ITSP® SOLUTIONS, INC.

Instrument Top Sample Prep
SmartSPE™

A consumable solution for automation and the best measurement performance!

Automated Online μSPE-LC/MS/MS for the Measurement of Basic Drugs in Blood

Developed in collaboration with
Shimadzu Scientific Instruments
Somerset, NJ

Introduction

Deaths caused by poisoning account for >20% of all deaths caused by injury in the United States and is second only to deaths caused by motor vehicle collisions, which is approximately 25%. These poisoning deaths include both intoxication from abuse of illicit drugs and deaths resulting from medication taken as medical therapy. [1] Since intoxication is involved in many motor vehicle collisions, the crucial role toxicology analysis plays in modern death investigation is clear.

Deaths related to drug overdoses in celebrities have brought mass media attention to the drug abuse pandemic. Historically drugs such as heroin and cocaine have been considered the "dangerous" drugs, but in recent years, prescription drug abuse has far surpassed "illegal" drugs as agents of lethal drug toxicity. This trend is expected to continue and the need for toxicology analysis has never been greater. Medical examiners' offices that routinely test for alcohol and drugs of abuse report finding some substance in toxicology analysis in at least 50% of their cases.[2]

Given the prevalence of substances in decedents examined by forensic pathologists, simply dying in circumstances that would bring one's body to the medical examiner's office for investigation seems to be sufficient indication to warrant toxicology analysis.[2] Nevertheless, factors such as cost or a prolonged lag in receiving toxicology results can make it impractical to perform a thorough analysis on every case. Then, history becomes the best guide to determining when and what tests to perform.

Cases where evidence of alcohol or drug abuse exists certainly merit toxicology analysis, as do cases where no visible cause of death can be found at autopsy. Toxicology analysis in all homicides and accidents is also wise, as questions about the presence or absence of intoxicating substances are likely from relatives of the decedent and in any legal proceeding that may arise later. Questions also are likely in suicides where 16% are found to be the result of poisoning.[3] However, multiple studies have reported that approximately 70% of suicides have some intoxicating substance detected in the body upon toxicology analysis.[4]

Blood, urine, bile, ocular fluid, gastric contents, liver, and brain tissue can all be useful specimens. Peripheral blood (femoral or subclavian) specimens are optimal for postmortem toxicologic quantification; urine is the primary body fluid used for drug screening. Specimens need not necessarily be tested automatically, but the best practice is to at least collect and hold the specimens until completion of the autopsy report and death certificate. Frequently, additional history does not become known until after the body is released, when it is too late to collect samples.

Interpretation of concentrations of drugs, whether legal or illicit, is properly made from blood or solid organ concentrations determined and confirmed by liquid chromatography/tandem mass spectrometry (LC/MS/MS) within the context of the circumstances that surrounded death.[5] By itself, a positive screening test of urine is insufficient to conclusively prove use of a drug by the decedent as cause of death, although it may strongly suggest recent use and can provide valuable information when taken in concert with the circumstances of death.

Clearly, there is great need for forensic toxicology testing, particularly for blood analysis. At the same time, there are limited resources available from the government for highly skilled toxicologists. This situation shifts the burden of need to the testing technology employed. The technology deployed must be thoroughly robust, accurate, and highly automated, so that it may be routinely performed quickly by laboratory technologists having limited expertise. This application note describes methodology that meets and exceeds these criteria. It uses a CTC/PAL autosampler / sample manager equipped LC/MS/MS for online SPE performed in parallel with LC/MS/MS to automatically measure basic drugs in blood. The method achieves the highest recoveries, accuracies, and reproducibility while thoroughly removing unwanted sample matrix components to maximize operational robustness. The outcome is a simplified single workflow delivering the highest possible quality test results without increasing operational costs. Since the workflow uses parallel automation, it is also possible to significantly increase the laboratory throughput without addition to staff. [6]

Online SPE Using the ITSP Cartridge

The heart of the ITSP form of SPE is the patented single use cartridge (Figure 1) containing customer-defined packed chromatographic media.

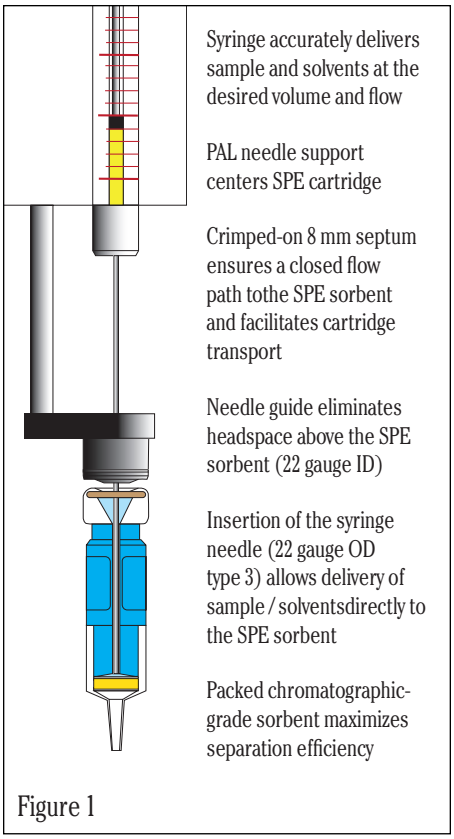


Figure 1

The crimped-on septum and needle guide (upper 80% of cartridge) enable automation by facilitating accurate cartridge transport on a syringe needle. The automated SPE process starts using the syringe to perform cartridge conditioning, sample loading, and sample washing steps over a waste receptacle. Automation continues using the syringe to perform the elution step(s) over a clean vial(s) or well(s) and then discarding the used cartridge in a different waste receptacle. The automation finishes using the syringe to mix the freshly eluted sample and then inject it into the LC/MS/MS or GC/MS/MS that will be used to measure the sample. The locations of these events on a PAL autosampler are shown in Figure 2.

This automation approach is a simple single workflow process with the SPE integrated directly into the LC/MS/MS or GC/MS/MS software and workflow. It requires no additional skills beyond those needed to operate the LC/MS/MS or

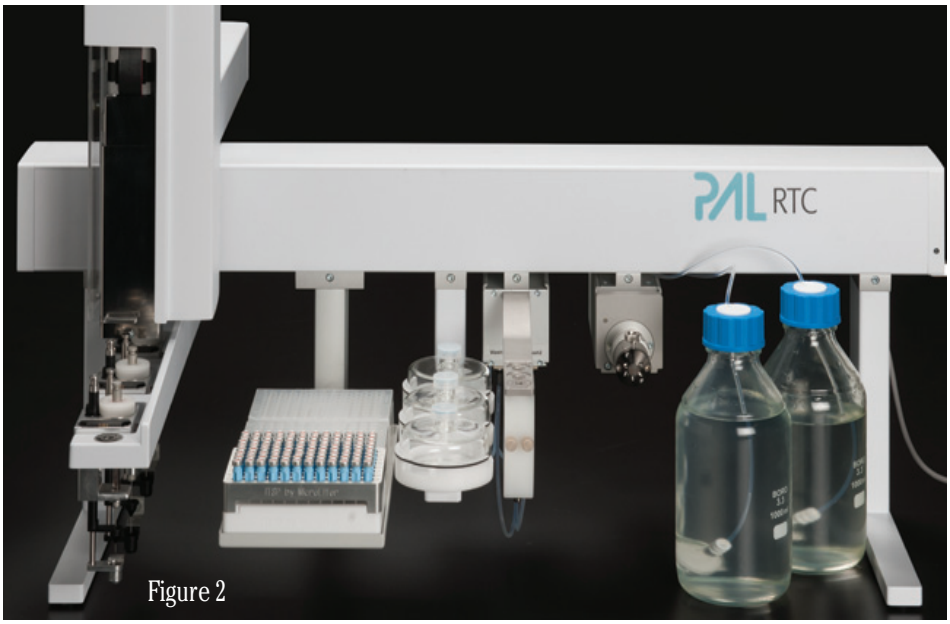


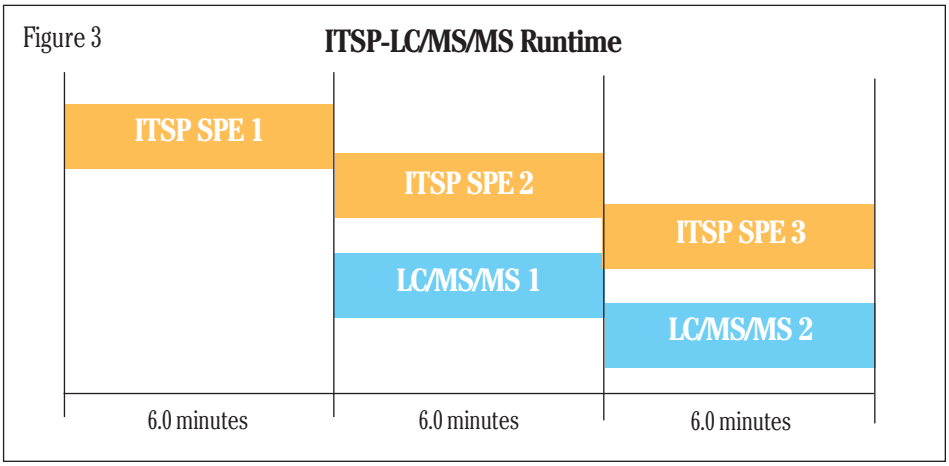
Figure 2

GC/MS/MS. Put simply, it is just a different method selection in the MS software already in use with any PAL autosampler equipped LC/MS/MS or GC/MS/MS.

In this online SPE by ITSP workflow, parallelism is achieved in a different way than it is with the two workflow process (robotic parallel SPE, followed by serial LC/MS/MS or GC/MS/MS). In the ITSP workflow, both SPE and LC/MS/MS (or GC/MS/MS) are performed serially, but in parallel with each other. This alternative approach to parallelism is depicted in the timeline shown in Figure 3.

In addition, this small volume SPE cartridge allows elution volumes of 50-100 µl, which facilitates significant control of the analyte concentrations delivered for measurement. As a result, the sample volume can be simply adjusted to match both the capability (sensitivity) of the measurement instrumentation and the specific needed cutoffs (LLOQs, S/N ≥20) for proper interpretation of the test results. This is achieved without the customary dry down step

Figure 3

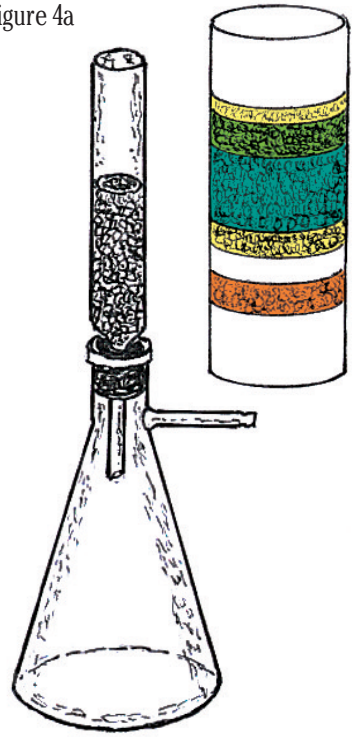


used with vacuum or pneumatically driven flow forms of SPE, which tend to require ≥5x larger elution volumes. The micro size of the ITSP SPE cartridge saves hours of time by eliminating the highly time consuming dry down step.

The above covered the benefits of the upper 80% of the ITSP SPE cartridge (Figure 1), which enables the ability to accurately move the SPE cartridge, thereby enabling precise automation. Although smaller, the bottom 20% of the cartridge is every bit as important as the top. This is the part that does chromatographic SPE, the part that expertly cleans and enriches the sample producing high quality results, and the part that one trusts to keep the LC/MS/MS or GC/MS/MS clean for many months of robust operation.

To understand the importance of chromatographic SPE, one needs to examine all other single use SPE devices which are all derived from the very first liquid chromatography (LC) experiments performed by Tswett more than 100 years ago.[7] These very first experiments used vacuum to drive liquid flow as shown in Figure 4a. Tswett quickly realized that neither constant nor controlled flow was possible with vacuum and the crucial importance of precisely controlled flow. So Tswett quickly adapted and built an apparatus utilizing positive pressure liquid flow (with pressure monitoring) in order to address this need (Figure 4b) and became the first to isolate chlorophyll. Nonetheless, conventional commonly used single use SPE devices that are common today (tubes, plates, pipette tips) still have not taken this crucial second step (flow control) that Tswett took more than a century ago.

Figure 4a



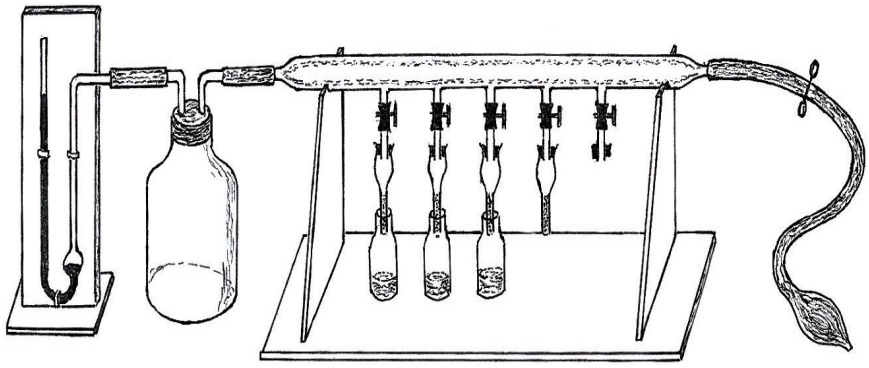


Figure 4b

It is important to understand the impact of the lack evolution in flow control for SPE. Figure 5a shows shows actual SPE recovery using conventional SPE syringe tubes[8]. The lack of a clearly defined optimum flow demonstrates the lack flow control or sufficiently packed sorbent, or both. In contrast, the expected flow profile of a chromatographic device is shown in (Figure 5b). Since the flow changes during the SPE process (due to liquid level changes), recovery is variable and so are the results. Internal standards are required to achieve meaningful results. In addition, flow is rarely, if ever, operated at flow slow enough to achieve high absolute recovery. So, method validation using comparison with external solvent only standards is generally not an

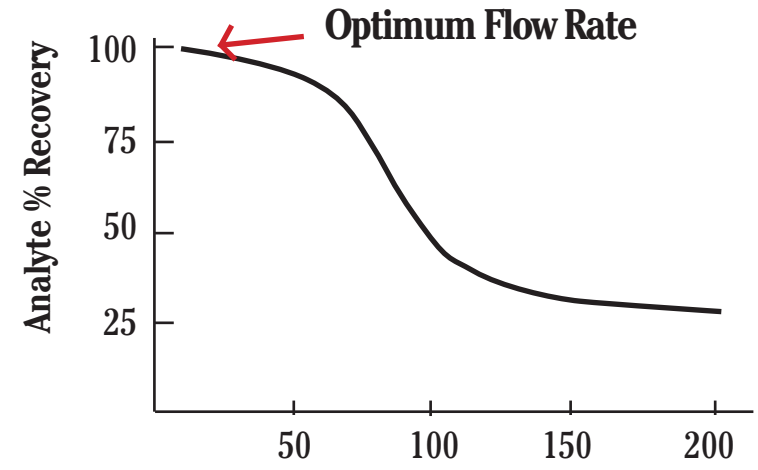


Figure 5a

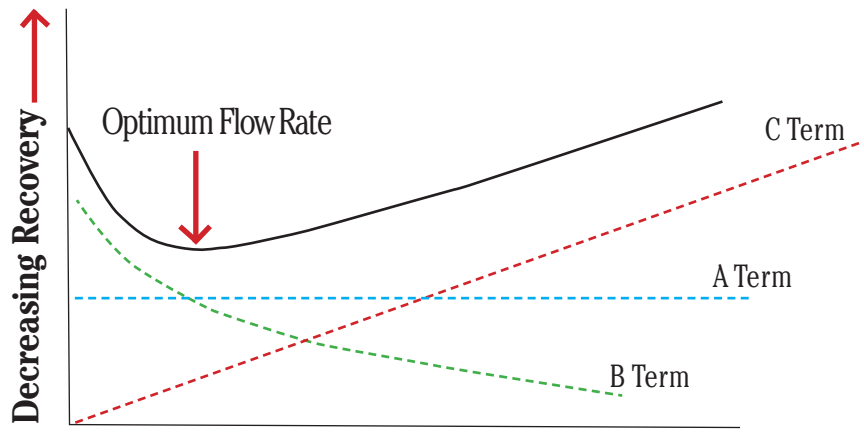


Figure 5b

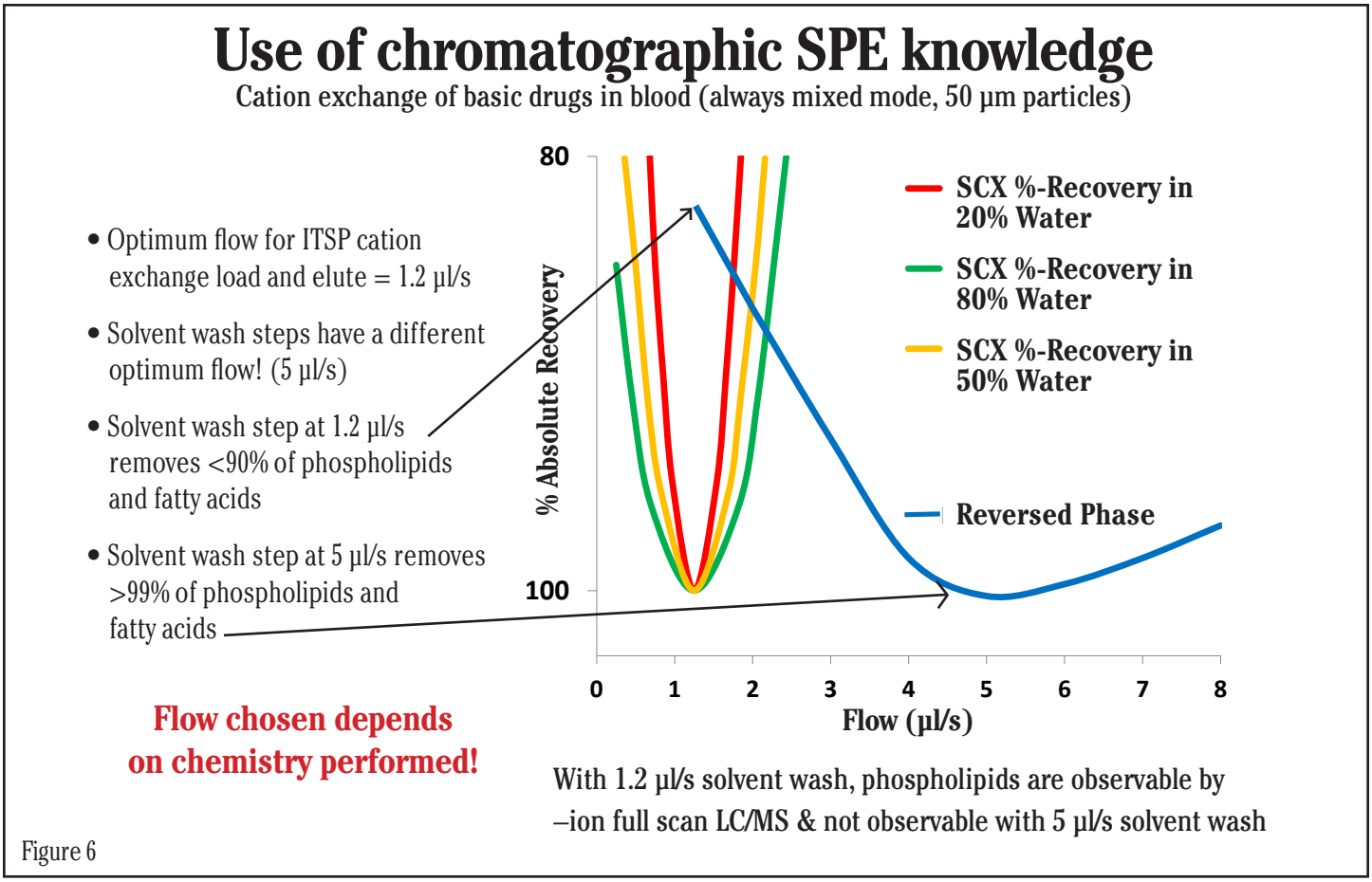


Figure 6

option for demonstrating the lack of matrix effects using SPE devices with vacuum or pneumatic driven flow.

In contrast, the ITSP SPE cartridge was designed to do chromatographic SPE well. This can be seen in the syringe needle placement shown in Figure 1 where needle placement is directly at the frit containing the packed chromatographic sorbent, thereby allowing delivery of the sample, without significant dispersion, directly to the packed sorbent. Flow of the sample and solvents is positive liquid pressure, syringe pump driven (flow adjustable at a resolution of +/-10 nl/s) and is both precise and accurate. The extra-column volume of the cartridge (Figure 1, below sorbent) is just 16 µl. As a result, the ITSP SPE cartridge is truly a unique micro-SPE device with significant operational and performance advantages.

The most unique aspect of ITSP single use SPE cartridges is the precise flow control and the ability to achieve LC column levels of performance. This is shown in Figure 6 where the flow profiles for the ITSP form of SPE are the expected van Deemter curves with clearly defined optima. The result of controlled flow at the optima is that absolute recoveries of >99% can be achieved (vs. external solvent only standards) and that >99% of targeted matrices can be removed.

This is achieved using chemistry specific optimal flow as shown in Figure 6 for basic drugs in blood using cation exchange sorbent. ITSP is high performance SPE. It uses all chromatographic knowledge given by Tswett as well as that of van Deemter, Giddings, Horvath, and Neue that followed. No other single use SPE device can match the chromatographic performance of ITSP.

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Sample Preparation

Blood samples require solvent crash and centrifugation. All standards and their isotopically labeled internal standards may be obtained from Cerilliant. Standards of the drugs should be prepared in clean blank blood at appropriate concentrations across the analytical range. Isotopically labeled internal standards also should be added to the specimen sample, calibrators, and QCs.

Step-by-Step Process:

1) To each vial (except for those containing double blanks), add 10µl (per 100µl sample) internal standard working solution (Concentration: 0.1 - 1 ng/ml)
2) To each labeled vial, add an aliquot (10-1000 µl, 100 µl typical) of the applicable blood sample (i.e., forensic specimen, standard, QC, or blank) [dried/weighed blood also can be used]
3) To each vial, add 200 µl (per 100 µl sample) MeOH (100 µl minimum) [For dried blood, add 300 µl 70/30 MeOH/H2O to 50 mg]
4) Cap vials and vortex vigorously for at least 15 s
5) Centrifuge for at least 15 minutes at ≥2000 g

Solvents:

Sample Diluent: MeOH (Honeywell part no.: 230-4 (VWR))

Condition / Wash: trace analytical grade water fresh from a Millipore Integral Water Purification System w/BioPak (or equivalent)

Solvent Condition / Wash: 70/30 MeOH/IPA (Honeywell part nos.: 230-4/ 324-4 (VWR))

Optional Buffer Wash: 0.02 M pH = 6.0 phosphate buffer in trace analytical grade water

Elution Solvent: 30 or 50% IPA/H_2O - 6% NH_3 (Fluka part no.: 44273) [30% IPA for opioids / basic illicits only, 50% for benzodiazepines and other high LogP drugs) Note: NH_3 evaporates quickly. Make fresh weekly and replace the septum on this container after 500 uses. Parafilm working and source bottles.

Elution pH Neutralizer: HOAc (atomic analysis grade, Fluka part no.: 07692. See the LC injection notes in the LC method below).

The ITSP Solution for Cation Exchange SPE Sample Preparation

Each sample is prepared using SPE individually for LC/MS/MS analysis. This process is performed for each vial (steps 6-16 continued in the next column) before moving to the next vial. The method operates concurrently with all successive LC/MS/MS analyses within a list, in parallel, as shown in Figure 3. The CTC/PAL autosampler / sample manager used is fully software compatible with all makes / models of LC/MS/MSs and can be purchased (with full warranty) from your LC/MS/MS manufacturer and/or other distributors. Also, the CTC/PAL can be operated for SPE with equal ease in a stand alone / offline manner, if desired. Clean samples are eluted into 2ml vials (w/inserts) and then injected by the PAL System into the LC/MS/MS.

Cation Exchange ITSP SmartSPE™ Cartridges Product Number:
10S-UCUPCX -T - United Chemical Technologies (UCT), n-propylsulfonic acid on silica, 10mg, 96/Tray

6) Condition ITSP cartridge with 100 µl 70/30 MeOH/IPA Wash Solvent at 5 µl/s
7) Condition ITSP cartridge with 100 µl water [steps 6-7 can be combined using the DLW option]
8) Load 150 µl of crashed / centrifuged blood (from step 5) at 1.2 µl/s on to ITSP cartridge (other sample volumes possible, 10-1000 µl whole blood, to match volume to sensitivity needs or sample availability)
9) If desired, wash the cartridge with 100µl Optional Buffer at 1.2 µl/s
10) Wash the cartridge with 100 µl Wash Solvent at 5 µl/s (removes triglycerides, fatty acids, and phospholipids)
12) Elute at 1.2 µl/s with 100 µl Elution Solvent into elution vial (w/ insert)
13) Add Optional Elution pH Neutralizer (see LC method notes below)
14) Mix elution thoroughly with syringe (5x at 50% volume [minimum] at 20 µl/s)
15) Inject onto LC/MS/MS system for analysis (>3x overfill recommended for precision; see LC method notes below)
16) Rinse syringe and LC injection valve with ACN/formic acid (99/1) and water/formic (99/1). Cleaning should consist of at least 3 full syringe volume rinses and >1ml each pushed through the LC injection valve. Then prepare (SPE) next sample in parallel to the ongoing LC/MS/MS analysis.

Outcome: Morphine can be measured at 1 ng/ml when using a 100 µl blood sample. Sensitivity will progressively improve with increasing LC retention time (%B) up to 100x better for later eluting opioids and all other drugs eluting later. There will be no measurable ion suppression from fatty acids and phospholipids.

ITSP® SOLUTIONS, INC.

Instrument Top Sample Prep® Is SmartSPE™

LC/MS/MS Conditions to cover a wide range of basic drugs after ITSP cation exchange SmartSPE

Mobile Phases:

A - Trace analytical grade water fresh from a Millipore Integral Water Purification System w/BioPak (or equivalent) with 1% ACN (LC/MS grade, Honeywell product no.: 015-4 (VWR)) buffered with 1% HOAc (atomic analysis grade, Fluka product no.: 07692)

B - ACN (LC/MS grade, Honeywell product no.: 015-4 (VWR)) with 1% HOAc (atomic analysis grade, Fluka product no.: 07692)

Column:

GL Sciences Inertsil C_{18} ODS3, 3µm particles, 2.1 x 50 mm (part no. 5020-04412) or Restek Pinnacle DB Biphenyl 2.1 x 50 mm 3 µm particles (part no. 9409352) held at 40°C using a heat exchanger in column oven to preheat mobile phase. [Install an Upchurch A318 precolumn filter housing (with A100X frit) inline prior to column oven.]

Gradient (0.5 ml/min):

Time = 0.00: 97% A, 3% B (start)
Time = 0.25: 97% A, 3% B (hold)
Time = 1.20: 80% A, 20% B (linear gradient)
Time = 4.50: 40% A, 60% B (linear gradient)
Time = 5.00: 5% A, 95% B (linear gradient)
Time = 5.35: 5% A, 95% B (hold)
Time = 5.36: 100% B (column clean up)
Time = 5.50: 100% B (column clean up)
Time = 5.51: 97% A, 3% B (column conditioning)
Time = 6.00: 97% A, 3% B (column conditioning)

Depending on the drugs measured, the gradient transition points and/or ramp rates may have to be adjusted to separate isobaric drugs and/or to achieve at least 16 data points across each LC peak. These (and all LC/MS/MS parameters) should be optimized, fully functional, and made routine based on solvent/standards-only solutions using the PAL autosampler prior to proceeding with on-line SPE with ITSP.

Sample Injection by PAL System

1) Fill valve/loop with trace analytical grade water prior to injection.
2) In all cases, a pre-cut and polished SS loop is used.
3) 1 or 2µl loop using 3x overfill will give optimal performance (the narrower LC peaks will improve speed and/or separation).
4) Injection loop volume should be held to a maximum of 5 µl when using a 2.1mm diameter LC column. Larger volumes increase peak area primarily in width, not height, and thus deteriorate the LC separation with little, if any, gain in sensitivity. Use of 5 µl loops usually will require careful pH adjustment of the sample prior to injection (see below). Always operate LC/MS/MS at optimal conditions. Use of a 1 or 2µl loop is highly recommended.
5) Chemical presentation of the sample from ITSP SPE to the LC is important. In LC analysis, control of the pH (ionization state) controls peak shape and the approach used depends on the LC column chosen. Biphenyl columns function best when the analytes are fully ionized in solution and will function best when the NH3 in the SPE elution is fully neutralized with an excess of acetic or formic acid. C_{18} columns function best when the analytes are not ionized in solution. This can be achieved with pH and/or ion pairing with acetate. Optimal separations, peak shape, and ion source cleanliness are often found with partial (ca. 80%) neutralization of the NH_3 in the SPE elution with acetic acid.

MS/MS Conditions:

Use all the usual MRMs for all drugs. Select MRMs based on signal to noise ratio to achieve both high signal and low background. Ion source conditions, MS/MS collision gas pressure, and initial MRM should be optimized using morphine only in 15%B/85%A. Then, all MRMs should be optimized using these ion source and MS/MS collision gas pressure conditions found to be optimal for morphine.

Maintenance recommendations:

Wipe off ion source entrance cone weekly with Kimwipes soaked in IPA with 5% HOAc to maintain steady MS/MS response.

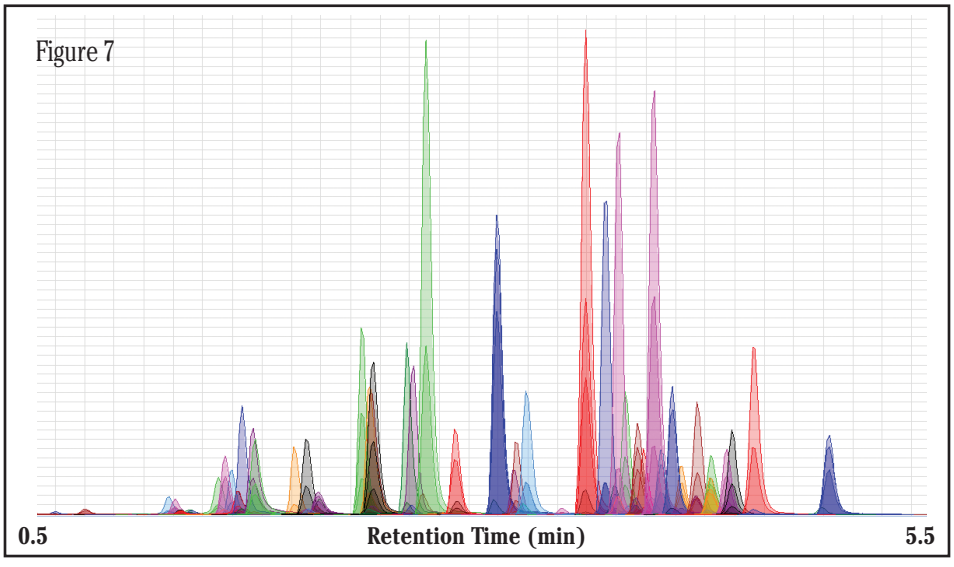
Change the A100X precolumn filter frit when LC pressure increases by 20% [approximately once per month during very heavy use].

Monitor LC peak width and asymmetry daily and replace LC column when these increase [approximately every 3 months during very heavy use].

Perform all manufacturers' recommendations for periodic maintenance annually for all components [PAL, LC, and MS/MS] and consider doing this every 6 months during very heavy use to avoid down time.

SPE Workflows

Figure 7 shows a chromatogram for 65 potentially lethal basic drugs and metabolites measured in blood using the above SPE sample preparation method. Such an approach might be used when case circumstances do not lead toward specific drug classes. The same SPE sample preparation method could be easily used



in a basic drug class specific way based on case information or screening results by simply selecting the desired drug class MRMs in the MS software.

ITSP also has a robust SPE (RP) method for drug and metabolite measurement in urine that is highly useful as a screening method prior to blood analysis. This method is thoroughly validated for production achieving 1 ng/ml LLOQs (or better) for all drugs (basic and acidic) in one method. Both methods (blood and urine) are easily performed on the same LC/MS/MS simply by exchanging the SPE cartridge rack and selecting the desired method in the MS software.

The same drugs in urine SPE method also is a powerful tool for oral fluid analysis that might be used in DUID cases. Since the sample amount is increased to 1 ml (from a Quantisal OF sampling device), it can easily measure all drugs achieving 0.2 ng/ml LLOQs (or better) including measurement of low dose benzodiazepines and their metabolites. Again, switching between any of these methods is simply a matter of making sure the correct SPE cartridges are in place and selecting the desired method in the MS software. In addition, all of the above can be performed using bar coded vials for total chain of custody needs.

Conclusions

The need for forensic toxicology testing has never been greater and all indications are that the need will continue to grow. However, the funding for more toxicologists is not growing nearly as fast. At the same time, the wide dissemination of scientific knowledge is allowing test results to be challenged more frequently and thoroughly. So simultaneously, the bar is being raised on the quality of the results. Given this seemingly impossible situation, the solutions apparently will have to come from new technology that delivers both automation and a new level of quality allowing greater, more thorough validation of test results.

The ITSP form of SmartSPE™ performed with a CTC/PAL autosampler / sample manager addresses both of these challenging needs. It is the only single use SPE device that is automated into a single simple and inexpensive workflow. All others require separate workflows for SPE and LC/MS/MS. At the same time, ITSP SPE is the only single use device that delivers chromatographic SPE capable of validation against external solvent only standards for >99% recoveries and clear, unambiguous validation of the absence of matrix effects. Can your toxicology lab afford to be without ITSP?

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US Patents: 6,859,615 & 7,001,774 • European Patents: EP 1 174 701 • Canadian Patent: 2,316,648