Rapid Identification and Quantification of Performance-Enhancing Stimulants in Human Urine Using High-Resolution Mass Spectrometry

Using SWATH® Acquisition on the SCIEX X500R QTOF System

Pierre Negri[1], Enrico Gerace[2], Daniele Di Corcia[2], Marco Vincenti[2,3], and Alberto Salomone[2,3]
[1]SCIEX, USA; [2]Centro Regionale Antidoping e di Tossicologia "A. Bertinaria", Orbassano, Turin, Italy; [3]Dipartimento di Chimica, Università degli Studi di Torino, Turin, Italy

Stimulants are performance-enhancing substances that are commonly used by athletes to reduce fatigue, improve endurance, alertness and competitiveness. These substances activate the central and peripheral nervous system resulting in blood vessel constriction and relaxation of smooth muscle as well as increased heart rate and blood pressure. Despite their psychotropic effects that may be perceived as ergogenic, stimulants represent a substantial risk to athletes as their misuse can potentially result in adverse health effects. For these reasons, stimulants have received considerable attention by several sporting federations and associations such as the International Olympic Committee (IOC) and the World Anti-doping Agency (WADA) which have taken measures to either prohibit or monitor the use of these substances.

Historically, detection of stimulants in biological samples was performed using high-performance liquid chromatography (HPLC) with ultraviolet-diode array detection (UV-DAD). However, this detection technique lacks specificity for the unequivocal identification of these substances. In recent years, gas chromatography coupled to mass spectrometry (GC-MS) has been the most widely used analytical technique for the detection of stimulants. This technique, however, requires sample derivitization and suffers from lengthy chromatographic runs. As a result, there is a need for rapid, robust and comprehensive detection methods that allow positive identification and accurate quantification of stimulants in biological samples.

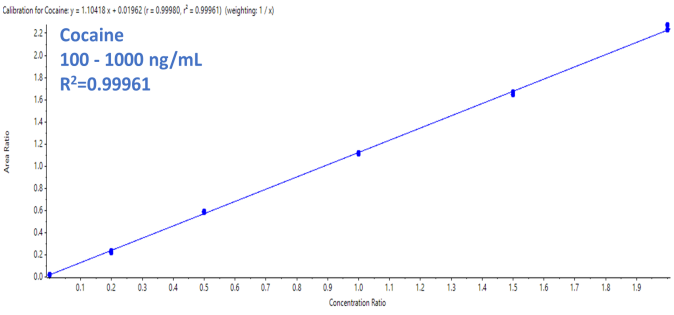

Figure 1. High Sensitivity and Linearity for the Detection of Stimulants in Human Urine. Calibration curve resulting from the calibration series of cocaine, one of the stimulants used in this study, from 100 to 1000 ng/mL.

The use of high-resolution mass spectrometry (HRMS) in the sports drug-testing laboratory enables toxicologists to rapidly screen for the presence of these substances by acquiring their complete chemical profile from biological samples. In this technical note, a comprehensive workflow combining the use of the SCIEX X500R QTOF System with a fast sample preparation procedure for the sensitive detection of a structurally-diverse panel of stimulants in human urine is described.

Key Features of SWATH Acquisition Method for Stimulant Detection in Urine Samples

- Simplified, dilute-and-shoot sample preparation provided a high-throughput and easily implemented procedure enabling rapid detection of stimulants listed on the World Anti-Doping Agency (WADA) Prohibited List
- SWATH Acquisition generated comprehensive and high-quality MS/MS spectra for screening and confirmation of every detectable stimulant from urine samples, creating a digital record of each urine sample analyzed
- Method showed excellent sensitivity with LODs and LOQs in the ng/mL range for all stimulants used in this study
- Intra-day precision (expressed as percent variation coefficient, CV%) and accuracy (expressed as bias%) were found to be below 20% across the calibration range at all three concentration levels (100, 500, and 1000 ng/mL)
- Assay showed excellent linearity with R2 value >0.99 across the calibrator series for all the stimulants used in this study

Experimental Details

Sample Preparation: A 10 µg/mL stock standard mixture, containing all the stimulants used in this study, was prepared by diluting stock standards with methanol. A series of six calibrator solutions were prepared by spiking blank urine samples with the stock standard mixture and the internal standards. Spiked urine samples were diluted 10-fold with a solution of acetonitrile/methanol (80/20, v/v) followed by ultracentrifugtion to give desired concentrations ranging from 100 to 1000 ng/mL. Two replicates for each concentration were analyzed to build the calibration curves and evaluate the dynamic range. The full list of the compounds and internal standards used in this study is detailed in Table 1.

Table 1. List of the 15 Stimulants and 4 Internal Standards Used in This Study.

Stimulants	Internal Standards
Cathine	*3,4-Methylenedioxymethamphetamine-D5*
Cathinone	*Cocaine-D3*
Amphetamine	*Amphetamine-D5*
Cocaine	*Diphenylamine*
Phendimetrazine	
Fenethylline	
Fenproporex	
Phentermine	
Mephentermine	
Methamphetamine	
Methylhexanamine	
Methylphenidate	
Pemoline	
Strychnine	
Benzphetamine	

Liquid Chromatography: UHPLC separation was performed on a Phenomenex C18 column (100 x 2.1 mm, 1.7 µm, 00D-4475-AN) at 45ºC on the SCIEX ExionLC™ AC System. Mobile phases used consisted of water, acetonitrile and modifiers. The LC flow rate was 0.5 mL/min and the total run time was 7 min. The injection volume was 1 µL.

Mass Spectrometry: MS and MS/MS data were collected for each sample using SWATH Acquisition on the SCIEX X500R QTOF System in positive mode. Data acquisition was TOF MS scan followed by 12 MS/MS scans using variably sizedQ1 windows covering a mass range from 100 to 350 m/z. The resulting cycle time was 0.555 sec. Data was acquired using SCIEX OS Software 1.5.

Data Analysis: Data processing was performed using SCIEX OS Software 1.5.

Developing a Comprehensive Workflow for Accurate Mass Detection of Stimulants in Human Urine

Blank human urine samples were spiked with the stock standard mixture containing all 15 target analytes at various concentration levels. The resulting samples were spiked with the internal standards, diluted 10-fold with a solution of acetonitrile/methanol (80/20, v/v) to final concentrations ranging from 100 to 1000 ng/mL then centrifuged prior to injection, to build a data processing method. Figure 2 shows the total ion chromatogram (TIC) for the 15 target compounds in a control human urine sample at a concentration of 1000 ng/mL. The chromatographic profile shows near baseline separation of the 15 stimulants, including structurally-related compounds eluting off the column at similar retention times.

Analytical Performance of the SCIEX X500R System for Detection for Stimulant in Urine

The same set of urine samples spiked with the 15 target analytes at various concentration levels was used to evaluate the robustness and quantification performance of the assay. Peak detection and integration was achieved using the AutoPeak algorithm, the automated peak integration software platform integrated in SCIEX OS Software. Calibration curves were generated to evaluate the response and quantitation performance for each of the 15 stimulants used in this study. Two replicates for each concentration were analyzed to build the calibration curves and evaluate the dynamic range.

Figure 3 shows calibration curves for benzphetamine and pemoline for the calibration levels ranging from 100 to 1000 ng/mL. The calibration curves showed excellent correlation and linearity with R^2 values of 0.99965 and 0.99844 for benzphetamine and pemoline, respectively. Similar linearity and correlation of the generated regression curves were observed for the other stimulants in the panel.

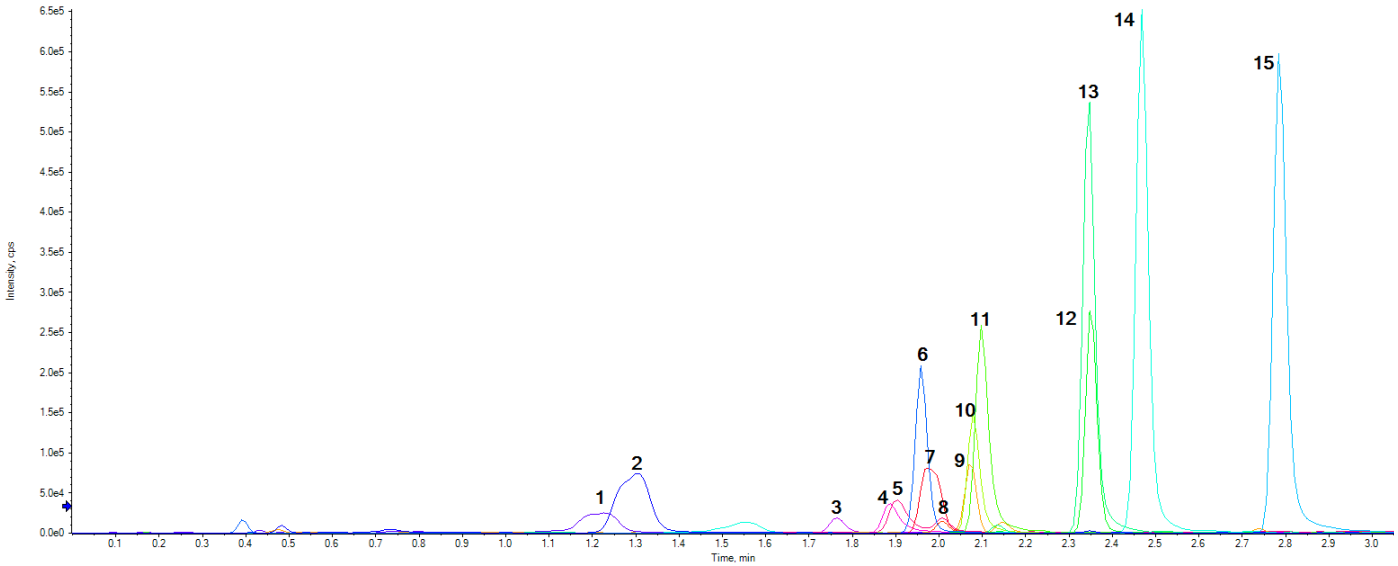

Figure 2: Chromatographic Profile for the 15 Stimulants in the Panel. Total Ion Chromatogram (TIC) resulting from near baseline separation of the 15 stimulants used in this study. The total LC runtime was 7 min. The numbered peaks are assigned as follows: 1. Cathine, 2. Cathinone, 3. Amphetamine, 4. Cocaine, 5. Phendimetrazine, 6. Fenethylline, 7. Fenproporex, 8. Phentermine, 9. Mephentermine, 10. Methamphetamine, 11. Methylhexanamine, 12. Methylphenidate, 13. Pemoline, 14. Strychnine, and 15. Benzphetamine.

In addition to determining linearity and correlation, the following validation parameters were calculated for the 15 stimulants in the panel: LODs, LOQs, inter- and intra-assay precision and accuracy as well as matrix effect. Table 2 shows the average (n=2) results from the validation study for the 15 stimulants spiked at three concentration levels (100, 500 and 1000 ng/mL) in blank urine samples.

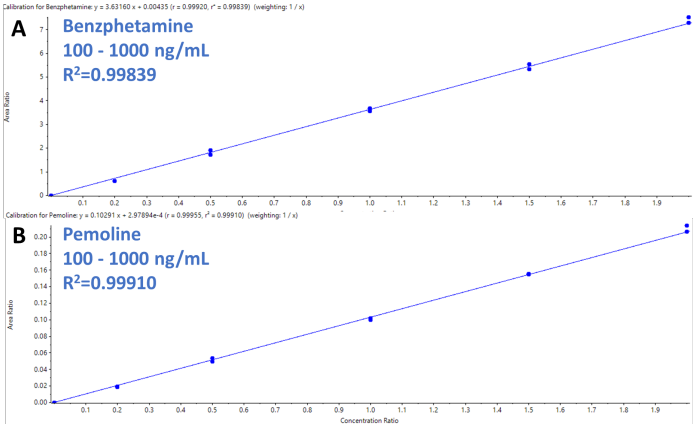

Figure 3. Excellent Sensitivity and Linearity for Stimulants in Urine. Calibration curves resulting from the calibration series for benzphetamine (A) and pemoline (B). Excellent linear response and sensitivity were observed with R^2 values of 0.99839 and 0.99910 for benzphetamine and pemoline, respectively

Limits of quantitation (LOQ) and detection (LOD) for the 15 stimulants in matrix ranged from 3.3 to 100 ng/mL and 1.0 to 30 ng/mL, respectively. These results demonstrate that the presented workflow shows excellent sensitivity and quantitative results for the detection of stimulants from urine samples.

Matrix effect was evaluated by comparing the signals between the duplicate set urine samples spiked with the 15 target analytes at 100 ng/mL and a duplicate set of reference samples prepared by spiking a solution of acetonitrile/methanol (80/20, v/v) with the stock standard mixture containing the 15 target analytes at the same final concentration (100 ng/mL). As seen in Table 2, matrix effects ranged between ±2.2 and ±35.7% for all 15 analytes, suggesting no significant ion suppression or enhancement was observed for any of the stimulants in the panel.

Lastly, intra-day precision (expressed as percent variation coefficient, CV%) and accuracy (expressed as bias%) were calculated for the detection of the 15 stimulants in urine samples. The values were found to be below 20% for both the CV% and the bias%, for the calibrators at 100, 500 and 1000 ng/mL concentration. These results demonstrate the robustness and reproducibility of the overall workflow, suggesting that this dilute-and-shoot method could potentially be extended to include a larger panel of stimulants. The results of this validation study are summarized in Table 2.

Table 2: Results of the Validation Study. Average (n=2) Results From the Validation Study Showing the Linearity Range, Correlation Coefficient, LOD, LOQ, Matrix Effect, as well as Intra-Day Precision (%CV) and Accuracy (bias%) Tested at Three Concentration Levels (100, 500, and 1000 ng/mL) for the 15 Stimulants Used in This Study.

Analyte	Linearity Range (ng/mL)	Correlation Coefficient (R^2)	LOD (ng/mL)	LOQ (ng/mL)	Matrix Effect (±%)	Concentration (ng/mL)	Precision (CV%)	Accuracy (Bias%)
Cathine	*100-1000*	*0.9997*	*30.0*	*100*	*27.9*	*100* *500* *1000*	*18.65* *9.90* *4.70*	*16.90* *-8.57* *-4.35*
Cathinone	*100-1000*	*0.9990*	*16.6*	*55.4*	*9.9*	*100* *500* *1000*	*14.58* *9.77* *3.19*	*18.65* *-6.02* *-2.51*
Amphetamine	*100-1000*	*0.9992*	*18.2*	*60.6*	*17.9*	*100* *500* *1000*	*12.96* *7.45* *1.97*	*17.33* *-6.77* *-3.25*
Cocaine	*100-1000*	*0.9996*	*1.0*	*3.4*	*2.2*	*100* *500* *1000*	*15.02* *7.86* *3.22*	*5.37* *-6.77* *-2.80*
Phendimetrazine	*100-1000*	*0.9993*	*6.2*	*20.6*	*18.7*	*100* *500* *1000*	*16.76* *6.52* *7.07*	*6.62* *-7.65* *-11.44*
Fenethylline	*100-1000*	*0.9995*	*1.0*	*3.3*	*35.7*	*100* *500* *1000*	*17.35* *4.36* *6.05*	*10.91* *-12.88* *-11.09*
Fenproporex	*100-1000*	*0.9983*	*1.4*	*4.8*	*21.8*	*100* *500* *1000*	*16.14* *5.89* *4.56*	*0.55* *3.23* *-3.13*
Phentermine	*100-1000*	*0.9980*	*12.9*	*43.0*	*5.4*	*100* *500* *1000*	*15.65* *5.55* *7.19*	*-3.26* *-4.69* *-11.43*
Mephentermine	*100-1000*	*0.9989*	*11.6*	*38.6*	*16.6*	*100* *500* *1000*	*16.39* *10.08* *6.14*	*16.06* *-8.10* *-4.41*
Methamphetamine	*100-1000*	*0.9992*	*8.0*	*26.8*	*14.7*	*100* *500* *1000*	*13.87* *8.80* *5.57*	*17.11* *-8.24* *-4.96*
Methylhexanamine	*100-1000*	*0.9969*	*10.4*	*34.7*	*29.9*	*100* *500* *1000*	*16.00* *6.60* *3.29*	*8.66* *-6.01* *-1.18*
Methylphenidate	*100-1000*	*0.9978*	*3.1*	*10.4*	*21.5*	*100* *500* *1000*	*13.71* *10.75* *4.65*	*16.18* *-5.31* *-10.4*
Pemoline	*100-1000*	*0.9991*	*10.6*	*35.1*	*9.8*	*100* *500* *1000*	*18.24* *7.97* *5.73*	*16.95* *-7.76* *-9.80*
Strychnine	*100-1000*	*0.9988*	*11.6*	*38.6*	*34.2*	*100* *500* *1000*	*17.89* *7.92* *4.33*	*8.23* *1.29* *2.21*
Benzphetamine	*100-1000*	*0.9984*	*4.6*	*15.2*	*23.7*	*100* *500* *1000*	*17.55* *7.02* *3.02*	*9.66* *-8.14* *-1.27*

Conclusions

A comprehensive workflow for the detection of stimluants in human urine was successfully developed using the SCIEX X500R System. A rapid sample preparation procedure in combination with a highly selective MS/MS method with SWATH Acquisition enabled robust and reproducible detection of a panel of 15 stimulants in human urine with ng/mL detection limits.

- A rapid sample preparation protocol consisting of a simple dilute-and-shoot procedure was implemented for the detection of stimulants in urine samples
- A 7-min LC method using the ExionLC AC HPLC system enabled near baseline separation of the 15 stimulants in the panel
- SWATH Acquisition enabled generation of a comprehensive digital archive of each urine sample via acquisition of high-quality MS/MS spectra for every detectable stimulant present in the sample
- Workflow showed excellent correlation and linearity in the panel, with R^2 values >0.99 for all the stimulants in the panel
- Assay showed excellent sensitivity and quantitative results, with LOQs and LODs ranging from 3.3 to 100 ng/mL and 1.0 and 30 ng/mL, respectively
- Matrix effect values generated suggested no significant ion suppression due to the dilute-and-shoot sample preparation procedure
- Intra-day precision and accuracy were both found to be below 20% for the calibrators at 100, 500 and 1000 ng/mL, proving the overall robustness and reproducibility of the developed workflow
- Results suggest that this high-throughput workflow could be easily implemented to include a larger number of stimulants listed on the World Anti-Doping Agency (WADA) Prohibited List

Headquarters
500 Old Connecticut Path | Framingham, MA 01701 USA
Phone 508-383-7700
sciex.com

International Sales
For our office locations please call the division headquarters or refer to our website at
sciex.com/offices