Rapid screening of 65 common drugs and drug metabolites in urine and blood using high-resolution mass spectrometry

Using the SCIEX X500R QTOF System with an ExionLC™ AD System

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Drug abuse has become one of the most serious social issues worldwide as drugs continue to pose a threat to social stability and economic development. As the surge of new designer drugs continues to pose public health and safety problems, drug testing remains one of the most effective measures for global drug control. As some drugs are rapidly metabolized in the body however, the ability to swiftly detect them and their metabolites in the blood and urine of drug users is paramount for law enforcement and testing departments who require comprehensive drug screening approaches with high-level sensitivity and specificity. Here, a method is described that provides reliable and accurate drug intake information to the public, prosecutorial, legal and medical sectors so that the appropriate course of action can be taken following the results of a drug test.

The SCIEX X500R QTOF System is a fast scanning, and high-resolution mass spectrometer that provides forensic laboratories accurate mass analysis for compound screening, and high-resolution secondary spectra for compound confirmation in a single injection. This high resolution mass spectrometer (HRMS) provides the ruggedness, speed, sensitivity and resolution that is essential to support field authority investigations. The X500R QTOF System is therefore ideally suited for trace analysis of drugs and drug metabolites as well as screening and confirmation in complex biological matrices.

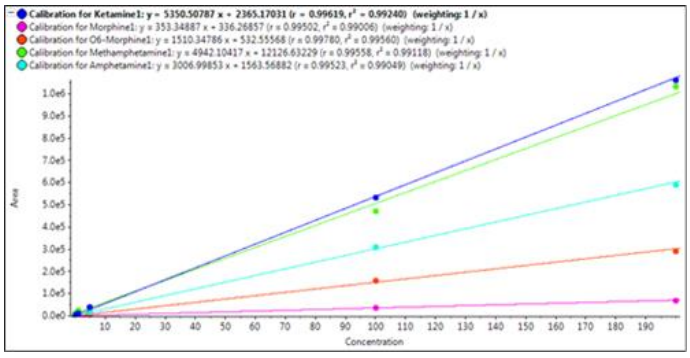


Figure 1. High sensitivity and linearity of the workflow enables accurate quantitation of drugs and drug metabolites from biological samples. Examples of the standard curves generated for five of the drugs in blood and urine samples (amphetamine, methamphetamine, ketamine, 6-monoacetylmorphine and morphine) at 5 ng/mL, 10 ng/mL and 50 ng/mL are shown.

In this technical note, SWATH® Acquisition is combined with MRM^{HR} workflow to generate a comprehensive acquisition method enabling rapid detection, identification, and quantitation of 65 drugs and drug metabolites from urine and blood samples.

Key features of combined acquisition method for detection of drugs and drug metabolites in blood and urine samples

- The X500R QTOF System is a high-resolution accurate mass LC-MS/MS instrument specifically designed for high-throughput testing
- MRM^{HR} acquisition provides accurate and sensitive quantitation of the drugs and drug metabolites such as morphine, 6-monoacetylmorphine, amphetamine, methamphetamine, and ketamine
- SWATH Acquisition generates comprehensive and high-quality MS/MS spectra for screening and confirmation of every detectable drug and metabolite in the sample, enabling confident identification using spectral library searching
- With one injection, the combined SWATH Acquisition with MRM^{HR} provides a reliable, high-speed profiling and quantitation method for 65 common drugs and drug metabolites from biological samples
- Excellent linearity from 0.1 ~ 200 ng/mL is observed for all 65 drugs accurately identified at low concentrations
- Sample preparation is convenient, rapid, and exhibits high recovery for drugs and drug metabolites in biological matrices
- Library matching enabled comprehensive and accurate identification of the 65 common drugs and drug metabolites

Methods

Sample preparation: A total of 65 drugs and drug metabolites were selected for this panel. Table 1 shows a subset of the drugs and drug metabolites analyzed in this study. Drugs and drug metabolites were extracted from blood and urine samples by using a protein precipitation procedure. In short, 500 µL of blood or urine were added to a centrifuge tube to which 1 mL of acetonitrile was added and vigorously vortexed for 2 minutes. The samples were centrifuged for 10 min at 12,000 rpm. 5 µL of the supernatant was injected for analysis. The protein precipitation procedure is summarized in Figure 2.

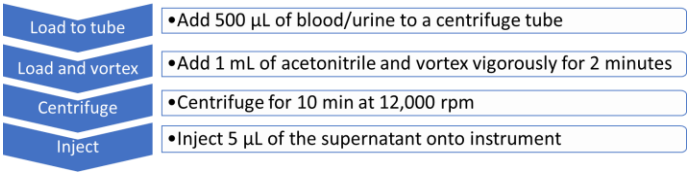


Figure 2. Protein precipitation procedure for blood and urine samples. A 4-step protein precipitation procedure was used for extracting the 65 drugs and drug metabolites from blood and urine samples for analysis with the X500R QTOF System.

Table 1. Subset of drugs and drug metabolites analyzed from blood and urine samples using the simple and fast sample preparation procedure.

Compound name	Molecular formula
Amphetamine	$C_9H_{13}N$
Methamphetamine	$C_{10}H_{15}N$
Pseudoephedrine	$C_{10}H_{15}NO$
Caffeine	$C_8H_{10}N_4O_2$
Ketamine	$C_{13}H_{16}ClNO$
Diphenhydramine	$C_{17}H_{21}NO$
Tetracaine	$C_{15}H_{24}N_2O_2$
Dextromethorphan	$C_{18}H_{25}NO$
Chlorpheniramine	$C_{16}H_{19}ClN_2$
Morphine	$C_{17}H_{19}NO_3$
Codeine	$C_{18}H_{21}NO_3$
Hydrocodone	$C_{18}H_{21}NO_3$
Cocaine	$C_{17}H_{21}NO_4$
Methadone	$C_{21}H_{27}NO$
Oxycodone	$C_{18}H_{21}NO_4$
Naloxone	$C_{19}H_{21}NO_4$
O6-Morphine	$C_{19}H_{21}NO_4$
Papaverine	$C_{20}H_{21}NO_4$
Buprenorphine	$C_{29}H_{41}NO_4$
Dezocine	$C_{16}H_{23}NO$
Meperidine	$C_{15}H_{21}NO_2$
Nortriptyline	$C_{19}H_{21}N$
…….	…….

Liquid chromatography: UHPLC separation was performed on a Phenomenex Kinetex C18 column (50 × 3 mm, 2.6µm, 00B-4462-Y0) held at 40 °C on a SCIEX ExionLC AC System. Mobile phases used consisted of ammonium acetate, acetonitrile, and appropriate additives. The flow rate was 0.3 mL/min. The injection volume was 5 µL and the total LC runtime was 8.5 minutes.

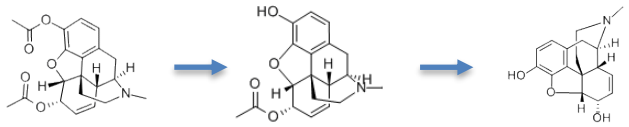
Mass spectrometry: MS and MS/MS data were collected using the SWATH Acquisition combined with MRM^{HR} workflow on the SCIEX X500R QTOF System with SCIEX OS Software 1.5. For the SWATH Acquisition method, data acquisition was TOF MS scan followed by 10 variable Q1 windows covering a mass range from 100 to 620 m/z.

Data analysis: Data processing was performed using SCIEX OS Software 1.5. For the SWATH Acquisition workflow, positive identification of analyte was accomplished based on confidence criteria as previously described.[1] The four main confidence criteria used include mass error (M), retention time (R), isotope ratio difference (I), and library score (L). For the MRM^{HR} workflow, positive identification of analyte was accomplished based on mass error (M) and isotope ratio difference (I) only.

Spectral library: An "in-house" MS/MS spectral library was developed by injecting neat standards of each drug and drug metabolite. The resulting MS/MS spectra were exported and saved to LibraryView™ Software to build the custom library for the 65 drugs and drug metabolites targeted in this study.

Simple and fast sample preparation leads to timely analysis of drugs and metabolites

Drugs can be rapidly metabolized in the human body after being ingested, so drug testing of biological samples such as blood and urine is extremely time sensitive and must be performed as quickly as possible. Moreover, in order to ensure accurate results, specific drug metabolites must also be monitored with (or instead of) the parent drug. For example, the drug heroin is rapidly metabolized into 6-monacetylmorphine by lipolipase and then further metabolized into morphine. Monitoring the presence of heroin only would be insufficient to confidently certify intake of the drug. Instead, drug tests must monitor the heroin metabolites 6-monoacetylmorphine and morphine. In particular, the metabolite 6-monoethylmorphine has special significance as it can indicate whether the subject has taken heroin recently. Figure 3 shows a depiction of the metabolism of heroin in the human body.



Heroin *6-Monoacetylmorphine* *Morphine*

Figure 3. Metabolism of heroin in the human body.

For those reasons, it is not uncommon for forensic toxicology laboratories to monitor drugs along with their metabolites to increase detection confidence. The resulting expanded panel of drugs and drug metabolites must be monitored and quantified to confirm drug intake by a subject. Luckily, a fast, simple, generic, and comprehensive sample preparation method can be applied to all drugs in the panel to minimize the time and effort required for sample preparation. Lasty, because no chromatographic clean-up or concentration is required, less time is spent preparing sample, thereby reducing analysis time to improve laboratory throughput.

Developing a combined SWATH Acquisition with MRM^{HR} Workflow for sensitive detection and quantitation of drugs and metabolites

Control blood and urine samples spiked with the 65 drugs and drug metabolites were prepared at various concentrations ranging from 5 to 50 ng/mL. These standard mixtures were extracted using the aforementioned procedure and injected to build a data processing method.

SWATH Acquisition was used in combination with MRM^{HR} workflow. SWATH Acquisition is a data-independent acquisition strategy that acquires MS/MS data on every detectable compounds in the sample. The acquired MS/MS spectra can be matched to spectral libraries for comprehensive and reliable identification of drugs and their metabolites in complex biological samples. MRM^{HR} is a high-resolution quantification strategy with selectivity and sensitivity similar to triple quadrupole MRM analysis. Because it is high resolution, the technique can effectively overcome matrix interferences from complex biological samples, reduce background noise, and make the quantitative results more accurate and reliable.

Figure 4 shows the extracted ion chromatogram (XIC) for the 65 drugs and drug metabolites. Near baseline separation of all 65 compounds was achieved, including structurally-related compounds eluting at similar retention times.

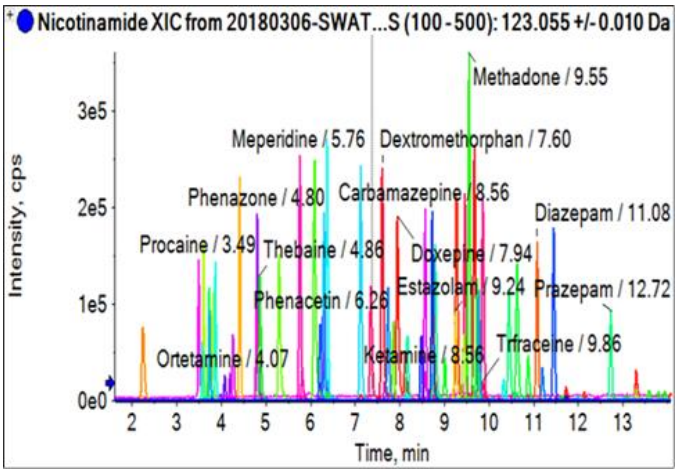


Figure 4. Chromatographic profile of the 65 drugs and drug metabolites targeted in this study. Extracted Ion Chromatogram (XIC) showing baseline separation of the 65 drugs and drug metabolites. The optimized LC conditions in conjunction with the choice of mobile phase composition and column selection resulted in total or near baseline separation of all analytes, including structurally-related compounds.

The quantitative performance of the assay was investigated by injecting a series of samples extracted from blood and urine samples at three concentration levels (5, 10 and 50 ng/mL). Calibration curves were generated to evaluate the sensitivity and linearity of the workflow. Figure 1 shows the calibration curves for amphetamine, methamphetamine, ketamine, 6-monoacetylmorhine and morphine. The curves show a high level of consistency and precision across the calibration series. In addition, excellent linearity across the calibration range was observed with R^2 values above 0.99 for all the drugs and drug metabolites in this panel. Full quantitative analysis was performed using SCIEX OS Software 1.5, designed for quick, intuitive and streamlined data processing with accurate and reliable results.

To assess the efficiendy of the protein precipitation procedure for the blood and urine samples, the recovery was calculated for each of the three concentration levels (5, 10 and 50 ng/mL). The protein precipitation procedure used in this experiment demonstrated recoveries between 77.0% and 118.8% for all the drugs and drug metabolites, which met the requirements for large scale detection of drugs from biological samples. The recovery values for all the drugs and drug metabolites in this panel allowed reliable quantitation, which is only possible through the implementation of an optimized protein precipitation procedure that is compatible for both blood and urine samples. Table 2 summarizes the recovery values at each of the three calibration levels for the 5 compounds shown in Figure 1.

Table 2. Recovery rates for five major drugs and metabolites.

Name	Concentration (ng/mL)	Blood (%)	Urine (%)
Amphetamine	*5*	*78.1*	*86.7*
	10	*105.7*	*104.5*
	50	*94.2*	*103.0*
Methamphetamine	*5*	*86.7*	*90.0*
	10	*106.7*	*103.8*
	50	*96.7*	*104.0*
Ketamine	*5*	*82.5*	*79.0*
	10	*101.1*	*92.8*
	50	*91.7*	*97.0*
6-Monoacetylmorphine	*5*	*84.0*	*83.3*
	10	*105.8*	*90.8*
	50	*95.0*	*96.0*
Morphine	*5*	*77.0*	*93.3*
	10	*111.8*	*107.2*
	50	*101.7*	*105.4*

Combined acquisition strategy leads to accurate identification and quantitation of drugs and drug metabolites in real-world biological samples

The overall robustness of the workflow was further investigated by analyzing five real-world biological samples. Of the five samples, one blood sample (S1) and two urine samples (S2 and S3) tested positive for several drugs and drug metabolites including 6-monoacetylmorphine, morphine, methamphetamine, and codeine. Table 3 summarizes the results from the analysis of the three samples that tested positive for the drugs and drug metabolites aforementioned.

Table 3. Test results for the blood and urine samples.

Name	Blood sample S1 (ng/mL)	Urine sample S2 (ng/mL)	Urine sample S3 (ng/mL)
Methamphetamine	*NA*	*62.3*	*NA*
Codeine	*NA*	*NA*	*4.7*
6-Monoacetylmorphine	*11.6*	*7.9*	*6.4*
Morphine	*27.2*	*24.1*	*34.5*

A few observation can be drawn from the results highlithted in Table 3. First, it should be noted that 6-monoacetylmorphine was detected in all three samples at low concentration, which may be related to the rapid metabolism of this drug metabolite. In addition, positive detection of 6-monoacetylmorphine in all three samples confirms heroin intake while detection of methamphetamine in sample S2 suggests crystal methamphetamine intake.

Another observation is the detection of codeine at low concentration in sample S3. SWATH Acquisition enabled generation of high-quality MS/MS spectra which allowed identification of codeine in sample S3 using spectral library searching. Figure 5 shows the XIC, TOF MS and MS/MS spectra with library search match showing positive identification of codeine from sample S3. The library fit scores (100.0%) provides excellent confidence for the definitive detection of codeine from sample S3. The presence of codeine in sample S3 may be derived from the intake of other drugs or from the consumption of heroin in the presence of monoacetyline and other by-products that may eventually be metabolized into codeine. As a result, the detection of codeine in sample S3 can also be used as a tertimony of heroin intake.

The results from the analysis of real-world biological samples show that the combined SWATH Acquisition with MRM^{HR} workflow enabled sensitive detection and accurate identification of drugs and drug metabolites. The information that can be inferred from the results offer a valuable insight on drug intake, providing health professionals a clearer picture of the long term use of these substances.

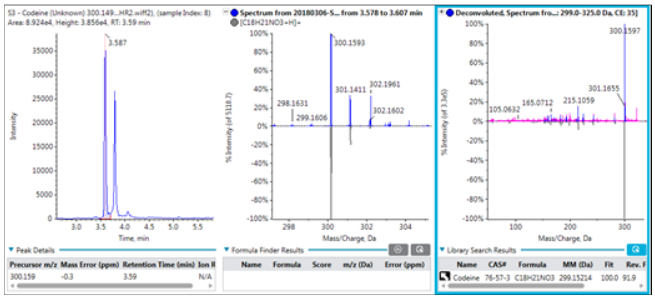


Figure 5. SWATH Acquisition leads to accurate identification of codeine in sample S3. Extracted Ion Chromatogram (XIC), TOF MS and MS/MS spectra showing confident and detailed identification of codeine in sample S3. The excellent MS/MS fit value of 100.0% provides abolsute confidence for the definitive identification of codeine from sample S3.

Conclusions

A comprehensive workflow for the detection of 65 drugs and drug metabolites in blood and urine samples was successfully developed using the SCIEX X500R QTOF System. The combination of a simple protein precipitation procedure and a unique SWATH Acquisition with MRM^{HR} workflow enabled high sensitivity detection of drugs and drug metabolites in biological samples.

- A simple sample preparation procedure enabled efficient extraction of drugs and drug metabolites from blood and urine samples
- The “in-house” MS/MS spectral library was used to identify the presence of drugs and drug metabolites using spectral library searching
- The combined SWATH Acquisition with MRM^{HR} workflow enabled sensitive and accurate detection of drugs and drug metabolites
- The high sensitivity and selectivity of MRM^{HR} allowed accurate quantitation of drugs and drug metabolites while SWATH Acquisition enabled generation of high-quality MS/MS spectra and accurate identification of the analytes using spectral library searching
- Analyte recoveries were found to be between 77.0% and 118.8% for all the drugs and drug metabolites, which met the requirements for large scale detection of drugs from biological samples
- The workflow showed excellent linearity resulting in R^2 values above 0.99 for all 65 drugs and drug metabolites
- This method was able to rapidly detect and quantify drugs in the blood and urine of real-worl-world subjects, providing evidence for drugs of abuse intake such as heroin and crystal methamphetamine

References

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