal presumptive screens are negative.

All differences in results between the methods can be explained.

Results



Totals

470	Not Detected by both SC and OpAns
32	Confirmed by both SC and OpAns
0	SC Confirmed & OpAns Detected
95	Confirmed by SC but not by OpAns
	OpAns Confirmed and SC Not Reported

Conclusions

More drugs were found using simultaneous screening/confirmation by ITSP/HPLC/MS/MS than traditional immunoassay screening with single drug class confirmation.

One operator can process 50 case samples per day through both methods on one ITSP/HPLC/MS/MS.

Current costs of expendable supplies for a five panel drug screen (FPIA) and a single confirmation utilizing traditional SPE and GC/MS or LC-MS/MS average $16.50. Supplies for additional confirmations average $7.00.

The total for all supplies to perform both ITSP/HPLC/MS/MS methods is $12 per sample.

Analysis using ITSP/HPLC/MS/MS produces comprehensive results in less time for less money than conventional screening with immunoassay followed by GC/MS and/or LC/MS/MS confirmation.

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