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Analysis of Drugs and Metabolites in Blood and Urine using Automated Disposable Pipette Extraction

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Abstract
In this work an automated DPX procedure is presented that enables the determination of acidic, basic, and neutral drugs in small blood or urine samples. The DPX method is based on a mixed-mode cation exchange sorbent (CX) with reversed phase characteristics. The automated extraction is shown to require only minimal manual labor when performed using the GERSTEL MPS 2 autosampler under MAESTRO software control. GC-MS data is provided showing that comprehensive, rapid and sensitive analysis can be performed using automated DPX combined with large volume injection. Automated sample preparation steps are performed during the GC/MS run of the preceding sample using the MAESTRO PrepAhead function. PrepAhead sample preparation enables high throughput analysis, improved productivity and “just in time” preparation of each sample.

INTRODUCTION

The analysis of blood or urine for the presence of drugs by chromatographic methods typically requires sample preparation prior to analysis. Solid Phase Extraction (SPE) methods with various adsorbents in traditional cartridge formats are often used for this sample cleanup, however traditional SPE requires relatively large volumes of solvents and is performed in multiple steps. After sample cleanup, a solvent evaporation step is often required to achieve the necessary detection limits. Finally, many analyte types require derivatization prior to GC analysis. This sample preparation is typically time-consuming and is widely considered to be the "bottle neck" for laboratory analysis throughput.

Disposable Pipette Extraction (DPX) was developed as an efficient and rapid extraction method alternative to conventional solid-phase extraction. The patented DPX tips comprise loose solid phase sorbent contained inside a pipette tip fitted with a lower screen and upper barrier. These tips do not require conditioning because the solution is mixed with the sorbent; channeling and flow rates have no impact on extraction efficiency for this type of dispersive solid-phase extraction technology. Therefore, extractions are much faster and require much less solvent. The barrier mounted in the upper opening of the DPX tip additionally acts as a transport adaptor, enabling the GERSTEL MPS 2 to move the tip around (patent pending).

After DPX sample cleanup, the time-consuming solvent evaporation step can be eliminated by using Large Volume Injection (LVI) to evaporate the solvent and concentrate the analytes directly in the GC inlet liner. For compounds that do not require derivatization, LVI provides the most efficient means to achieve high sample throughput combined with low detection limits.

Some compounds, such as benzodiazepines and opiates, must be derivatized prior to GC analysis. Two different methods were used to perform this task: Analytes were either derivatized in the sample vial prior to injection or directly in the inlet by delivering reagent into the GC inlet immediately following the sample using a "sandwich" injection. Both derivatization methods are performed automatically by the MPS 2 autosampler.

This research was performed with the aim to develop a faster, better and less labor intensive method for analysis of drugs and metabolites in blood and urine by GC/MS. Using the LVI technique, limits of detection comparable to those found in typical HPLC/MS/MS applications were achieved. One of the primary goals of this research was to provide "just in time" sample preparation. Every sample is prepared at the exact same point in time, just prior to analysis. This reduces sample to sample variation by reducing the risk of analyte decomposition since derivatized samples are not waiting for a long period of time in the autosampler prior to injection. Furthermore, each sample is extracted and prepared for injection while the previous sample is being chromatographically analyzed, providing the highest throughput possible.

EXPERIMENTAL

Materials. BSTFA and MTBSTFA and ß-Glucuronidase (Type H-2, from Helix pomatin) were purchased from Sigma Aldrich (St. Louis, MO). DPX-CX 1 mL tips were obtained from DPX Labs, LLC (Columbia, SC). Acetonitrile, ethyl acetate, hexane, methylene chloride, isopropanol, ammonium hydroxide, and hydrochloric acid (all analytical grade) were purchased from Fisher Scientific (Pittsburg, PA). A 'drug-free' urine sample was obtained from a lab volunteer. Ampoules of 6-monoacetylmorphine (6-MAM, 0.1 mg/mL), 6-MAM-d_3 (0.1 mg/mL), morphine (1.0 mg/mL), morphine-d_3 (0.1 mg/mL), codeine (0.1 mg/mL), codeine-d_3 (0.1 mg/mL), oxycodone (1.0 mg/mL), oxycodone-d_3 (0.1 mg/mL), PCP-d_5 (0.1 mg/mL), and pentobarbital-d_5 (0.1 mg/mL) were purchased from Cerilliant (Round Rock, TX). Quick-check drug solutions (0.5 mg/mL) containing 20 acidic, neutral and basic general drugs were purchased from Alltech (State College, PA).

Instrumentation. Analyses were performed using a 6890N GC equipped with a 5975 (inert XL) mass selective detector (Agilent Technologies), PTV type inlet Cooled Injection System (CIS 4, Gerstel) and MPS 2 Prepstation with DPX option under MAESTRO software control (Gerstel).

Analysis conditions.

PTV:	1 min solvent vent (150 mL/min) splitless 80°C (1 min); 12°C/min; 300°C (3 min)
Column:	30 m HP 5 (Agilent) d_i = 0.25 mm d_f = 0.25 µm
Pneumatics:	He, constant flow = 1.5 mL/min
Oven:	100°C (0.5 min); 20°C/min; 300°C (12.5 min)
MSD:	Full Scan or SIM mode

SIM parameters for the determination of benzodiazepines.

Group	Analyte	Target Ions (m/z)	Qualifier Ions (m/z)
1	Diazepam	256	283, 284
1	Nordiazepam-TBDMS	327	328, 329, 332*
2	Flunitrazepam	312	286, 294
2	7-aminoflunitrazepam	255	283, 254
2	Oxazepam-2TBDMS	457	513, 459
3	Nitrazepam-TBDMS	338	292, 394
3	Temazepam-TBDMS	357	283, 359
3	Lorazepam-TBDMS	491	513, 493
4	Clonazepam-TBDMS	372	374, 326
4	Alprazolam	279	204, 308
5	α-OH-alprazolam-TBDMS	381	382, 383, 386*

SIM parameters for the determination of opiates.

Group	Analyte	Target Ions (m/z)	Qualifier Ions (m/z)
1	Hydrocodone	299	242, 302*
2	Codeine-TMS	371	234, 178, 374*
3	Morphine-2TMS	429	236, 432*
4	6-MAM-TMS	399	340, 402*
5	Oxycodone-TMS	387	390*

Initial Sample Preparation.

- Deuterated internal standards were added to the sample solutions. D_5-PCP was added to the basic blood samples, bringing the final concentration to 0.2 ppm. For the benzodiazepine determination, d_5-nordiazepam and d_5-OH-alprazolam were added bringing the final concentration to 0.2 ppm. For opiates, deuterated analogues of each drug at a final concentration of 0.1 ppm were added.
- Preparing blood samples: 0.25 mL of whole blood sample was vortex mixed with 0.5 mL acetonitrile. The samples were subsequently centrifuged and the supernatant transferred to clean labeled tubes, each containing 0.1 mL of 0.1 M HCl.
- Preparing urine samples for benzodiazepine determination: Hydrolysis was performed by adding 10 µL ß-glucuronidase and 50 µL of 0.1 M sodium phosphate buffer pH 4 to 0.2 mL urine and then heating samples at 55°C for 2 hours. The samples were then cooled to room temperature and 0.25 mL acetonitrile was added to precipitate the enzyme. The solutions were centrifuged, and the resulting supernatants transferred to clean, labeled sample tubes. 200 µL of 0.1 M HCl was then added to the supernatant in each sample.
- All tubes were then placed onto the GERSTEL MPS 2 sample tray.

Automated DPX extraction procedure. Automated DPX was performed using 1 mL DPX-CX (mixed-mode) tips from DPX Labs, LLC.

- 250 µL of 30 % acetonitrile in water was dispensed from above onto the DPX sorbent.
- The DPX tip was inserted into the sample solution, which was aspirated and mixed with the sorbent.
- After equilibrating for 30 seconds, the solution was dispensed to waste.
- The sorbent was washed with 0.5 mL of 30 % acetonitrile in water and then with 0.5 mL of 100 % acetonitrile. Both wash solvents were dispensed to waste.
- Elution of the analytes was accomplished using 0.7 mL of elution solvent (2 % NH_4OH : 78 % CH_2Cl_2 : 20 % isopropanol). The eluent was dispensed directly into the pre-labeled GC vial.
- The total time required for extraction and liquid handling was approximately 6 minutes per sample.

Automated injection with concentration and derivatization steps. The derivatizing reagent contained 50 % MTBSTFA in acetonitrile for the analysis of benzodiazepines. A “sandwich” injection of derivatizing reagent and eluent was performed by the left tower of the MPS 2 by first aspirating 20 µL of the derivatizing reagent, then aspirating 20 µL of air, then aspirating 50 µL of the eluent from the DPX extraction. The injection was performed relatively slowly at 1.32 µL/s. This allowed the solvent to evaporate from the inlet through the split vent at approximately the speed of injection, eliminating any risk of flooding. The solvent was almost entirely kept outside the GC column and analytes were concentrated inside the injection port .

Derivatization in the sample vial. The eluent from the DPX extraction was dried with nitrogen, then 50 µL of MTBSTFA or BSTFA and 50 µL ethyl acetate were added. The solutions were heated at 70°C for 20 minutes. After cooling, each solution was introduced directly into the inlet using large volume injection (50 µL).

Results and Discussion

The DPX method was chosen for automated extractions because it can be performed in just a few minutes (6 min). Rather than extract and prepare a large number of samples in one batch and letting them wait for analysis with the associated risk of decomposition, only one sample is extracted at a time. Sample extraction is performed "just in time" during the GC or LC run of the previous sample. When the system becomes ready for the next injection, the next sample has been prepared and is ready to be introduced. This provides the highest possible throughput and system utilization while ensuring that all samples are treated exactly the same. The GC/MS or LC/MS system is never waiting idly for the next sample. Figure 1 shows a picture of the dual rail GERSTEL MPS 2 PrepStation that was used for the automated DPX and large volume injection (LVI).

Figure 1. A picture of the dual rail GERSTEL MPS 2 with DPX automation platform. The left MPS is equipped with a 100 uL syringe for injecting eluent and derivatizing reagents, and the right MPS is equipped with a 2.5 mL syringe for performing DPX extractions.

The automated extractions were performed using DPX-CX tips, which have previously been shown [1] to provide high recoveries of acidic, neutral and basic drugs in spiked urine (Table 1). It should be noted that the spiked urine samples in the previous study were not hydrolyzed. Some urine samples require hydrolysis in

Table 1. Statistical data of basic, acidic, and neutral drugs extracted by DPX-CX with just 0.2 mL of "non-hydrolyzed" urine spiked at 0.5 ppm.

Compound	Ion [m/z]	Internal Standard	Recovery [%]	RSD [%]	R2	*LOD* [µg/mL]
Butabarbital	156	Pentobarbital-d_5	96.3	2.63	0.9988	0.0120
Amobarbarbital	156	Pentobarbital-d_5	99.6	2.57	0.9988	0.0106
Pentobarbital	156	Pentobarbital-d_5	98.6	1.58	0.9985	0.0152
Secobarbital	168	Pentobarbital-d_5	97.6	1.41	0.9995	0.0261
Phenobarbital	204	Pentobarbital-d_5	95.3	2.54	0.9988	0.0129
Glutethimide	189	Pentobarbital-d_5	99.4	2.74	0.9980	0.0216
Merpridine	247	PCP-d_5	93.2	1.43	0.9931	0.0099
Methadone	72	PCP-d_5	96.4	2.65	0.9992	0.0051
Methaqualone	235	PCP-d_5	99.4	1.85	0.9902	0.0134
Amitriptyline	58	PCP-d_5	95.6	1.99	0.9990	0.0038
Cocaine	182	PCP-d_5	103	1.31	0.9961	0.0146
cis-Doxepin	58	PCP-d_5	98.5	1.18	0.9988	0.0200
Imipramine	234	PCP-d_5	96.0	3.72	0.9984	0.0099
trans-Doxepin	58	PCP-d_5	98.5	2.30	0.9987	0.0146
Pentazocine	217	PCP-d_5	95.7	4.37	0.9946	0.0104
Codeine	299	PCP-d_5	105	6.85	0.9864	0.0520
Oxycodone	315	PCP-d_5	111	8.80	0.9761	0.0638
Desipramine	234	PCP-d_5	91.5	9.60	0.9522	0.0742
PCP	200	PCP-d_5	96.8	1.04	0.9992	0.009

order to enable detection of various metabolites. We found that using high buffer concentrations (such as 1 M) for the hydrolysis resulted in poor analyte recovery. We therefore used 0.1 M buffer, which provided ample buffer capacity for hydrolysis of urine specimens.

Whole blood samples are too viscous to be aspirated through the screens of the DPX tips. These samples were first protein precipitated using acetonitrile and then centrifuged, after which the samples could readily be extracted using DPX technology.

When relying on a conventional split/splitless inlet, only a small volume of eluent can be introduced to the GC without impacting the quality of the analysis. This means that a concentration step is often needed prior to injection in order to achieve the required low detection limits. Performing evaporative concentration can be time consuming, even when only small elution volumes are involved.. Instead, we chose to perform Large Volume Injection (LVI) using the GERSTEL Cooled Injection System (CIS). A large volume (50 µL) of the DPX eluent was introduced directly into the CIS and the solvent was evaporated through the split vent, leaving the concentrated analytes in the inlet. To use LVI successfully for high throughput analysis, the eluent must be fairly clean. Otherwise, matrix residue will accumulate in the inlet leading to reduced analyte recovery due to adsorption or breakdown, which will have a negative effect on sensitivity and reproducibility If significant matrix build-up in the inlet liner is experienced, automated liner exchange can be performed after a user defined number of injections. This is possible when using the MPS 2 autosampler equipped with Automated Liner EXchange (ALEX) option. In this case, fortunately, the extracts from the DPX-CX method were very clean; multiple injections could be made with good reproducibility without replacing the inlet liner.

Figure 2 shows total ion chromatograms from the 5th and the 20th injection respectively in a series of injections of whole blood extracts produced using automated DPX. Resulting peak intensities were shown to be comparable. Minimal decline in performance was experienced after approximately 50 such injections.

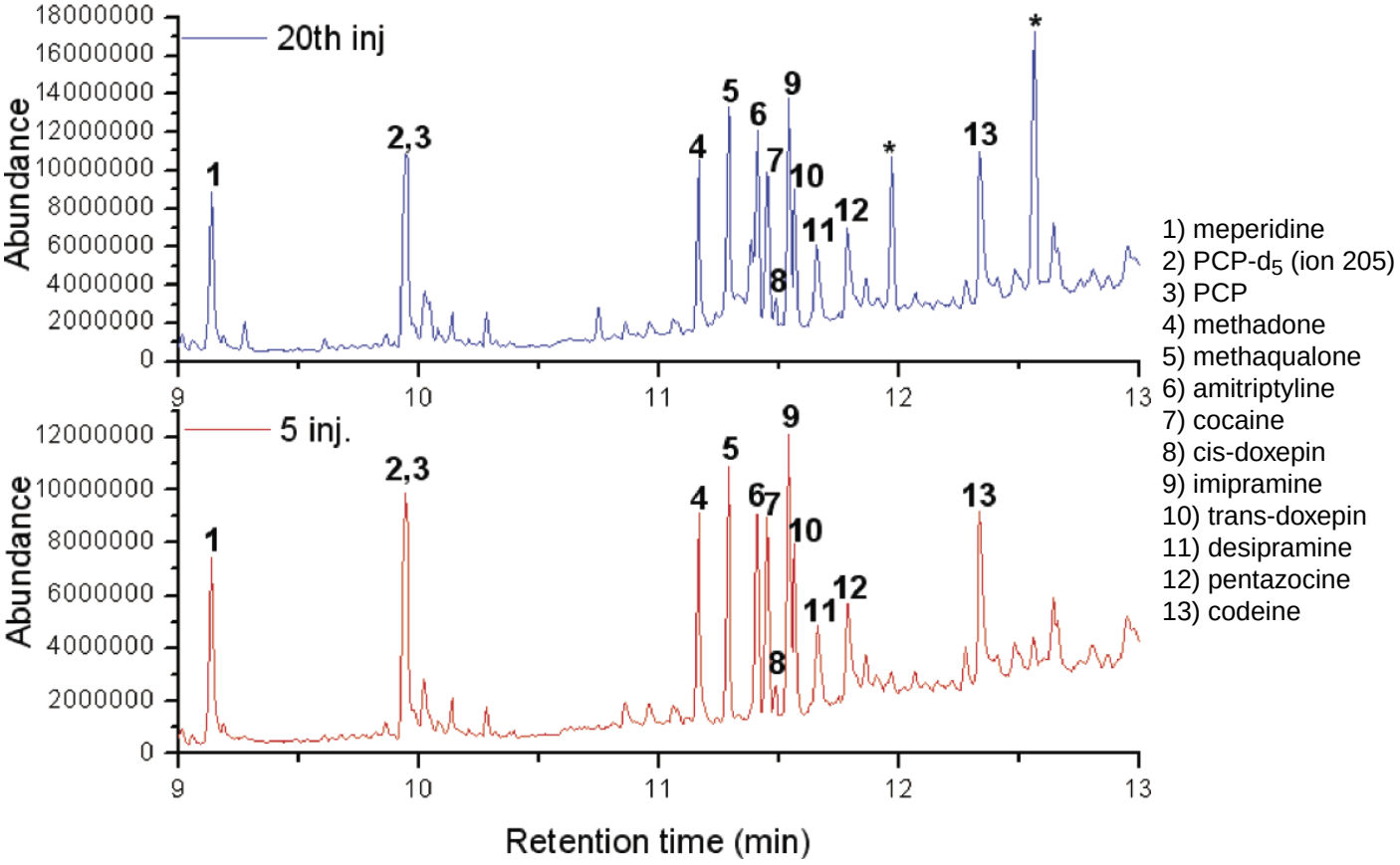


Figure 2. Total ion chromatograms of basic drugs extracted from 0.25 mL of whole blood spiked at 0.5 ppm. The top chromatogram represents the 20th large volume injection of 50 µL of whole blood extracts, and the bottom chromatogram represented the 5th injection. *-Denotes septum bleed from the vial cap from multiple injections.

It should be noted that the chromatograms shown in Figure 2 are obtained from extracts of just 0.25 mL whole blood spiked at 0.5 ppm. Furthermore, the data was acquired in FULL SCAN mode using a GC/MS instrument (Table 2). Based on this small sample volume, limits of detection below 0.1 ppm were obtained for all determined drugs. SIM analysis would allow the limits of detection to be reduced even further. The limited amount of time required per sample makes it possible to perform high throughput analysis "one sample at a time", which again means that samples are not prepared until they can be injected into the analysis system, reducing the wait-time for prepared samples as well as the risk of analyte decomposition.

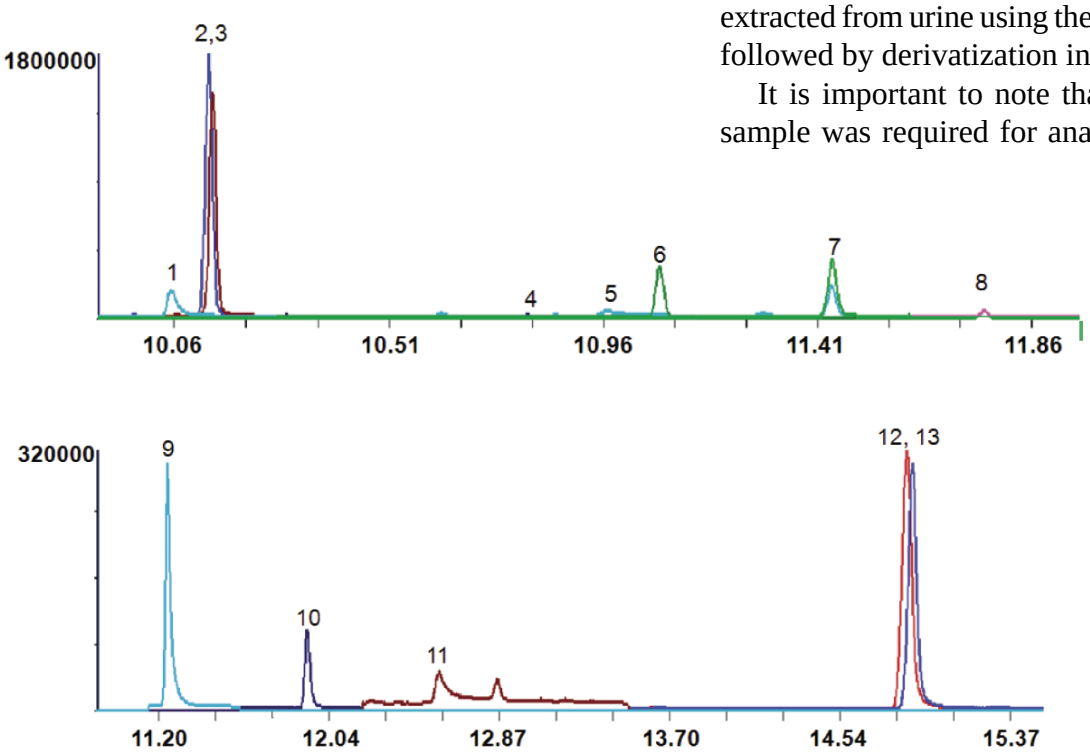
Many analytes require chemical derivatization prior to GC analysis. The CIS inlet provides an inert and heated environment that is well suited for chemical derivatization. Additionally, it has the capability to purge and remove volatile compounds such as the solvent eluent and reagent to ensure that these are not introduced to the GC column. We decided to perform both solvent evaporation and analyte derivatization in the CIS inlet. To derivatize benzodiazepines, 20 µL of 50 % MTBSTFA in acetonitrile was aspirated into the the injection syringe followed by 20 µL of air and finally 50 µL of eluent from the automated DPX sample preparation. The resulting "sandwich" injection was performed slowly in a programmed stop-flow procedure to ensure that the solvent was purged through the split vent prior to introduction of the derivatizing reagent. After the derivatizing reagent had been introduced a further purge followed. The split vent was then closed and the temperature ramped rapidly to 300°C to derivatize and transfer analytes to the GC column in the splitless mode.

Table 2. Statistical data of basic drugs extracted from whole blood using FULL SCAN GC/MS analysis..

Compound	Ion [m/z]	Internal Standard	Recovery [%]	*C.V.* [%]
Meperidine	247	PCP-d_5	94.1	9.20
PCP	200	PCP-d_5	72.5	2.20
Methadone	72	PCP-d_5	69.7	6.30
Methaqualone	235	PCP-d_5	78.9	7.70
Amitriptyline	58	PCP-d_5	70.3	7.20
Cocaine	182	PCP-d_5	67.9	7.70
cis-Doxepin	58	PCP-d_5	78.2	8.80
Imipramine	234	PCP-d_5	64.3	6.30
trans-Doxepin	58	PCP-d_5	110	3.20
Desipramine	234	PCP-d_5	52.0	14.1
Pentazocine	217	PCP-d_5	86.9	16.8
Codeine	299	PCP-d_5	67.0	9.60

Derivatization in the inlet was successful for several benzodiazepines in blood, but a few benzodiazepines were not easily derivatized using this method and derivatization of these was therefore performed in the sample vial. Figure 3 shows results for benzodiazepines extracted from urine using the automated DPX method followed by derivatization in the sample vial.

It is important to note that only 0.2 mL of urine sample was required for analysis at 0.2 ppm. LODs



1) diazepam
2) nordiazepam
3) d_5-Nor TBDMS
4) flunitrazepam
5) 7-aminoflunitrazepam
6) oxazepam-2TBDMS
7) temazepam-TBDMS
8) lorazepam-2TBDMS
9) nitrazepam-TBDMS
10) clonazepam-TBDMS
11) alprazolam
12) OH-alprazolam
13) d_5-OH-alprazolam-TBDMS

Figure 3. Extracted ion chromatograms of benzodiazepines extracted from 0.2 mL of hydrolyzed urine sample at 0.2 ppm. This derivatization was performed with MTBSTFA.

for most of the benzodiazepines were approximately 1 ng/mL and LODs were below 20 ng/mL for all 22 drugs determined (Table 3).

Extraction of opiates using the automated DPX method was successful and resulted in high recoveries for all compounds in the test. For opiates, inlet derivatization using BSTFA was attempted, but proved unsuccessful. Figure 4 shows chromatograms of opiates from whole blood samples, and Table 4 shows the statistical results from the opiate analysis. The majority of the resulting limits of detection were calculated to be less than 1 ng/mL based on analysis of just 0.25 mL of whole blood sample. Derivatization of opiates was performed in the sample vial rather than in the GC inlet, the process was automated using the MPS 2.

Table 3. Statistical data of benzodiazepines extracted from "hydrolyzed" urine samples and derivatized with MTBSTFA.

Compound	Recovery [%]
Diazepam	96.7
Nordiazepam	82.1
Flunitrazepam	35.6
Oxazepam	54.4
Temazepam	52.0
Lorazepam	15.8
Alprazolam	62.1
OH-Alprazolam	71.3
7-Amino-flunitrazepam	38.3
Nitrazepam	79.1
Clonazepam	52.3

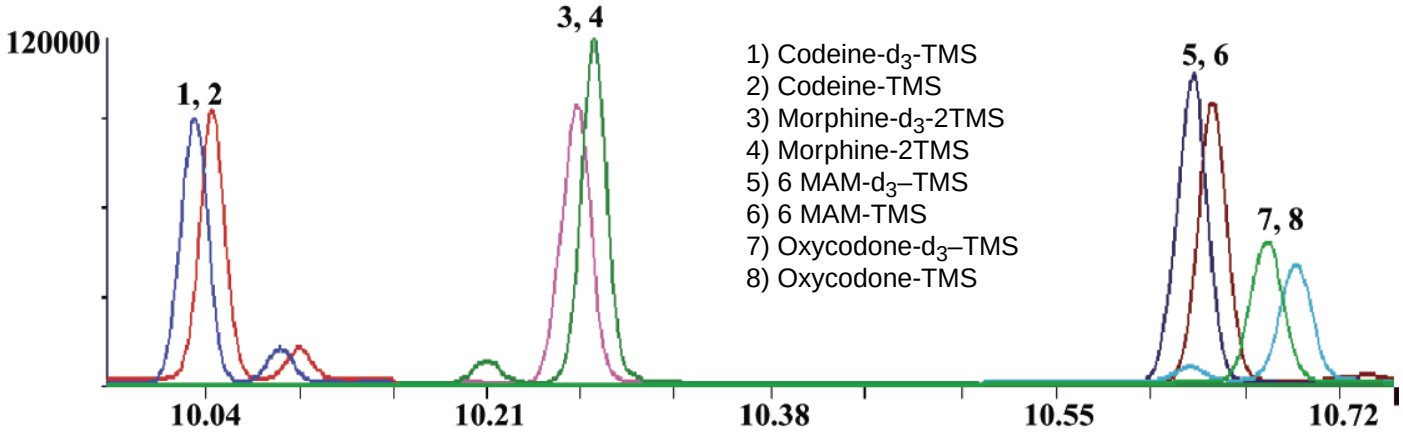


Figure 4. Extracted ion chromatograms of opiates extracted from 0.25 mL of whole blood at 0.1 ppm. This derivatization was performed with BSTFA.

Table 4. Statistical data of opiates extracted from whole blood using just 0.25 mL with extracts derivatized with BSTFA.

Compound	Ion [m/z]	External Standard	Recovery [%]
Hydrocodone	299	Hydrocodone-d_3	99.9
Codeine-TMS	371	Codeine-d_3	89.3
Morphine-2TMS	429	Morphine-d_3	85.6
6-MAM-TMS	399	6-MAM-d_3	87.7
Oxycodone-TMS	387	Oxycodone-d_5	86.2

Minimal sample pre-treatment was required prior to automated DPX. Whole blood specimens required protein precipitation with acetonitrile, while urine specimens required hydrolysis. The rest of the analysis was completely automated using a dual rail GERSTEL MPS 2 PrepStation with a cooled injection system (CIS) used for large volume injection (LVI). The automated extractions were performed using 1 mL DPX-CX (mixed-mode) tips. The time required per sample for complete DPX extraction was approximately 6 minutes.

Conclusions

- The DPX technique enables rapid sample preparation and it is readily automated using the GERSTEL MPS 2; the MAESTRO software makes it easy to set up the DPX methods for "Ready for Analysis" sample preparation.
- DPX was used successfully to determine numerous basic drugs that don't require derivatization, such as cocaine, methadone, PCP, TCAs, and meperidine.
- Most benzodiazepines could readily be determined using derivatization with MTBSTFA in the GC inlet. These include diazepam, nordiazepam, oxazepam, temazepam, alprazolam, and α-OH-alprazolam.
- Automated DPX combined with GC/MS was shown to provide excellent results for the determination of 11 benzodiazepines in urine.
- High recoveries and high sensitivity was shown for the determination of opiates; for most of these, limits of detection were below 1 ng/mL in whole blood.
- The GERSTEL CIS inlet was shown to provide excellent results, when used for combined concentration and derivatization steps during the injection process.
- Automated DPX and sample introduction using the dual rail GERSTEL MPS 2 PrepStation provides the highest throughput available.

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[1] S. T. Ellison, W. E. Brewer, and S. L. Morgan, "Comprehensive Analysis of Drugs and Metabolites in Urine with Disposable Pipette Extraction", PittCon 2008, New Orleans, LA.

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