Forensic Sciences and Toxicology

Drugs of abuse: Extraction in seconds

In Forensic Science and in Toxicology, body fluids are regularly analyzed for residues of drugs of abuse, therapeutic drugs and of their metabolites. In general this type of analysis requires extensive sample preparation. At PittCon 2009, GERSTEL is presenting automated Disposable Pipette Extraction (DPX). DPX is a fast and efficient SPE technique used for a wide range of applications such as drugs of abuse, therapeutic drug monitoring, comprehensive screening, pharmacology studies (NNK), as well as pesticides in fruit and vegetables. DPX is based on unique and patented SPE devices: Pipette tips that incorporate loosely contained sorbent material, which is mixed with the sample solution. Turbulent air bubble mixing creates a suspension of sorbent in the sample ensuring optimal contact and highly efficient extraction. The extraction is performed much faster than with traditional SPE techniques.

Body fluids represent a complex and heterogeneous matrix. Accurate determination of drugs, pharmaceuticals and metabolites in blood and urine requires both a suitable chromatographic system and adequate sample preparation. Sometimes more than one extraction technique is needed for a successful result. Solid Phase Extraction (SPE) is among the most widely used sample clean-up and analyte extraction techniques in forensic and toxicology laboratories.

Traditionally, SPE requires the use of significant quantities of solvent, some of which are toxic. Following several labor intensive steps, many methods require that the solvent be evaporated in order to concentrate the analytes of interest and achieve the necessary detection limits. Depending on the chemical properties of the analytes, a chromatographic determination may also require further sample preparation steps such as derivatization. The sum total of sample preparation steps can amount to a significant bottleneck for laboratory productivity and a risk to occupational health unless adequate and costly safety precautions are taken.

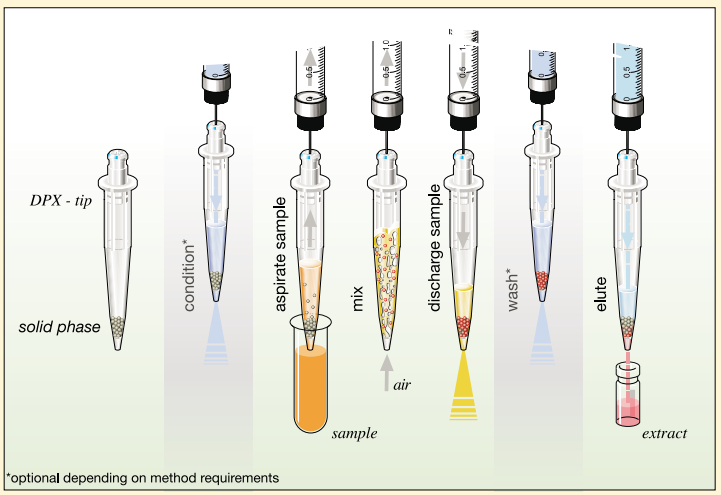
If the tedious and labor intensive steps can be eliminated, the overall task can be performed more efficiently and faster while producing accurate results and using only a fraction of the amount of solvent normally used. All this is possible thanks to the novel Disposable Pipette Extraction (DPX) technique developed by Professor William

Automated DPX process

All steps are performed automatically by the MPS. If needed, the sorbent is conditioned with solvent prior to the extraction process.

1. Sample is drawn into the pipette tip for direct contact with the solid phase sorbent. There is no contact between the sample and the syringe used to aspirate the sample and therefore no risk of cross contamination.
2. Air is drawn into the pipette tip from below through the frit. Turbulent air bubble mixing creates a suspension of sorbent in the sample, ensuring optimal contact, highly efficient extraction, and high recovery.
3. The extracted sample is discharged, typically after 30 seconds.

If needed, the sorbent can be washed to remove unwanted residue.



4. Extracted analytes are eluted using a suitable solvent, which is added from above for most efficient elution. The eluate is collected in a vial for subsequent sample introduction to LC/MS or GC/MS.

The total time required for extraction in the examples shown in this article was always less than 6 minutes. Sample preparation and GC/MS or LC/MS determination can be performed in parallel for best possible throughput and system utilization.

Disposable Pipette Extraction (DPX)

(Bill) E. Brewer, Ph.D. from the University of Southern Carolina. Professor Brewer is the Owner-President of DPX Labs (www.dpxlabs.com).

The DPX technique has now been automated by GERSTEL, a leader in automation of sample preparation and sample introduction for GC/MS and LC/MS. GERSTEL is presenting automated DPX at PittCon 2009 in Chicago after it was initially introduced at the annual meeting of the Society of Forensic Toxicologists (SOFT) in Phoenix, Arizona (USA) in October 2008.

„The reactions we got from the forensic scientists at the SOFT meeting", says Robert J. Collins, Ph.D., President of GERSTEL, Inc. in Baltimore, MD, "lead us to believe that automated DPX is seen by experts as a very promising alternative to standard extraction techniques".

In order to provide an efficient solution for the determination of drugs and metabolites in blood in a routine laboratory environment, GERSTEL and DPX Labs LLC collaborated on automating the DPX technique. "DPX immediately struck us as the right solution", says Bob Collins. Furthermore, for this application, samples should be prepared and derivatized just prior to analysis. Just in time sample preparation eliminates analyte degradation and underreporting of concentration levels since no sample is waiting for an extended period of time in the autosampler before being analyzed. Also, in order to optimize GC/MS system utilization and sample throughput, a sample should be ready for introduction every time the GC/MS finishes its run and becomes ready for the next sample. In summary, performing GC/MS analysis and sample preparation carefully synchronized and in parallel benefits both the quality of results and the throughput. The MAESTRO software with its PrepAhead and Scheduler functions makes it extremely easy for the

In contrast to conventional SPE, DPX does not rely on standard cartridges with a packed sorbent bed. DPX is based on unique and patented SPE devices: Pipette tips that incorporate loosely contained sorbent material, which is mixed with the sample solution. DPX is a dispersive SPE technique, turbulent air bubble mixing creates a suspension of sorbent in the sample ensuring optimal contact and highly efficient extraction. Extractions are performed much faster than with traditional SPE techniques. Elution requires only a small amount of solvent, which means that DPX effectively provides a concentration step: For many applications, such as pesticides in fruit and vegetables, solvent evaporation is not required.

DPX methods are readily automated using the GERSTEL MPS, which can introduce the extract into a GC/MS or LC/MS system. Additional sample preparation steps can be performed, including derivatization or adding an internal standard. The analyst only needs to place the samples in the MPS autosampler and activate the sequence table from the MAESTRO software. Everything else is performed automatically including GC/MS and LC/MS analysis.

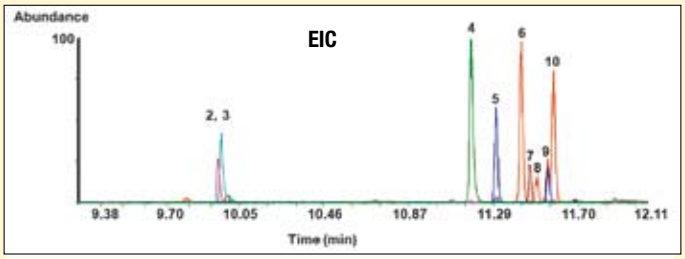
Ralf Bremer, GERSTEL Managing Director for R&D and production

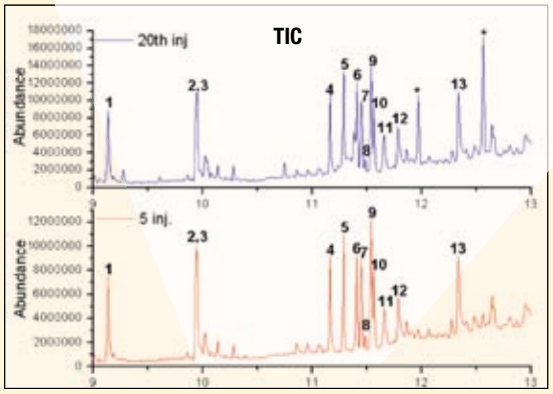
The patented DPX tips comprise loose solid phase sorbent contained inside a pipette tip fitted with a frit at the bottom and a polymer barrier at the top. The barrier mounted in the upper opening of the DPX tip has some additional functions: It is used as a transport adaptor, enabling the GERSTEL MPS to move the tip around and fully automate the DPX process. An orifice in the transport adapter enables the autosampler syringe to deposit liquids and to aspirate air through the sample/sorbent suspension for highly efficient mixing and extraction. „DPX is a unique and patented extraction technique that provides the user a previously unheard of level of automation, efficiency and throughput", says Ralf Bremer, Managing Director for R&D and production. GERSTEL has exclusive rights to sell automated DPX instrumentation world-wide.

The DPX process is wonderfully simple: DPX tips are placed on the sample tray of the MPS. Aspirating the sample; adding liquids for conditioning, rinsing or elution; and aspirating air through the sample/sorbent suspension for efficient mixing – all this is performed by the microliter syringe in the autosampler. Depending on the application, the sorbent is conditioned using a suitable solvent: This is aspirated into the DPX tip out of a vial or added from above. The defined amount of sample is aspirated into the DPX tip without making contact with the syringe or syringe needle. "There is no cross-contamination between samples", says Ralf Bremer and notes: "Furthermore, since DPX is a dispersive SPE technique, it is not affected by flow rates or by changes in the flow path (i.e. channelling) through the sorbent. Such things have no impact on the extraction efficiency."

When the DPX tip is safely retracted from the sample vial, air is aspirated through it, through the polymer frit and through the sample inside. "Fine air bubbles quickly rise through the sample / sorbent suspension resulting in extremely efficient, turbulent mixing. This significantly accelerates the extraction of analytes onto the sorbent", says Bremer. The extraction is performed under optimal conditions while requiring much less sorbent material than other SPE techniques. The extracted sample is now discarded into a waste vial. A rinse step follows and the extracted analytes are then eluted into an empty vial using a suitable solvent. The DPX tip is discarded and the MPS is ready for the next sample.

When performing SPE extractions manually, it is often necessary to concentrate the extract by evaporating the solvent under an inert atmosphere and then adding a keeper solvent to take up the residue. Such a labor intensive and time consuming step is not required when performing fully automated DPX: "The extract is simply introduced into the GC/MS system using a Large Volume Injection (LVI). The solvent is evaporated in the GC inlet and the analytes are concentrated inside the inlet liner, provided you have a temperature programmable GC inlet such as the GERSTEL Cooled Injection System (CIS)", says Ralf Bremer.





Drug	Average RSD
Amphetamine	13.9 %
Methamphetamine	14.4 %
Meperidine	5.8 %
PCP	2.2 %
Methadone	3.3 %
Methaqualone	3.6 %
Amitriptyline	3.1 %
Cocaine	3.8 %
Cis Doxepin	2.8 %
Imipramine	3.3 %
Trans Doxepin	3.2 %
Pentazocine	5.3 %
Codeine	4.2 %
Desipramine	6.4 %

Drugs of abuse in blood: Total ion chromatogram of a DPX extract of 250 µL whole blood spiked at 0.5 ppm with drugs of abuse using d5-PCP as internal standard. Chromatograms from the 5th and 20th injections are shown alongside each other to demonstrate the ruggedness of the analysis. The insert shows the extracted ion chromatogram (EIC) from the 5th injection. The sample was protein precipitated with 0.5 mL acetonitrile, and the supernatant was transferred to clean tubes. After adding 0.1 mL of 0.1 M HCl, automated DPX was performed. 1) Meperidine, 2) d5-PCP (ion 205), 3) PCP, 4) Methadone, 5) Methaqualone, 6) Amitriptyline, 7) Cocaine, 8) cis-Doxepin, 9) Imipramine, 10) trans-Doxepin, 11) Desipramine, 12) Pentazocine, 13) Codeine (Septum bleed from vial cap after repeat injections from the same vial).

analyst to plan, optimize, and set up the whole process.

From theory to practice

In order to test the MPS-DPX-CIS-GC/MS system in practice, the scientists analyzed blood and urine samples that had been spiked with different drugs and pharmaceuticals. Compounds determined included amphetamines, benzodiazepines, cocaine and methadone as well as tetrahydrocannabinol (THC) and metabolites. For details, please see the graphic representations on this page. The analysis was performed using deuterated internal standards: For blood samples, d5-PCP (0.2 ppm) was used. For the determination of benzodiazepines, d5-Nordiazepam (0.2 ppm) and d5-OH-Alprazolam (0.2 ppm) were used. For the opiates, equivalent deuterated compounds were used (each at a level of 0.1 ppm).

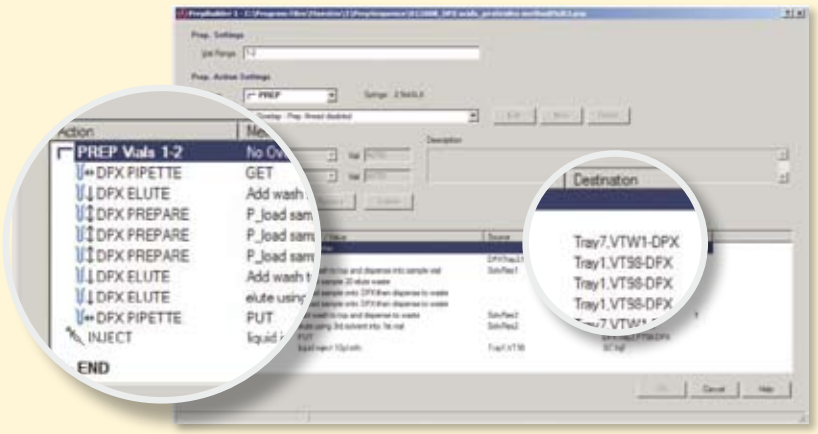
Required manual sample preparation steps

Preparing blood samples: 0.5 mL of acetonitrile was added to a 0.25 mL sample of whole blood followed by mixing to precipitate proteins in the sample. The mixture was centrifuged and the supernatant transferred to a clean labelled test tube containing 0.1 mL of 0.1 M HCl.

Preparing urine samples for the determination of benzodiazepines: In order to determine the total level of free, bound and metabolized residues of drugs and pharmaceuticals in urine, hydrolysis must be performed of the respective conjugates, such as for example glucuronides of benzodiazepines, which are metabolites of the drugs that are formed to facilitate excretion of the substances from the body.

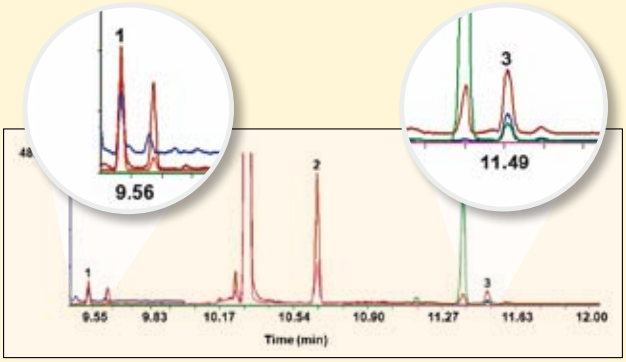
The hydrolysis reaction is started by adding 10 µL of a solution of the enzyme *β*-glucuronidase and 50 µL of a 0.1 M sodium phosphate buffer with pH 4 to a 0.2 mL sample of urine. The mixture is kept at 55 °C for two hours and is then allowed to cool to room temperature. Acetonitrile (0.25 mL) is then added in order to precipitate the enzyme. Following centrifugation, the supernatant is transferred to a clean, labelled test tube and 200 µL of 0.1M HCl is added. The prepared samples are then placed in the MPS sample tray.

Automated Disposable Pipette Extraction (DPX) is subsequently performed on the prepared samples using the MultiPurpose Sampler (MPS) equipped with 1 mL CX tips from DPX Labs (www.dpxlabs.com). As the name suggests, CX tips contain a novel and unique cation exchange material with additional slightly apolar characteristics. The DPX process is completely automated: 250 µL of a 30 % solution of acetonitrile in water is dispensed onto the DPX sorbent inside the tip for condi-

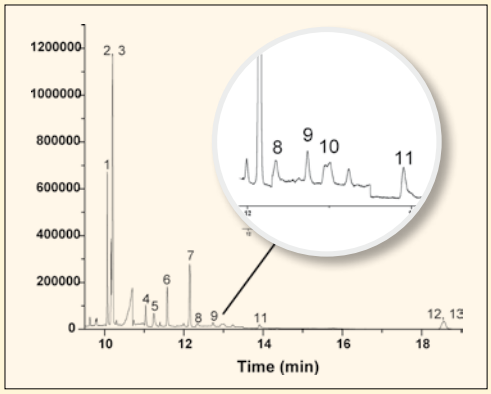


Analysis conditions

CIS:	1 min solvent purge (150 mL/min); splitless; temperature program: 80 °C (1 min) – 12 °C/min – 300 °C (3 min)
Column:	30 m HP5 (Agilent Technologies); di = 0.25 mm; df = 0.25 µm
Pneumatics:	He, constant flow, 1.5 mL/min
GC oven:	100 °C (0.5 min) – 20 °C/min – 300 °C (12.5 min)
MSD:	Selectable Full Scan or SIM mode



THC and metabolites in blood: Total ion chromatogram of 10 ng/mL THC and metabolites extracted from 0.5 mL whole blood following protein precipitation, centrifugation and DPX-RP. Derivatization was performed in the CIS inlet by injecting 50 µL of DPX eluent together with 20 µL of 50/50 BSTFA/acetonitrile. No additional solvent evaporation or derivatization step was performed. Analytes: 1) THC-TMS, 2) OH-THC-TMS, 3) COOH-THC-2TMS



Benzodiazepines in urine: Total ion chromatogram of 0.2 ppm benzodiazepines in 0.2 mL urine following enzymatic hydrolysis and DPX. Derivatization was performed in the CIS inlet by injecting 50 µL of DPX eluent together with 20 µL of 50/50 MTBSTFA/acetonitrile. No separate solvent evaporation step was performed. Increasing the sample volume to 0.5 mL and performing multiple DPX extractions would increase the sensitivity. 1) Diazepam, 2) Nordiazepam-d5-TBDMS, 3) Nordiazepam-TBDMS, 4) Flunitrazepam, 5) 7-aminoflunitrazepam, 6) Oxazepam-2TBDMS, 7) Temazepam-TBDMS, 8) Nitrazepam, 9) Lorazepam-2TBDMS, 10) Clonazepam-TBDMS, 11) Alprazolam, 12) α-OH-Alprazolam-d5-TBDMS, 13) α-OH-Alprazolam-TBDMS

tioning. The conditioning solvent is subsequently discarded to waste. The DPX tip is then immersed into the sample and a defined volume is aspirated into the tip. Air is then aspirated into the tip, causing turbulent mixing and efficient extraction of analytes into the sorbent. Following a 30 second equilibration time, in which the sorbent is allowed to settle, the extracted sample is discarded to waste. The sorbent is rinsed twice, first with 0.5 mL of a 30 % solution of Acetonitrile in water and then with 0.5 mL acetonitrile. The extracted analytes are eluted using 0.7 mL of a solution consisting of 2 % concentrated ammonia, 78 % CH_2Cl_2 and 20 % isopropanol.

The eluate was dispensed directly into an autosampler vial. The total amount of time required for extraction and liquid handling was less than 6 minutes per sample.

Instrumentation

The analysis was performed on a 6890N/5975 (inert XL) GC/MS system from Agilent Technologies. The GC was fitted with a Cooled Injection System (CIS 4) PTV-type inlet. An MPS PrepStation with DPX option and MAESTRO Software control was used for automated sample preparation and sample introduction. The complete system including GC/MS was operated using one integrated method and one sequence table directly from the Agilent Technologies ChemStation Software, operated integrated with the MAESTRO software.

Derivatization

Some analytes must be derivatized to enable GC/MS determination. „The Cooled Injection System (CIS) inlet offers an inert and temperature programmable environment", says Prof. Brewer, „which is highly suited to evaporating and purging excess solvent while simultaneously, or at least sequentially, performing derivatization of analytes."

For the derivatization of benzodiazepines, 20 µL of 50 % N-(t-butyldimethylsilyl)-N-methyl-trifluoracetamide (MTBSTFA) in acetonitrile was aspirated into the autosampler syringe followed by 20 µL of air and 50 µL of the DPX eluate. „The resulting „Sandwich" injection was performed slowly, using a programmed stop flow method to ensure that the solvent was completely removed through the split vent prior to the derivatization step", the application specialist explains. The CIS temperature quickly ramped to 300 °C, which started the derivatization process and helped transfer the derivatized analytes to the GC column in splitless mode for highest possible recovery and lowest limits of determination.

"Automated analyte derivatization in the GC inlet proved to be both simple and highly practical", said Prof. Brewer. "The method was successfully applied to the determination of benzodiazepines in blood. Compounds that were not successfully derivatized in this way were derivatized directly in the sample vial". For this approach, the DPX eluate was evaporated to dryness under a flow of nitrogen in the sample vial. 50 µL of MTBSTFA and 50 µL of ethyl acetate were added and the mixture kept at 70 °C for 20 minutes. When the extract had cooled off, 50 µL of the solution was introduced to the CIS inlet using the Large Volume Injection (LVI) technique.

The conclusion reached by the experts

"As we had expected, the analysis based on automated DPX delivers excellent results", said Bob Collins. Even though all analyses were performed on very small sample volumes (250 µL blood or 200 µL urine), the resulting peak intensities were highly satisfactory – even in full scan mode. The MultiPurpose Sampler (MPS) with automated DPX enables fast sample preparation of difficult samples while delivering high sensitivity and accurate results. The additional liquid handling capabilities of the dual rail MPS PrepStation enabled full automation of all the required liquid handling steps such as derivatization and addition of an internal standard. This added level of automation provided best possible productivity and throughput. The instrument combination used proved to be especially useful for the determination of basic drugs such as cocaine, methadone, PCP, TCAs and Meperidine.

Most benzodiazepines were easy to determine using MTBSTFA derivatization in the GC inlet. The following were determined: Diazepam, Nordiazepam, Oxazepam, Temazepam, Alprazolam and α-OH-alprazolam. "DPX combined with GC/MS determination provided excellent results for the 11 listed benzodiazepines in urine", Bob Collins notes, "plus we got good recovery and great sensitivity for the opiates. For most opiates, we achieved limits of determination under 1 ng/mL in whole blood". Ralf Bremer, General Manager for production and R&D, is thrilled about the use of the CIS 4, PTV-type inlet for evaporative concentration and analyte derivatization: "This year, we are celebrating the 25th anniversary of the introduction of the first GERSTEL CIS. It is very reassuring to see that the constant improvements we have engineered into the CIS over the years enable us to stay well ahead of the competition".