thermo scientific

TECHNICAL NOTE 65008

Targeted forensic screening and semi-quantitation of drugs in plasma using high-resolution, accurate-mass detection and online sample preparation

Authors

Valérie Thibert, Peggy Regulus, Bénédicte Duretz, Wei Xing,

Thermo Fisher Scientific, France

Keywords

Q Exactive Focus, TraceFinder, screening, forensic toxicology, Transcend, TurboFlow technology

Application benefits

- Rapid screening method with a possibility of online extraction using Thermo Scientific™ TurboFlow™ technology
- More than 1500 compounds in positive and negative ionization mode
- Separation of isomers of interest in forensic toxicology
- Sensitivity assessed for online extraction approach
- Semi-quantitation capabilities for part of a complete screening panel

Goal

Evaluation of a screening method for a large panel of compounds deploying the high-resolution, accurate-mass Thermo Scientific™ Q Exactive Focus™ hybrid quadrupole-Orbitrap™ MS. Drug screening is based on the use of a spectral library and a database containing retention times, chemical formulas, and fragment ions. Testing of both quantitation and confirmation capabilities of Thermo Scientific™ TraceFinder™ software with the screening method developed.

ThermoFisher SCIENTIFIC

Introduction

In forensic toxicology, it is of high importance to be able to screen a large panel of compounds on a single injection of sample for further confirmation by more specific methods. Methods developed for this purpose need to use a low volume of sample and to include the capability of monitoring a very large panel of compounds; it is also desirable to reduce the runtime of these methods to increase throughput. The development of a spectral library and compound database for the screening and semi-quantitation of more than 1500 compounds in plasma samples, but which is applicable to other biological matrices, is reported. For each compound, the database includes the exact mass, chemical formula, retention time, and exact masses of its main fragments. Two different analytical methods were used, one based on high-performance liquid chromatography (HPLC), and the other based on online extraction using TurboFlow technology prior to HPLC separation. Both methods were previously reported.[1,2] For both approaches, a Thermo Scientific™ Transcend™ II TLX-1 system coupled to a Q Exactive Focus Orbitrap high-resolution, accurate-mass spectrometer with a heated electrospray ionization source was used. The current work consisted of the injection of more than 1500 standard solutions to define a database and spectral library of compounds for forensic screening. For the online extraction method, a panel of 41 compounds was selected to evaluate the sensitivity of the method in plasma. TraceFinder 4.1 software was used for the processing of all these data. The calibration curves obtained in this process were then stored within TraceFinder 4.1 software to be able to further use them to do semi-quantitative work.

Experimental

Target analytes

A total of 1513 compounds consisting of drugs of abuse, antidepressants, neuroleptics, beta-blockers, antibiotics, antihistaminics, some novel psychoactive substances, synthetic cannabinoids, and other drugs and metabolites were used as target analytes.

Sample preparation

Standard solutions for library generation were prepared in groups of 20 compounds at a concentration of 0.1 µg/mL in methanol/water 30:70 v/v solution.

Plasma samples were prepared only for the testing of the method based on TurboFlow extraction. Calibrators were prepared by spiking the compounds into blank plasma matrix from Innovative Research (Le Perray-en-Yvelines, France). The 41 compounds used for this stage were divided in three groups for the preparation of the calibrators according to the levels of concentration of the analytes to be assessed in plasma samples. Calibrators had concentrations ranging from 0.1 ng/mL to 250 ng/mL for compounds in group A, and from 10 ng/mL to 5000 ng/mL for groups B and C. Sample preparation previous to injection consisted of the precipitation of proteins as follows: 25 µL of a solution containing isotopically labeled internal standards (2 mg/L amphetamine-d5, 1 mg/L THC-COOH-d_3, 5 mg/L haloperidol-d_4, prazepam-d_5 and morphine-d_3, and 0.2 mg/L trimipramine-d_3 in methanol) and 100 µL of acetonitrile were added to 100 µL of calibrator. After vortex mixing, the calibrators were centrifuged and the supernatant was transferred to a vial for sample injection. The selected internal standards covered the entire time range of the chromatographic run as well as positive and negative polarities.

Liquid chromatography

Gradient elution was performed using a Transcend II TLX-1 system, either by direct injection onto the analytical column or TurboFlow column for online sample cleanup prior to HPLC separation. Figure 1 presents the instrument configuration used for this work. Mobile phases consisted of 2 mM ammonium formate with 0.1% of formic acid in water for phase A (both loading and elution) and 2 mM ammonium formate with 0.1% of formic acid and 1% of water in methanol/acetonitrile 50:50 v/v for phase B (both loading and elution), and acetonitrile/isopropanol 50:50 v/v for phase C (only for the loading pump). The column used for HPLC separation was a Thermo Scientific™ Accucore™ Phenyl-Hexyl column, 100 × 2.1 mm (2.6 µm). For the TurboFlow extraction, one Thermo Scientific™ Cyclone™ and one Thermo Scientific™ Phenyl TurboFlow column (both 50 × 0.5 mm) coupled in series were used. The HPLC screening method duration was 15.5 minutes while the method including online extraction was 16.75 minutes long. Gradient details are presented in Table 1 for the HPLC-only method, and Table 2 for the TurboFlow extraction method.[1,2] For the online TurboFlow extraction method, the plumbing of the system was operated in Focus Mode, in a configuration named “pseudo quick elute”.

Figure 1. Q Exactive Focus MS coupled with a Transcend II TLX-1 system.

Table 1. Gradient conditions for the HPLC screening method.

Step	Duration(s)	Loading Pump					Tee	Loop	Eluting Pump			
		Flow	Grad	%A	%B	%C			Flow	Grad	%A	%B
1	60	0	Step	100	-	-	-	Out	0.5	Step	99	1
2	540	0	Step	100	-	-	-	Out	0.5	Ramp	1	99
3	90	0	Step	100	-	-	-	Out	0.5	Step	1	99
4	240	0	Step	100	-	-	-	Out	0.5	Step	99	1

Table 2. Gradient conditions for the TurboFlow extraction coupled to HPLC screening method.

Step	Duration(s)	Loading Pump					Tee	Loop	Eluting Pump			
		Flow	Grad	%A	%B	%C			Flow	Grad	%A	%B
1	20	2	Step	100	-	-	-	Out	0.5	Step	99	1
2	5	0.5	Step	100	-	-	-	In	0.5	Step	99	1
3	60	0.5	Step	99	1	-	T	In	0.05	Step	99	1
4	540	0.5	Ramp	1	99	-	T	In	0.05	Ramp	1	99
5	90	0.5	Step	1	99	-	T	In	0.05	Step	1	99
6	10	1	Step	-	-	100	-	In	0.5	Step	-	100
7	10	1	Step	100	-	-	-	In	0.5	Step	-	100
8	10	1	Step	-	-	100	-	In	0.5	Step	-	100
9	10	1	Step	100	-	-	-	In	0.5	Step	-	100
10	10	1	Step	-	-	100	-	In	0.5	Step	-	100
11	60	0.3	Step	100	-	-	T	In	0.05	Step	99	1
12	180	1	Step	100	-	-	-	In	0.5	Step	99	1

Mass spectrometry

Data were acquired on a Q Exactive Focus Orbitrap mass spectrometer equipped with a Thermo Scientific™ Ion Max™ source and heated electrospray ionization (HESI-II) sprayer. The detection was performed by Full Scan in data-dependent MS^2 acquisition mode with an inclusion list. Full Scan data were acquired in both positive and negative mode with a resolution of 35,000 FWHM at *m/z* 200, and the MS^2 spectra for confirmation were acquired with a resolution of 17,500 FWHM at *m/z* 200. The inclusion lists, including retention time windows, had 1513 compounds for the HPLC-only method and 1433 compounds for the method with online extraction. External mass calibration was performed once a week during this study to provide high mass accuracy and stability during the experimental work.

Method development

For generation of the spectral library and compound database, the 1513 standard solutions were injected with the HPLC method to obtain retention times and MS/MS spectra. The 1513 corresponding spectra were imported into a Thermo Scientific™ mzVault™ library. The mzVault library employs a new library search algorithm based on the mzCloud database (www.mzCloud.org) that improves library matching.

The information obtained from the injections (retention times and exact masses of the main fragments) was used to create the compound database. For the TurboFlow compound database, the same panel of compounds was injected and only analytes presenting a peak were included in the database.

Method evaluation

For the TurboFlow approach, the limit of quantitation (LOQ), the limit of detection (LOD), and the limit of identification (LOI) were determined for spiked plasma samples. The LOD was obtained as the lowest concentration for which a peak is still observed for three different plasma matrices tested. The LOQ was obtained as the lowest concentration for which a quantitation has an accuracy with a bias less than 20% and a %RSD less than 20% for three repeated injections in three different plasma matrices. The bias determination was based on the calibration curves generated from 0.1 to 250 ng/mL for group A compounds, and from 10 ng/mL to 5000 ng/mL for groups B and C. Finally, the LOI was determined as the lowest concentration for which a compound can be identified based on the following conditions: *m/z* of the parent (< 5 ppm), isotopic pattern match, fragment ion presence, and MS^2 spectra matching. The detailed criteria for LOI determination are reported in Table 3. The high mass accuracy performance of the Q Exactive Focus mass spectrometer gives additional confirmation points for the screening based on the high resolution and the precision of the measured masses.

Data analysis

Data were acquired and analyzed with TraceFinder 4.1 software. TraceFinder software uses a database that contains compound-related information for identification and confirmation. It also uses proprietary MS^2 spectral libraries containing the spectra of the 1513 compounds tested. Within TraceFinder software, a master method is created that includes the acquisition parameters as well as the processing parameters for the screening method. The software allows use of either a screening-only approach or a combination of screening and quantitation parameters according to the needs of the user. An example of the results review modality for quantitation and screening approaches is presented in Figure 2.

Table 3. Criteria used for the determination of LOI.

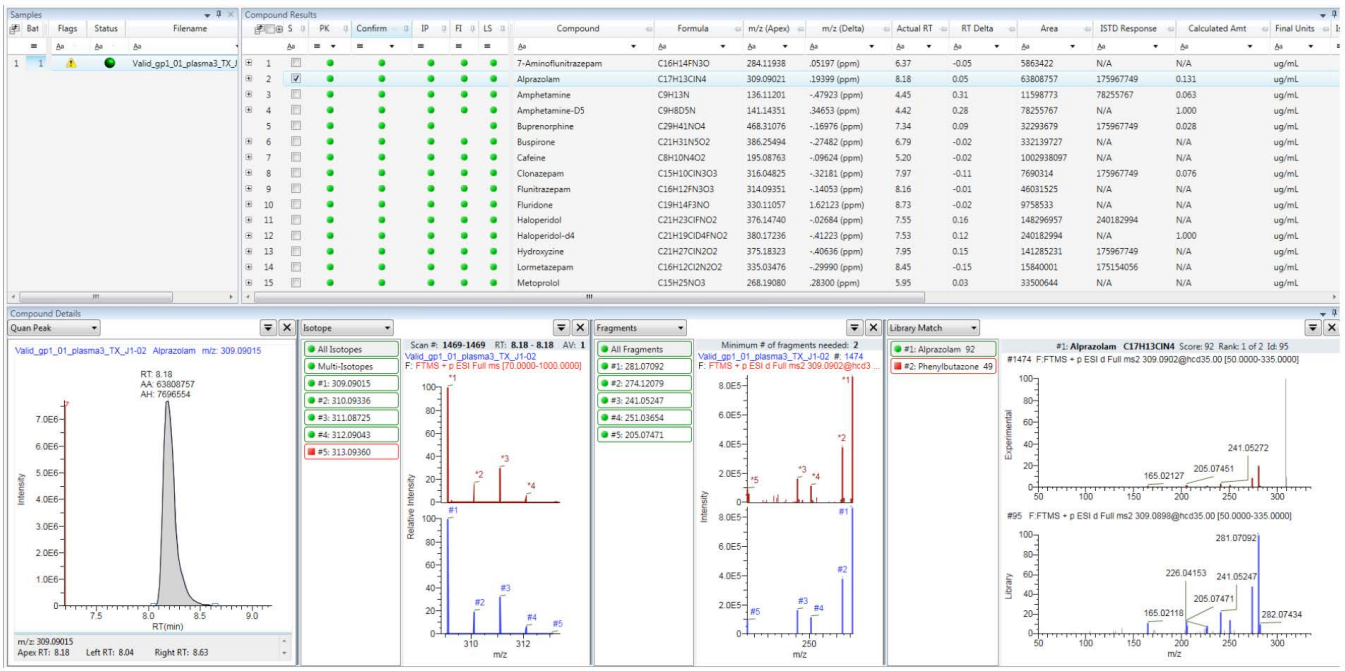
Parameter	Criteria
m/z of the parent ion	< 5 ppm mass deviation for an intensity threshold set at 5000 au
Retention time	Within a 30 s window
Isotopic pattern match	< 5 ppm mass deviation, < 30% intensity deviation, fit > 70%
Fragment ion match	At least 2 fragments with < 5ppm deviation and an intensity threshold of 5000 au
Spectral mzVault library matching	< 500 mmu precursor tolerance, passing value > 50%

Results and discussion

A database containing compound-related information was created for both methods, one using HPLC-only and one using TurboFlow technology online extraction on a Transcend II TLX-1 system. For the development of the database, concentrated solutions were used.

Out of 1513 injected solutions, 1433 were detected in both approaches. The somewhat lower number for the TurboFlow approach is due to poor retention of some of the analytes in the extraction columns. An example of the review of the data oriented to a screening approach is presented in Figure 2A.

A



B

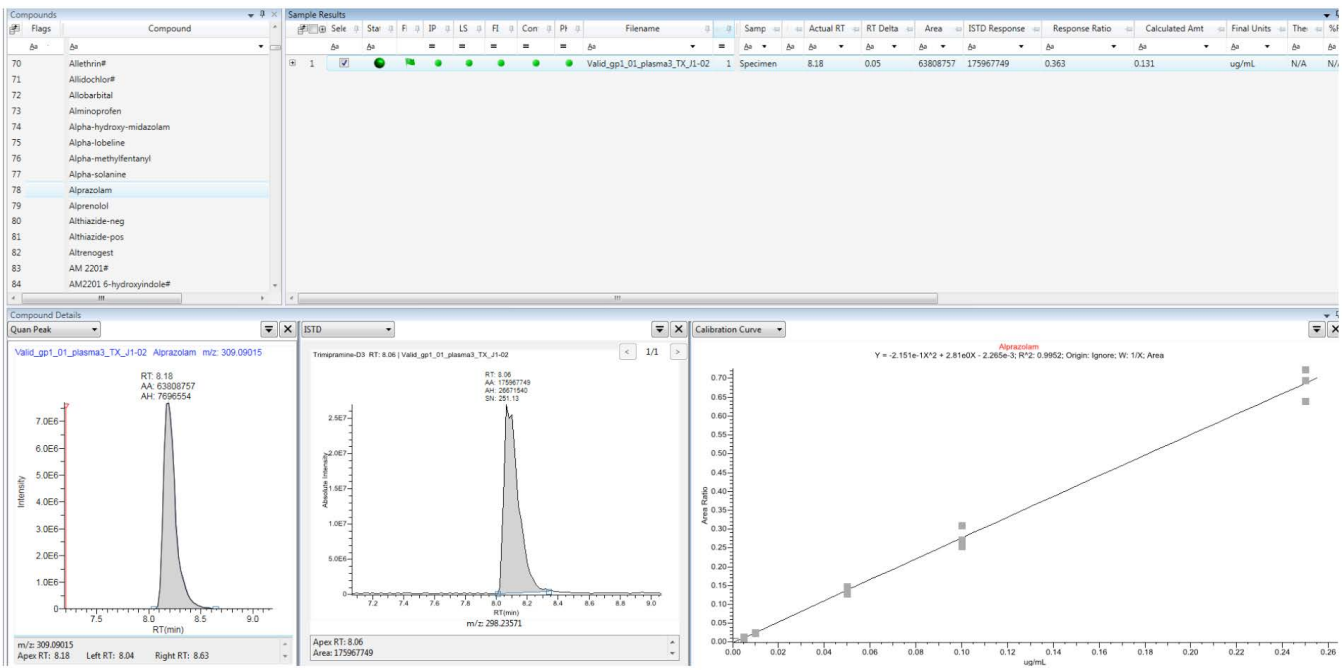


Figure 2. Data review page for TraceFinder 4.1 software in (A) screening oriented approach, or (B) a combination of screening with quantitation.

For a quantitation method, analytical validation is generally based on the evaluation of the LOQ and the intra-day and inter-day accuracy and precision. The approach is difficult to apply in this case considering the large number of compounds in the panel. This would suggest the preparation, injection, acquisition, and processing of data for more than 1400 compounds. Moreover, there are no official guidelines regarding the analytical validation of a screening method. A possible solution consists of selecting some compounds that are representative of different drug groups that appear in the complete retention time window of the chromatographic run, and that can present different polarities.

For this work, 41 compounds were selected from the panel, covering different compound classes, retention times, and polarities. LOD, LOQ, and LOI were evaluated for the 41 selected compounds using the TurboFlow approach. The results of this study are presented in Table 4. The recovery was not evaluated at this stage.

TraceFinder software offers the opportunity to save the calibration curves for future semi-quantitation work. As reported in Figure 2B, it is possible not only to perform a screening workflow with identification and confirmation of compounds, but also to obtain a semi-quantitative result based on pre-acquired calibration curves. The method presented here is suitable for a quantitative approach considering that the calibration curves were linear from LOQ up to 250 ng/mL for group A compounds, and from LOQ up to 5000 ng/mL for groups B and C. These results confirm the suitability of the Q Exactive Focus mass spectrometer for quantitative work, both for low and high concentrations of analytes, with an excellent dynamic range and an accurate-mass measurement for the different concentrations tested.

Table 4. LOD, LOQ, and LOI obtained for 41 compounds with the TurboFlow method.

Group	Compound	LOD (ng/mL)	LOQ (ng/mL)	LOI (ng/mL)
A	Alprazolam	5	50	50
	Amphetamine	50	50	100
	Buprenorphine	5	5	50
	Buspirone	10	10	10
	Clonazepam	10	50	100
	Flunitrazepam	5	50	50
	Haloperidol	1	1	50
	Hydroxyzine	1	5	10
	Lormetazepam	10	10	100
	Mianserine	0.5	0.5	5
	Morphine	50	100	250
	Olanzapine	5	50	50
	Prazepam	5	5	50
	Zopiclone	50	50	100
B	Amoxapine	50	100	100
	Chlordiazepoxide	50	100	100
	Chlorpromazine	50	500	500
	Doxepine	50	50	50
	EDDP	50	100	100
	Estazolam	50	100	100
	Fluoxetine	50	1000	1000
	Norclobazam	100	1000	1000
	Nordiazepam	50	100	100
	Nortriptyline	50	100	100
	Temazepam	50	500	500
C	Amitriptyline	10	50	50
	Bisoprolol	10	50	50
	Clobazam	10	10	50
	Clomipramine	10	50	50
	Clozapine	10	10	50
	Codeine	10	10	50
	Cyamemazine	10	10	50
	Desipramine	10	10	10
	Doxylamine	10	50	50
	Fluvoxamine	50	50	50
	Imipramine	10	50	50
	Levomepromazine	10	50	50
	Metformin	50	250	500
	Methadone	10	50	50
	Tramadol	10	50	50
	Trimipramine	10	50	50

Conclusions

A compound database and a spectral library for the screening of 1513 compounds were implemented on a Transcend II TLX-1 system coupled to a Q Exactive Focus Orbitrap high-resolution, accurate-mass spectrometer. The panel includes both positively and negatively ionized compounds such as drugs of abuse and metabolites, antidepressants, beta-blockers, antibiotics, pesticides, and other classes. An HPLC-only approach and a TurboFlow extraction method coupled to HPLC were used.

The drug screening method presented in this work covers a large panel of compounds with a short run time of 15.5 minutes and an option for an online extraction approach of only 16.75 minutes. TraceFinder 4.1 software is a simple interface to quickly review screening results, which can also be used for a semi-quantitative workflow for screening purposes.

Analytical validation for the TurboFlow method was performed on 41 compounds spiked in plasma matrix. The 41 selected compounds could reliably be quantified in plasma matrix with a simplified sample pre-treatment consisting of protein precipitation followed by online extraction coupled to HPLC separation. The compounds can be used as a basis for the method validation since they cover different drug classes, retention times, and polarities.

References

1. Helfer A.G.; Michely J.A.; Weber A.A.; Meyer M.R.; Maurer H.H. Orbitrap technology for comprehensive metabolite-based liquid chromatographic-high resolution-tandem mass spectrometric urine drug screening - exemplified for cardiovascular drugs. *Anal. Chim. Acta,* **2015,** *891,* 221–233.
2. Helfer A.G.; Michely J.A.; Weber A.A.; Meyer M.R.; Maurer H.H. LC-HR-MS/MS standard urine screening approach: Pros and cons of automated on-line extraction by turbulent flow chromatography versus dilute-and-shoot and comparison with established urine precipitation. *J, Chromatogr, B Analyt, Technol, Biomed, Life Sci.* **2017,** *1043,* 138–149. DOI 10.1016/j.jchromb.2016.06.036.

For Forensic Use Only.

Find out more at **thermofisher.com/forensics**

ThermoFisher SCIENTIFIC