

Application Note

Utilizing an Automated Platform to Streamline Workflow Processes: Sample Preparation and Extraction for Definitive Drug Testing on the Hamilton Microlab STAR

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For forensic toxicology use only.

This is an Application Brief and does not contain a detailed Experimental section.

Abstract

This work aims to provide a practical and broadly applicable, automated SPE strategy for the accurate and reproducible quantification of drugs of abuse and pain management drugs from urine samples in support of clinical research in forensic toxicology. A previously validated method was transferred to the Hamilton Microlab STAR which performed all sample pretreatment in addition to the full SPE procedure. These automation strategies simplify and streamline the sample preparation workflow, maximize productivity, and reduce risk of human error, while ensuring peak analytical performance.

Benefits

- Automation of repetitive tasks
 - Reduces risk of manual error
 - Robust and reproducible method for SPE
 - Frees up scientist time to perform other tasks
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Introduction

Liquid chromatography coupled with mass spectrometry (LC-MS/MS) is now a common technique for rapid analysis of multiple compounds in a single acquisition. However, despite advances in instrumental analysis, sample preparation can be a rate limiting step and a source of errors in the overall bioanalysis workflow.¹

Driven by analytical sensitivity, selectivity, and robustness requirements for LC-MS/MS bioanalysis, the choice of sample preparation techniques commonly include simple dilution, protein precipitation (PPT), liquid-liquid extraction (LLE), supported liquid extraction (SLE), and solid-phase extraction (SPE).

Development, optimization, and implementation of these methods, especially for larger analyte panels, can prove to be time consuming and difficult to transfer between scientists and laboratories. Employing fully automated devices such as the Hamilton Microlab STAR frees up the analyst to concentrate on other tasks by streamlining the sample preparation process. Perhaps more important is the possibility of reducing

human errors such as mis-spikes, internal standard addition errors, inconsistencies in technique, and sample transfer errors.²⁻⁵ In turn this improves analytical method reproducibility and consistency.

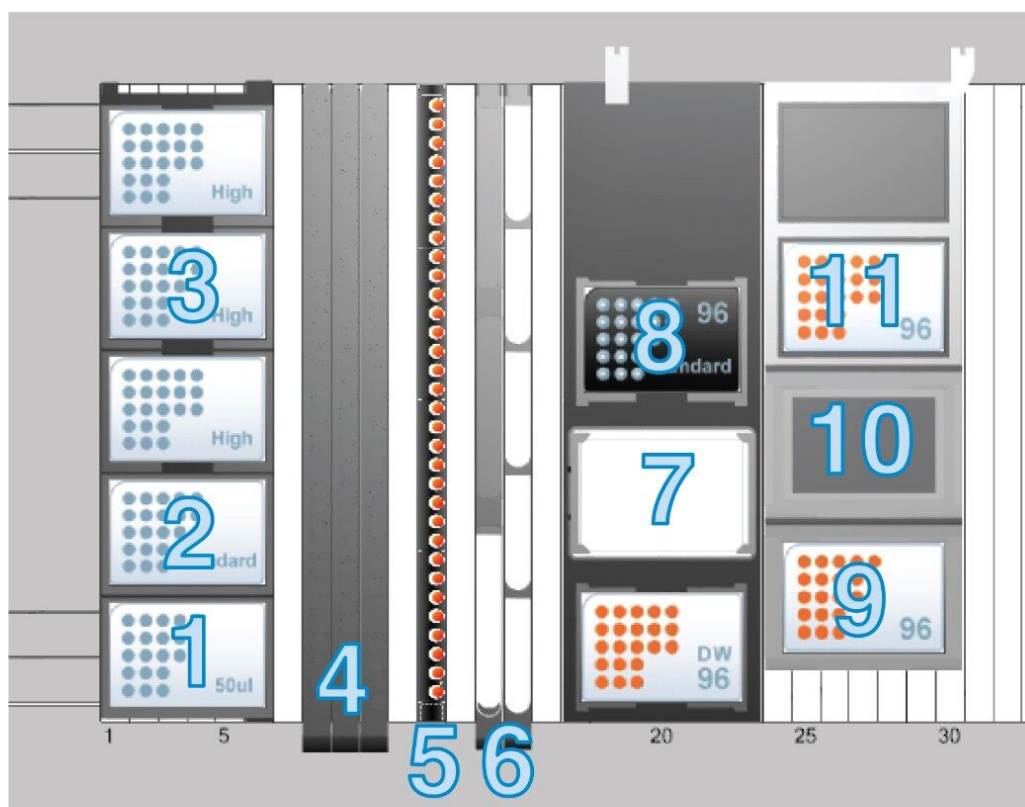
This work aims to provide a practical and broadly applicable, automated SPE strategy for the accurate and reproducible quantification of drugs of abuse and pain management drugs from urine samples in support of clinical research in forensic toxicology. A previously validated method was transferred to the Hamilton Microlab STAR which performed all sample pretreatment in addition to the full SPE procedure.⁶ These automation strategies simplify and streamline the sample preparation workflow, maximize productivity, and reduce risk of human error, while ensuring peak analytical performance.

Experimental

All standards were obtained from Cerilliant (Round Rock, TX) and Cayman Chemical (Ann Arbor, MI). A mixed stock solution was prepared in methanol at concentrations of 2, 10, and 25 µg/mL, depending upon the analyte. Stable isotope labeled standards were used as internal standards (IS). The IS stock solution was prepared in methanol at a concentration of 1 µg/mL.⁶ Samples were prepared by diluting stock solutions into pooled, blank urine.

Sample Extraction

All calibration standards and quality control samples were prepared manually in pooled blank urine prior to solid-phase extraction. The Hamilton Microlab STAR deck layout and accessories used for this extraction process are shown in Figure 1. The extraction process was also carried out manually to compare accuracy and precision across the four QC levels. For pretreatment, the STAR liquid handler adds 100 µL of IS in hydrolysis buffer followed by 100 µL of urine into individual wells of the Oasis MCX µElution Plate and aspirates to mix the samples. After incubation, the STAR adds 200 µL of 4% H₃PO₄ and aspirates to mix. The samples are loaded onto the sorbent bed under vacuum on the STAR deck and subsequently washed with 200 µL of 20% MeOH in H₂O. The sorbent is dried under high vacuum. The samples are eluted with 2 x 25 µL of 50:50 ACN:MeOH containing 5% strong ammonia solution (28-30%). All samples are diluted with 150 µL of 97:2:1 H₂O:ACN:formic acid and mixed on the heater-shaker for 3 minutes prior to removal for analysis on LC-MS/MS. A detailed visual of the workflow process can be seen in Figure 2. The entire automated SPE process explained in detail above comes as a readily available script that can be easily implemented to any Hamilton Microlab STAR or STARlet configuration.



- | | |
|---------------------------------|---|
| 1. 50 µL Tip carrier | 7. Hamilton Heater Shaker (HHS) |
| 2. 300 µL Tip carrier | 8. Tip storage location |
| 3. 1000 µL Tip carrier | 9. Vacuum manifold |
| 4. Sample tube rack | 10. MCX µElution SPE plate place holder |
| 5. IS Buffer tube rack | 11. 700 µL Round-well collection plate |
| 6. MCX reagent reservoir holder | |

Figure 1. A representation of the layout for Hamilton Microlab STAR with accessories.

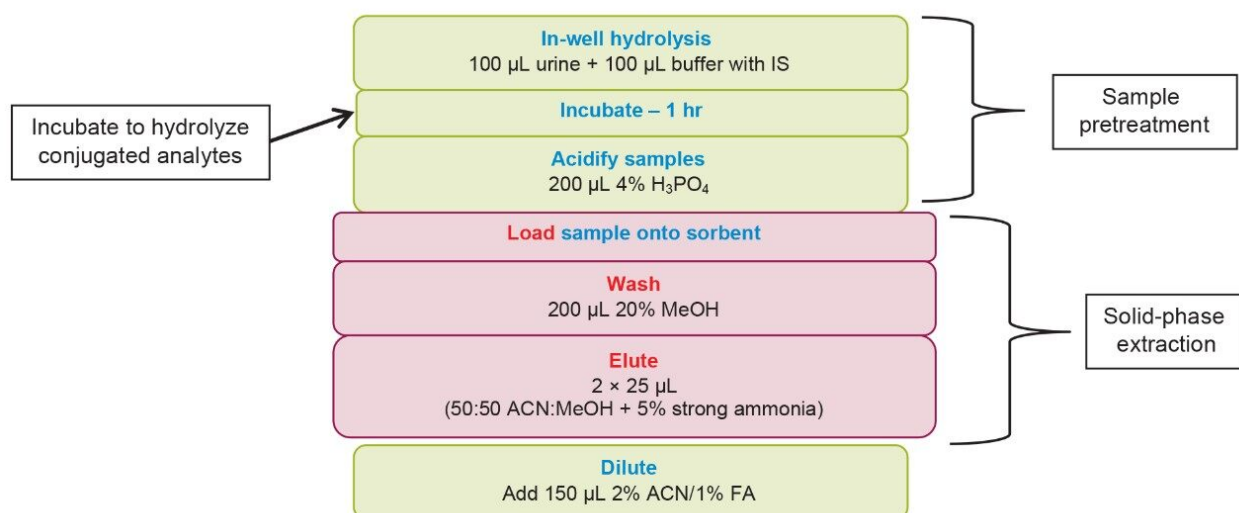


Figure 2. The workflow for the addition of internal standard, incubation, and entire SPE process.

LC-MS/MS Conditions

LC system:	ACQUITY UPLC I-Class (FTN)
Detection:	Xevo TQ-S micro ESI+
Column:	ACQUITY UPLC BEH C ₁₈ , 1.7 µm, 2.1 x 100 mm (p/n: 186002352)
Temp.:	40 °C
Sample temp.:	10 °C
Injection volume:	5 µL
Mobile phases:	A: 0.1% formic acid in water B: 0.1% formic acid in acetonitrile
Purge solvent:	50% methanol in H ₂ O

Wash solvent:

25:25:25:25 MeOH:H₂O:IPA:ACN

Gradient:

Time (min)	Flow (mL/min)	%MPA	%MPB
0.00	0.6	98	2
3.33	0.6	33	67
3.50	0.6	10	90
3.60	0.6	98	2
4.00	0.6	98	2

MS Conditions

Capillary: 1.0 kV

Desolvation temp.: 500 °C

Cone gas flow: 150 L/Hr

Desolvation gas flow: 1000 L/Hr

The following parameters were optimized for specific compounds: Acquisition range, cone voltage, MRM transitions, and collision energy. These parameters can be found in Appendix 1 of Waters application note 720006187EN <<https://www.waters.com/waters/library.htm?cid=511436&lid=134965859>> .

Data Management

Hamilton control software:	Venus 3
Instrument control software:	MassLynx v4.2
Quantification software:	TargetLynx

Results and Discussion

Quantitative Analysis

Prior to quantitative analysis, an analyte recovery experiment was conducted for comparison on a manual platform against the Hamilton Microlab STAR to prove robustness of the validated method on an automated platform. The recovery results were comparable between the two platforms deeming the use of automation for sample extraction to be as effective as manual extraction.

Pooled urine samples were extracted in three batches on three different days. A seven-point calibration curve was extracted in duplicate and quality control samples at four different concentrations were extracted in replicates of six. For most compounds, quality control samples were prepared at 15, 75, 250, and 750 ng/mL, with compounds in the lower concentration range at 3, 15, 50, and 150 ng/mL, and compounds in the higher concentration range at 37.5, 187.5, 625, and 1875 ng/mL. The calibration ranges for each compound can be found in Table 1 of Waters application note [720006187EN <https://www.waters.com/waters/library.htm?cid=511436&lid=134965859>](https://www.waters.com/waters/library.htm?cid=511436&lid=134965859). The acceptance criteria for each individual calibrator was within $\pm 15\%$ of target values, except for the lowest point at 20%. Acceptance criteria for quality control samples was within 15% except for the lowest QC at 20%. These are in line with SWGTOX guidelines⁷ and FDA bioanalytical method validation requirements.⁸ A summary of inter-day results across the three batches can be seen in Appendix 1. All compounds met the criteria above and %RSDs for most compounds were less than 5%. A summary of intra-day results for batch #3 can be seen in Appendix 2. All compounds (except for 7-aminoclonazepam 117%, buprenorphine 131%, diazepam 116%) met criteria with %RSDs for most compounds less than 5%. All compounds had R^2 values of greater than 0.99.

Comparative Analysis

All individual samples were extracted twice for every batch. One aliquot of each calibrator and quality control sample was processed using the Hamilton Microlab STAR, and one aliquot of each calibrator and quality

control sample was processed manually. The purpose of this was to prove the reliability of the automated platform to perform an extraction that will give accuracy and precision within the expected range of acceptance criteria. The results for QC 2 across the three batches for manual versus automated sample preparation is shown in Figure 3. When comparing manual versus automated sample extraction, the results are comparable if not better for accuracy and %RSD for individual compounds on the STAR.

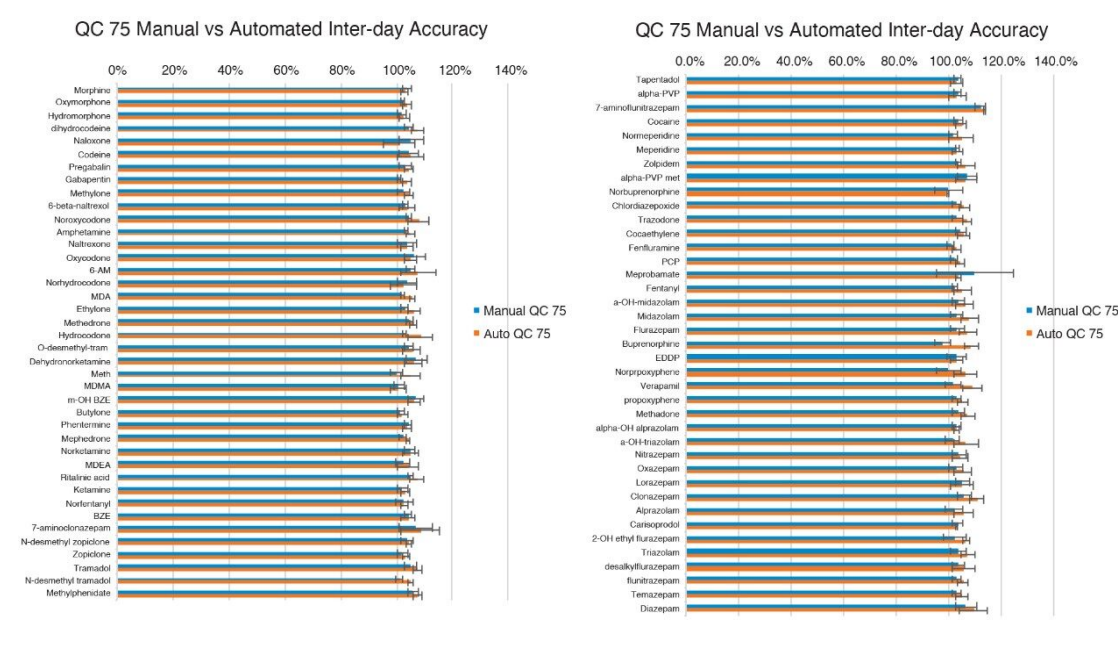


Figure 3. The comparison of accuracies and %error for QC2 between the manual and automated platform.

Conclusion

To address the issue of the rate limiting step that is sample preparation, the use of the Hamilton Microlab STAR to automate the pretreatment and subsequent extraction process of a large panel of drugs of abuse and pain management drugs in urine proved most effective. The solution offers a simple and fast SPE process for a comprehensive panel of toxicological compounds. A ready-made script, in combination with a previously validated SPE and analytical method enables rapid implementation of the entire process. This fully automated sample preparation approach provides robust and reproducible quantitative performance with R^2 values greater than 0.99, QC accuracies between 85-115% (80-120% for low QC) for all compounds, and %RSDs under 10% for most compounds. Analyst associated errors, such as analyst inconsistency, sample

transfer, IS additions, and labelling errors, are effectively minimized. Accurate quantification was achieved using a simple yet robust fully automated sample preparation and SPE protocol.

References

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Appendix 1

Compound name	Inter-day accuracy											
	QC 15			QC 75			QC 250			QC 750		
	Mean	SD	%RSD	Mean	SD	%RSD	Mean	SD	%RSD	Mean	SD	%RSD
Morphine	102.9%	0.5%	0.5%	102.8%	1.2%	1.1%	102.2%	1.5%	1.4%	101.2%	1.5%	1.5%
Oxymorphone	102.6%	1.5%	1.5%	103.6%	1.7%	1.6%	102.4%	0.6%	0.6%	102.8%	1.3%	1.3%
Hydromorphone	101.7%	3.0%	2.9%	102.7%	1.7%	1.7%	101.1%	1.0%	1.0%	102.5%	0.2%	0.2%
Dihydrocodeine	100.5%	5.6%	5.6%	107.7%	2.3%	2.1%	102.7%	2.8%	2.7%	101.2%	1.4%	1.4%
Naloxone	103.7%	16.2%	15.6%	101.0%	5.6%	5.6%	97.7%	4.0%	4.1%	104.6%	8.4%	8.0%
Codeine	100.7%	4.7%	4.7%	105.0%	4.9%	4.6%	100.6%	4.1%	4.0%	104.7%	3.4%	3.2%
Pregabalin	99.1%	7.2%	7.3%	104.2%	1.6%	1.5%	103.4%	0.4%	0.3%	102.6%	4.3%	4.2%
Gabapentin	101.9%	2.4%	2.4%	103.6%	1.5%	1.4%	101.9%	2.2%	2.1%	102.1%	1.3%	1.3%
Methylone	103.1%	2.1%	2.1%	104.5%	1.5%	1.5%	102.7%	1.8%	1.8%	101.2%	1.3%	1.3%
6-beta-naltrexol	100.9%	2.3%	2.3%	103.7%	3.1%	3.0%	99.9%	3.0%	3.0%	100.9%	1.7%	1.7%
Noroxycodone	105.2%	7.4%	7.0%	107.8%	3.9%	3.6%	101.7%	2.6%	2.5%	99.0%	0.8%	0.6%
Amphetamine	101.0%	2.1%	2.1%	105.1%	1.7%	1.6%	102.8%	3.3%	3.2%	100.2%	0.8%	0.8%
Naltrexone	99.6%	7.1%	7.2%	103.7%	2.2%	2.1%	98.4%	2.5%	2.6%	100.3%	1.5%	1.5%
Oxycodone	103.5%	2.6%	2.5%	105.0%	2.3%	2.2%	100.1%	2.3%	2.3%	97.3%	0.9%	0.9%
6-AM	103.0%	2.6%	2.5%	107.6%	6.6%	6.1%	104.8%	5.5%	5.3%	103.4%	1.2%	1.2%
Norhydrocodone	100.3%	6.0%	5.9%	102.3%	4.8%	4.7%	102.0%	3.8%	3.7%	103.2%	6.4%	6.3%
MDA	100.3%	6.5%	6.5%	105.6%	1.0%	0.9%	102.9%	2.0%	2.0%	101.3%	1.4%	1.4%
Ethylone	101.6%	1.1%	1.1%	106.0%	2.4%	2.2%	103.2%	2.2%	2.1%	102.0%	0.8%	0.8%
Methedrone	102.4%	3.1%	3.0%	105.9%	1.2%	1.2%	102.3%	0.3%	0.3%	102.1%	1.9%	1.9%
Hydrocodone	104.3%	1.5%	1.4%	108.6%	4.8%	4.4%	104.1%	3.0%	2.9%	102.7%	2.6%	2.6%
O-desmethyl-tramadol	103.4%	2.8%	2.7%	105.3%	3.0%	2.9%	101.9%	2.2%	2.2%	100.9%	1.3%	1.3%
Dehydronorketamine	103.4%	2.0%	1.9%	106.0%	3.3%	3.1%	102.4%	2.9%	2.8%	101.8%	1.0%	1.0%
Meth	100.9%	2.4%	2.4%	105.2%	3.7%	3.5%	103.2%	5.6%	5.4%	99.8%	3.6%	3.6%
MDMA	101.8%	4.5%	4.4%	100.5%	3.0%	3.0%	101.1%	4.5%	4.5%	102.4%	3.3%	3.2%
m-OH BZE	100.2%	2.0%	2.0%	106.3%	2.4%	2.2%	100.8%	1.1%	1.1%	101.0%	1.2%	1.1%
Butylone	103.6%	1.9%	1.8%	102.1%	2.1%	2.1%	101.1%	1.9%	1.9%	102.8%	1.5%	1.5%
Phentermine	99.0%	1.6%	1.6%	103.7%	1.6%	1.5%	101.5%	2.2%	2.1%	101.0%	4.2%	4.1%
Mephedrone	102.8%	3.0%	2.9%	104.1%	0.6%	0.6%	102.7%	1.3%	1.3%	103.1%	1.7%	1.6%
Norketamine	101.1%	2.9%	2.8%	105.1%	2.8%	2.7%	102.6%	2.5%	2.4%	102.1%	1.7%	1.7%
MDEA	103.1%	2.3%	2.2%	104.2%	3.7%	3.6%	101.4%	4.5%	4.4%	101.7%	3.5%	3.4%
Ritalinic Acid	101.5%	0.9%	0.9%	107.4%	2.7%	2.5%	102.8%	2.2%	2.2%	100.5%	2.6%	2.6%
Ketamine	102.6%	1.2%	1.2%	103.2%	1.8%	1.6%	101.7%	1.1%	1.0%	102.5%	1.8%	1.8%
Norfentanyl	100.7%	3.2%	3.2%	102.7%	1.3%	1.3%	101.1%	2.7%	2.7%	102.8%	2.6%	2.5%
BZE	102.9%	1.2%	1.2%	104.1%	2.8%	2.7%	101.6%	2.1%	2.1%	102.1%	0.9%	0.9%
7-aminoclonazepam	99.0%	2.1%	2.1%	108.6%	7.4%	6.8%	103.4%	2.4%	2.3%	98.1%	3.0%	3.1%
N-desmethyl zopiclone	105.7%	3.6%	3.4%	104.4%	1.0%	1.0%	102.7%	1.6%	1.5%	104.3%	5.2%	5.0%
Zopiclone	104.0%	2.4%	2.3%	103.3%	1.3%	1.3%	100.2%	0.9%	0.9%	102.4%	0.2%	0.2%
Tramadol	101.6%	1.6%	1.5%	107.3%	1.6%	1.5%	102.5%	2.1%	2.0%	100.5%	2.8%	2.8%
N-desmethyl tramadol	102.8%	2.1%	2.1%	105.0%	1.0%	0.9%	103.3%	2.0%	1.9%	101.3%	1.7%	1.7%
Methylphenidate	103.4%	1.8%	1.7%	107.5%	1.6%	1.5%	102.3%	2.5%	2.5%	96.4%	2.1%	2.2%
Tapentadol	102.6%	0.7%	0.7%	103.1%	2.7%	2.6%	102.2%	2.0%	1.9%	102.3%	1.0%	1.0%
Alpha-PVP	102.4%	1.2%	1.1%	103.5%	3.6%	3.5%	102.5%	3.0%	2.9%	102.1%	0.2%	0.2%
7-aminoflunitrazepam	103.1%	6.5%	6.3%	114.2%	0.4%	0.3%	100.8%	2.6%	2.6%	95.0%	2.6%	2.7%
Cocaine	102.3%	1.1%	1.1%	105.0%	2.1%	2.0%	102.4%	2.3%	2.2%	102.0%	1.6%	1.5%
Normeperidine	104.1%	1.8%	1.8%	105.2%	5.0%	4.7%	103.1%	3.7%	3.6%	103.4%	3.0%	2.9%
Meperidine	102.8%	1.8%	1.8%	103.5%	1.9%	1.8%	101.6%	1.6%	1.6%	102.2%	0.7%	0.7%
Zolpidem	105.8%	2.6%	2.5%	106.7%	3.6%	3.4%	104.6%	2.9%	2.7%	103.9%	2.3%	2.2%
Alpha-PVP mot	109.6%	3.9%	3.5%	106.7%	4.2%	3.9%	104.9%	3.7%	3.5%	108.2%	3.1%	2.9%
Norbuprenorphine	102.2%	20.0%	19.6%	99.4%	0.2%	0.2%	102.4%	6.2%	6.0%	106.5%	2.2%	2.1%
Chlordiazepoxide	104.8%	1.2%	1.1%	106.0%	2.1%	2.0%	104.1%	3.3%	3.2%	104.1%	2.7%	2.6%
Trazodone	111.1%	6.8%	6.1%	107.2%	2.0%	1.9%	105.9%	3.0%	2.8%	106.4%	2.3%	2.2%
Cocaehtylene	102.1%	1.1%	1.0%	105.8%	2.4%	2.3%	102.0%	1.2%	1.2%	102.2%	1.7%	1.6%
Fenfluramine	103.4%	2.0%	1.9%	103.4%	1.8%	1.7%	102.2%	1.8%	1.8%	102.7%	1.3%	1.3%
PCP	102.6%	2.1%	2.1%	104.4%	1.8%	1.7%	102.5%	2.0%	1.9%	101.0%	1.4%	1.4%
Meprobamate	103.5%	3.0%	2.9%	104.0%	0.9%	0.9%	98.2%	2.0%	2.7%	106.4%	1.7%	1.6%
Fentanyl	107.0%	3.5%	3.3%	106.4%	3.7%	3.5%	103.3%	1.9%	1.9%	104.5%	2.2%	2.1%
a-OH-midazolam	110.3%	3.3%	3.0%	106.3%	3.6%	3.4%	104.1%	1.6%	1.5%	103.1%	2.1%	2.1%
Midazolam	111.9%	4.6%	4.1%	108.1%	3.7%	3.4%	106.8%	3.2%	3.0%	106.3%	3.5%	3.3%
Flurazepam	109.0%	4.1%	3.8%	107.3%	3.7%	3.4%	108.3%	3.9%	3.6%	105.8%	1.7%	1.6%
Buprenorphine	120.7%	10.6%	8.7%	108.8%	2.8%	2.5%	106.8%	0.7%	0.7%	109.7%	2.4%	2.2%
EDDP	104.1%	1.3%	1.2%	103.3%	2.3%	2.3%	103.0%	3.3%	3.2%	101.8%	1.8%	1.8%
Norprorxyphene	107.2%	4.0%	3.7%	106.6%	4.8%	4.5%	103.7%	4.0%	3.8%	102.5%	5.0%	4.9%
Verapamil	117.5%	4.8%	4.1%	109.5%	4.0%	3.7%	111.2%	2.6%	2.4%	109.6%	2.4%	2.2%
Propoxyphene	110.1%	3.0%	2.7%	105.3%	2.1%	2.0%	104.8%	1.6%	1.5%	105.2%	2.4%	2.3%
Methadone	112.5%	3.6%	3.2%	107.3%	2.9%	2.7%	107.1%	2.6%	2.4%	105.3%	2.8%	2.6%
Alpha-OH alprazolam	106.1%	7.5%	7.1%	103.3%	0.9%	0.9%	103.2%	2.8%	2.7%	103.6%	6.0%	5.8%
a-OH-triazolam	105.5%	4.5%	4.3%	106.6%	5.0%	4.7%	105.2%	2.4%	2.3%	102.3%	2.4%	2.4%
Nitrazepam	108.1%	0.7%	0.6%	104.3%	3.1%	3.0%	102.3%	1.7%	1.6%	104.3%	2.7%	2.6%
Oxazepam	101.6%	5.9%	5.8%	105.8%	3.6%	3.4%	104.3%	2.5%	2.4%	102.5%	3.6%	3.5%
Lorazepam	102.7%	4.5%	4.4%	105.1%	4.5%	4.3%	103.5%	3.2%	3.1%	103.4%	1.6%	1.5%
Clonazepam	107.1%	3.7%	3.4%	111.1%	3.0%	2.7%	105.8%	3.6%	3.4%	104.4%	2.4%	2.3%
Alprazolam	103.0%	4.8%	4.7%	105.9%	3.6%	3.4%	101.7%	2.8%	2.7%	100.8%	2.2%	2.2%
Carisoprodol	101.3%	3.6%	3.5%	102.9%	0.4%	0.4%	102.1%	0.7%	0.6%	102.7%	1.2%	1.1%
2-OH ethyl flurazepam	105.6%	5.0%	4.7%	106.9%	1.2%	1.1%	104.4%	1.8%	1.7%	104.0%	2.6%	2.5%
Triazolam	105.1%	2.8%	2.6%	107.5%	3.0%	2.8%	105.3%	1.6%	1.6%	105.6%	1.0%	0.9%
Desalkylflurazepam	107.0%	3.9%	3.6%	105.7%	4.4%	4.2%	103.5%	3.5%	3.4%	102.6%	1.4%	1.3%
Flunitrazepam	103.3%	2.0%	2.0%	105.6%	2.3%	2.1%	102.8%	1.8%	1.8%	102.4%	1.8%	1.8%
Temazepam	104.3%	1.8%	1.7%	105.1%	2.6%	2.4%	103.7%	2.7%	2.6%	103.2%	1.6%	1.6%
Diazepam	106.8%	2.1%	2.0%	109.7%	6.0%	5.4%	104.4%	2.7%	2.5%	104.3%	0.7%	0.7%

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