Proof of Concept/Comparison of Automated SPE/HPLC/MS/MS Methods to Traditional Immunoassay with MS Confirmation in Death Investigation Cases

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Introduction

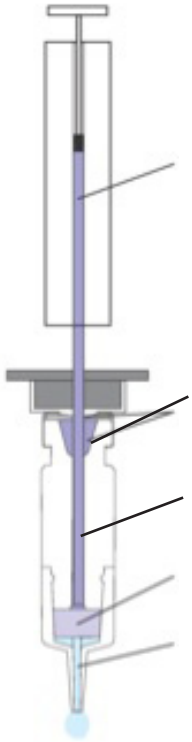
Immunoassay for screening followed by solid phase extraction (SPE) coupled with GC/MS or LC/MS/MS is well established for identification and confirmation/quantification of drugs and/or poisons from complex biological matrices submitted to forensic laboratories. However, reduced budgets and staffing necessitate improved operational efficiency. This poster provides a detailed comparison of operational efficiency using in-line automated SPE HPLC/MS/MS, versus traditional methods, for the analysis of postmortem blood samples submitted in death investigation 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. In a previous comparison, Instrument Top Sample Preparation (ITSP) coupled to liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) has proven itself a viable option for analysis of urine specimens 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. In this study, blood samples from death investigation 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 cocaine metabolite (benzoylecgonine) and opiates using Abbott Diagnostics fluorescence polarization immunoassay (FPIA). Additionally, samples were screened for amphetamine, methamphetamine, benzodiazepines, oxycodone, and cannabinoids (THC-A) using Immunalysis enzyme-linked immunosorbent assays (ELISA) 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 screening panel. Aliquots of confirmed positive samples were supplied to OpAns for testing utilizing the ITSP system.

Samples were stored refrigerated at 2-6°C for up to 12 months prior to retrieval and analysis using ITSP methods.



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

ITSP is protected under the following US PTO Patents: 6,859,615,7,001,774 & 7,798,021.
European Patents 1 74 701 and 1 808 700. Canadian Patents 2,316,648. Other patents pending.

Apparatus

Autosampler:	CTC Analytics PAL System HPLC auto sampler or Gerstel MPS with ITSP hardware kit and centrifuge
HPLC:	Agilent Model 1200 SL with Binary Pump
MS:	Agilent Model 6430 QQQ



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Opiate 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 opiates were assembled for the PAL by combining 25 µL of internal standard and 200 µL blood in standard 12x32mm vials (2mL) with the metal ring caps (for transport). Once loaded on the CTC auto sampler, 600 µL of 0.33 N HCl was added to each sample on the plate. As part of the automated extraction, plates were mixed via vortexing for 30 seconds followed by centrifugation for 3 minutes at approximately 2000g.

1. Load 700 µL of sample on the ITSP SPE cartridge.
2. Wash the ITSP cartridge with 100 µL Acetate buffer pH 4.5.
3. Wash the ITSP cartridge with 100 µL of water.
4. Wash the ITSP cartridge with 100 µL of methanol.
5. Move ITSP cartridge over collection vial and Elute with 100 µL of elution solvent (3:7:0.2 Water:Acetonitrile:Ammonium Hydroxide).
6. Dilute extract with 100 µL of water 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.

Opiates of Interest Include:

RT	Compound	RT	Compound
1.34	Morphine	2.30	Hydrocodone
1.44	Oxymorphone	2.52	6-MAM
1.55	Hydromorphone	4.05	Fentanyl
1.96	Codeine	4.15	Methadone
2.15	Oxycodone		

Analysis Conditions (Opiates)

ITSP Cartridges: UCT Styre Screen Strong Cation Exchange 10 mg (MicroLiter 07-UBCXP10-20A)

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

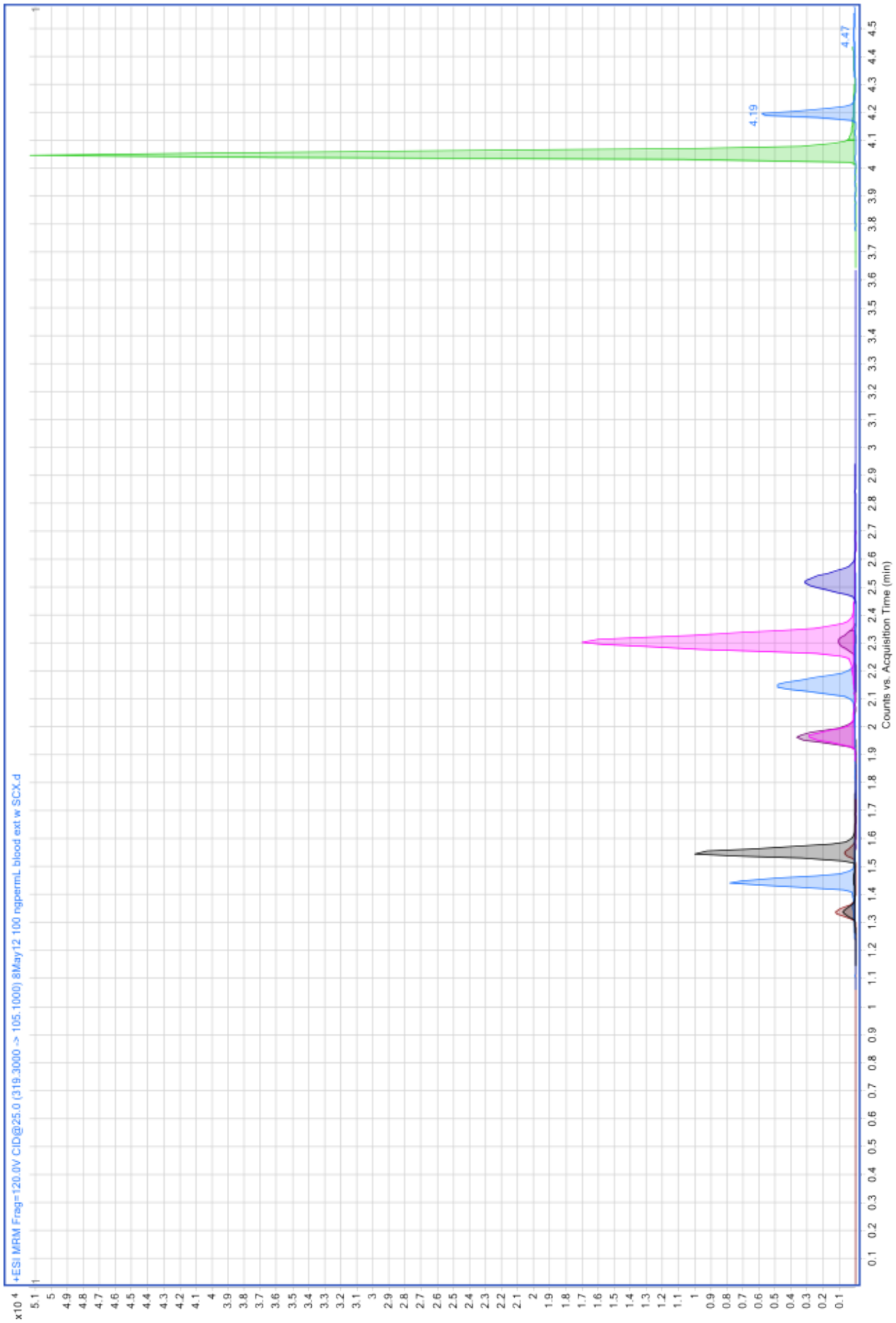
Gradient:

Time (min)	0.0	0.50	1.00	3.00	4.00	6.00	6.5
%B	3	3	15	20	100	100	3

MS Conditions:

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

Opiates


+ESI MRM Frag=120.0V CID@25.0 (319.3000 -> 105.1000) 8May12 100 ngpermL blood ext w SCX.d
x10 4
Counts vs. Acquisition Time (min)
4.19
4.47

Benzodiazepine Sample Preparation

Samples to be analyzed for benzodiazepines were assembled for the PAL by combining 25 µL of internal standard and 200 µL blood in standard 12x32mm vials (2mL) with the metal ring caps (for transport). Once loaded on the CTC auto sampler, 600 µL of 0.01 N Acetic Acid was added to each sample on the plate. As part of the automated extraction, plates were mixed via vortexing for 30 seconds followed by centrifugation for 3 minutes at approximately 2000g.

1. Load 700 µL of sample on the ITSP SPE cartridge.
2. Wash the ITSP cartridge with 200 µL 0.01 N Acetic Acid.
3. Wash the ITSP cartridge with 100 µL of Water:0.01N Acetic Acid:Methanol (2:2:1)
4. Move ITSP cartridge over collection vial and Elute with 100 µL of elution solvent A (1:1:1 Methanol: Acetonitrile:Tetrahydrofuran with 2% Ammonium Hydroxide).
5. Elute with 100 µL of elution solvent B (Tetrahydrofuran with 2% Ammonium Hydroxide
1. Dilute extract with 100 µL of water into the same vial.
2. Mix by aspirate/dispense.
3. Inject for LC/MS/MS analysis.
4. Peak areas were determined using Agilent MassHunter software.

Benzodiazepines of Interest Include:

RT	Compound	RT	Compound
0.75	7-Amino Clonaepam	2.75	2-OH Ethyl Flurazepam
1.03	7-Amino Flunitrazepam	2.76	Alprazolam
1.73	Chlordiazepoxide	2.79	Oxazepam
1.83	Midazolam	2.79	Lorazepam
2.50	Clonazepam	2.91	Temazepam
2.52	a-OH Triazolam	2.99	Nordiazepam
2.61	a-OH Alprazolam	3.17	Diazepam

Analysis Conditions (Benzodiazepines)

ITSP Cartridges: UCT Styre Screen Strong Cation Exchange 10 mg (MicroLiter 07-UBCXP10-20A)

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

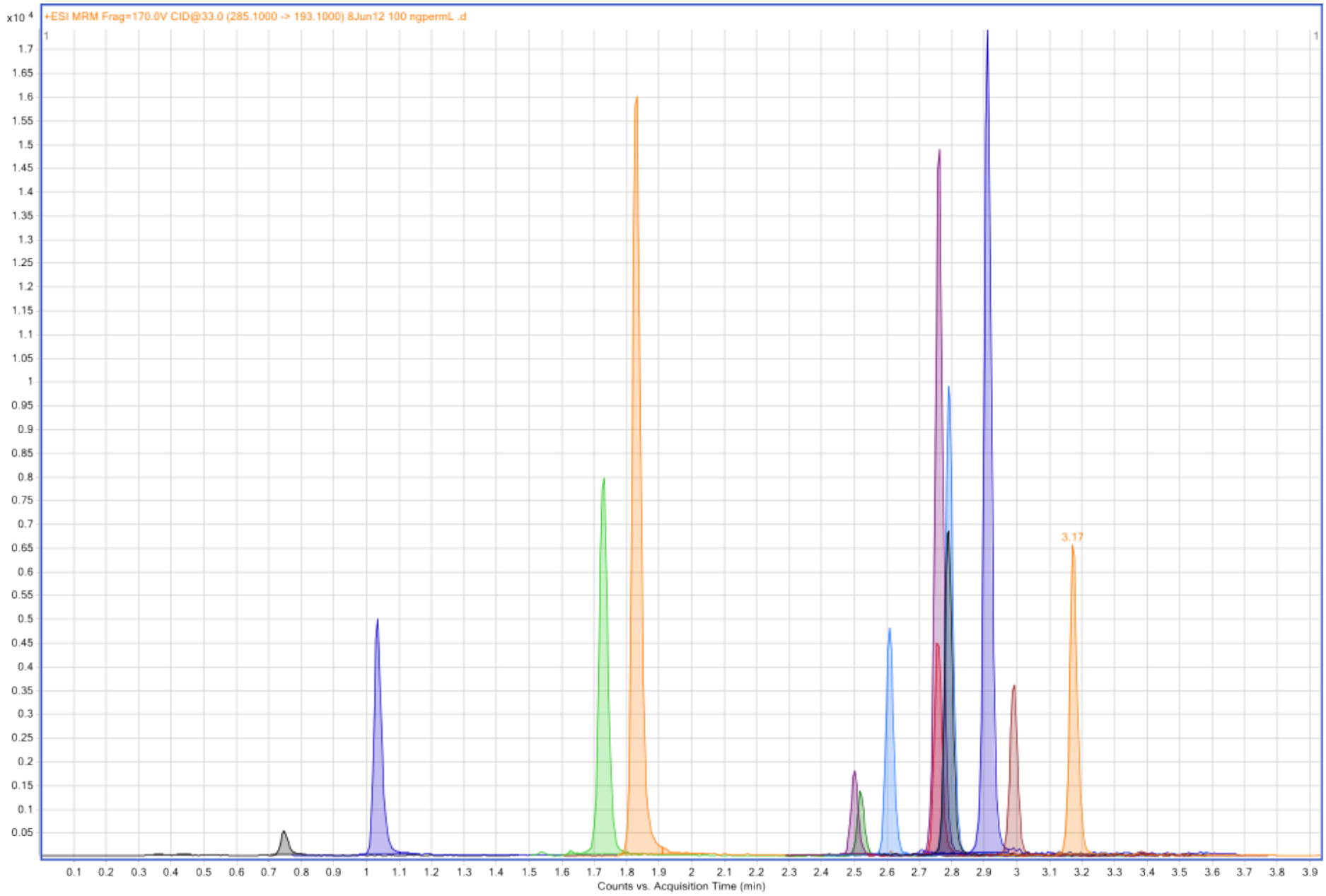
Gradient:

Time (min)	0.0	0.50	5.0	7.00	7.5
%B	30	30	100	100	30

MS Conditions:

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

Benzodiazepines



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Benzodiazepine Test Results

Sample #	SLED Results	OpAns/ITSP Results
1002	30 mg/mL Oxazepam 40 ng/mL Temazepam 120 ng/mL Diazepam 450 mg/mL Nordiazepam	24 ng/mL Oxazepam 43 ng/mL Temazepam 133 ng/mL Diazepam 731 ng/mL Nordiazepam
1017	Negative	Negative
1023	Negative	Negative
1024	< 10 ng/mL Clonazepam 50 ng/mL 7-aminoclonazepam	9 ng/mL Clonazepam 30 ng/mL 7-aminoclonazepam
1032	10 ng/mL Diazepam < 10 ng/mL Nordiazepam	18 ng/mL Diazepam 9 ng/mL Nordiazepam
1035	10 ng/mL Lorazepam < 10 ng/mL Oxazepam 60 ng/mL Temazepam	14 ng/mL Lorazepam 5 ng/mL Oxazepam 70 ng/mL Temazepam
1047	< 10 ng/mL Clonazepam 40 ng/mL 7-aminoclonazepam	14 ng/mL Clonazepam 36 ng/mL 7-aminoclonazepam
1049	50 ng/mL Diazepam 140 mg/mL Nordiazepam 120 mg/mL Oxazepam Clonazepam Qualifier out	63 ng/mL Diazepam 155 ng/mL Nordiazepam 147 ng/mL Oxazepam 5 ng/mL Clonazepam
1051	110 ng/mL Alprazolam	148 ng/mL Alprazolam
1057	130 ng/mL Diazepam 240 ng/mL Nordiazepam <10 ng/mL Oxazepam <10 ng/mL Temazepam	140 ng/mL Diazepam 203 ng/mL Nordiazepam 8 ng/mL Oxazepam 12 ng/mL Temazepam
1060	180 ng/mL Lorazepam	166 ng/mL Lorazepam
1063	50 ng/mL Alprazolam	78 ng/mL Alprazolam
1064	10 ng/mL Lorazepam	12 ng/mL Lorazepam
1067	290 ng/mL Diazepam 20 ng/mL Nordiazepam	423 ng/mL Diazepam 28 ng/mL Nordiazepam
1069	410 ng/mL Diazepam 130 ng/mL Nordiazepam < 10 ng/mL Oxazepam <10 ng/mL Temazepam	517 ng/mL Diazepam 131 ng/mL Nordiazepam 7 ng/mL Oxazepam 11 ng/mL Temazepam
1070	80 ng/mL Diazepam 60 ng/mL Nordiazepam	105 ng/mL Diazepam 88 mg/mL Nordiazepam

Opiate Test Results

Sample #	SLED Results	OpAns/ITSP Results
1001	Neg Drug Screen	5.6 ng/mL Fentanyl
1002	200 ng/mL Hydromorphone	227 ng/mL Hydromorphone
1015	41 ng/mL Fentanyl	35 ng/mL Fentanyl
1020	200 ng/mL Morphine (free)	295 ng/mL Morphine (free)
1023	Negative	Negative
1031	25 ng/mL Fentanyl 30 ng/mL Hydrocodone	25 ng/mL Fentanyl 31 ng/mL Hydrocodone
1034	6.8 ng/mL Fentanyl 180 ng/mL Oxycodone	7.6 ng/mL Fentanyl 183 ng/mL Oxycodone
1042	50 ng/mL Oxycodone	73 ng/mL Oxycodone
1063	50 ng/mL Codeine	78 ng/mL Codeine
1064	< 50 ng/mL Morphine (LOQ for batch)	69 ng/mL Morphine
1066	10 ng/mL Hydrocodone (LOQ) 460 mng/mL Oxycodone	17 ng/mL Hydrocodone 541 ng/mL Oxycodone
1069	200 ng/mL Hydrocodone	168 ng/mL Hydrocodone

Discussion

The ITSP methods used in this poster were originally developed for application to the clinical field of pain management. In a previous comparison of ITSP with more traditional extraction methods, ITSP has proven to be sufficiently robust to process forensic urine samples resulting in reduced cost of analysis and faster turn around time. With the exception of a few cases, the application of these methods to post mortem blood samples provided results that were generally in good agreement with original quantitative data (+ 30%). Storage conditions and elapsed time between the original quantitation and the subsequent analysis by OpAns with ITSP may have contributed to the unusually large differences seen in a few of the samples.

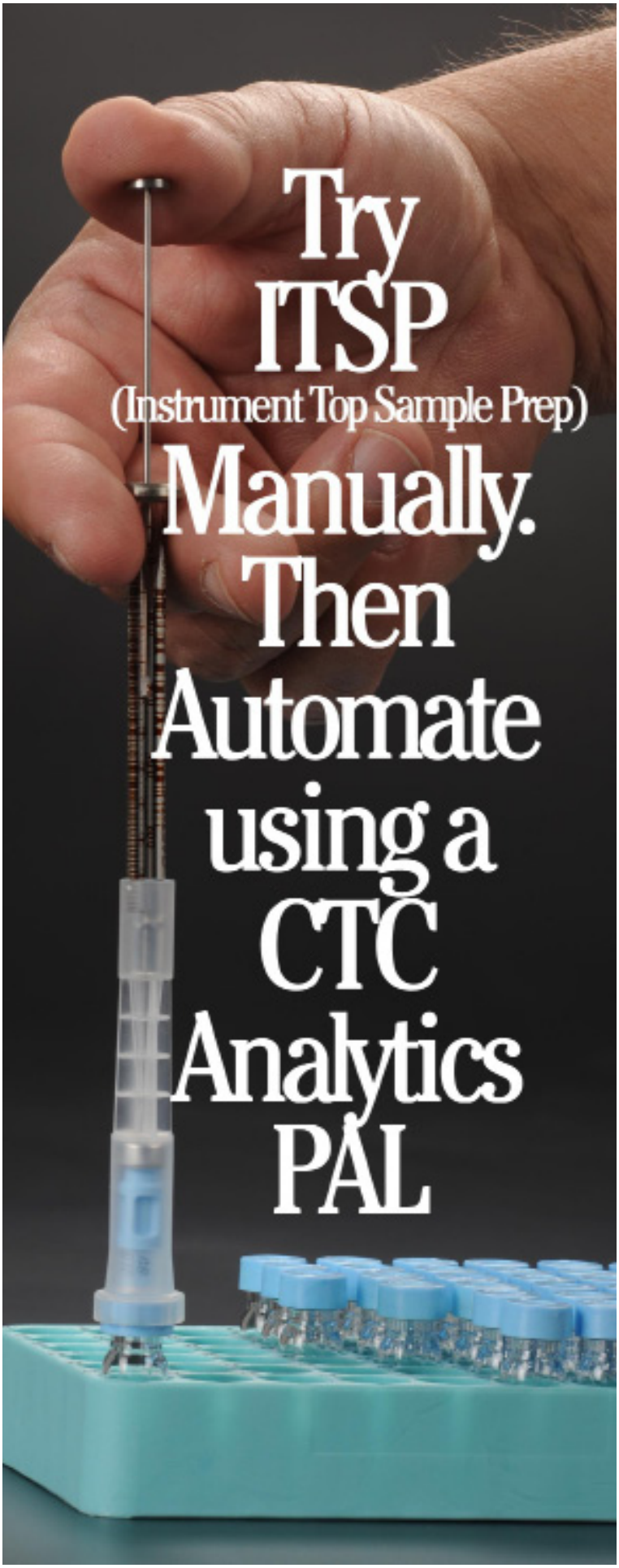
The lower limit of quantitation for benzodiazepines analyzed by SLED has been identified as 10 ng/mL. During analysis by OpAns using ITSP, several samples were found to contain one or more benzodiazepines with a concentration of 10 ng/mL or less. The lower limit of quantitation for all analytes using the ITSP methods is 5 ng/mL. With the exception of sample 1001, all analytes of interest were identified by both methods. Sample 1001 originally screened negative by immunoassay was found to contain Fentanyl when analyzed using ITSP and LC/MS/MS.

Conclusions

In this comparison of ITSP with traditional sample preparation and confirmation techniques, ITSP has proven itself to be sufficiently robust to enable analysis of post mortem blood specimens even after extended storage of specimens. Appropriate sample dilution prior to extraction and the use of positive pressure for sample application and elution resulted in an automated method capable of extracting difficult biological specimens. Use of automated sample preparation coupled with quantitation/confirmation by LC/MS/MS should reduce analyst work load related to sample preparation thereby reducing turn around time and allowing the analyst to concentrate on other tasks. Future work should involve a larger set of actual cases and provide evaluation of additional drugs and drug classes as well as the ability of the ITSP system to process additional specimen types.

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