Confident Drug Identification via Metabolites Using an Innovative Ion Mobility-HRMS Workflow

Abstract

Numerous novel psychoactive compounds emerge annually on the illicit drug market. These substances undergo frequent chemical alterations, rendering conventional detection methods such as immunochemical or targeted LC-TQ testing ineffective. This study demonstrates an innovative workflow, very similar to that developed to screen for inborn errors of metabolism in neonates, for the identification of drugs of abuse through their metabolites. HRMS was combined with ion mobility and a sophisticated software package.

Keywords:
timsTOF Pro 2, MetaboScape, metabolites, human liver microsomes

Introduction

The challenge of detecting drug abuse

The ever-changing landscape of drugs of abuse, including misuse of therapeutic drugs, creates significant analytical challenges for forensic science and health care fields. The chemical modification of existing drugs and the production of new recreational drugs, including New Psychoactive Substances (NPS), have created an exploding illicit drug market that exceeds the pace of development of conventional analytical methods, such as immunoassay or routine testing by Liquid Chromatography - Triple Quadrupole-Mass Spectrometry (LC-TQ-MS). Usually, these methods cannot be used for retrospective analysis as well.

Advantages of extending detection to drug metabolites

Extending the detection procedure of illicit drugs to their metabolites has a lot of inherent benefits and advantages. Since metabolites are mainly present in urine, this non-invasive matrix can be used for the testing instead of blood samples. This largely facilitates sampling and eliminates ethic concerns.

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The non-invasive sample collection and analysis of urine with a relatively wide window of detection is usually the method of choice for abstinence control anyway. Additionally, drug metabolites can still be detected in the body for an extended period of time, thus offering a more robust and confident way of drug detection in suspects.

Leveraging metabolite profiles for drug identification

If two compounds yield similar metabolites and the parent drug is not detectable in urine (e.g. synthetic cannabinoids), it's impossible to determine which drug was taken. Hence, differences in the observed metabolite profile may help to elucidate which compound was actually taken.
This also applies to 'unknown' compounds with small structural changes compared to a known compound. The development of an accurate metabolic profile of new synthetic drugs which is included in drug profiling panels is therefore of high interest.

Utilizing pooled human liver microsomes for metabolite prediction

The metabolites of some drugs are well characterized, and many others can be chemically predicted. In addition, and in cases where no authentic human sample material with confirmed uptake of the particular drug is available, the use of pooled Human Liver Microsomes (pHLM) is a rapid and inexpensive alternative. It can determine potential phase I metabolites *in vitro* as they would be generated *in vivo* in the human body. This approach proves advantageous in the context of ethical considerations, as it underscores the imperative to abstain from animal testing, acknowledging the ethical mandate to minimize the utilization of sentient beings in scientific experimentation, and does not require ethic committee consent regarding (self-) administration studies.

Case study: Quetiapine as a model substance

Quetiapine was used in this study as a model substance demonstrating the power of this novel workflow. Quetiapine is an atypical neuroleptic and FDA approved for schizophrenia, acute manic episodes, and adjunctive treatment for major depressive disorder. The drug is widely used, its metabolism is well understood and published, and many quetiapine metabolites are listed in the Maurer/Meyer/Helfer/Weber (MMHW) library. There are some cases reported on lethal intoxication.

Enhancing metabolite characterization with TIMS-HRMS

Dealing with sample complexity, especially concerning biological samples, is imperative for accurately detecting these metabolites analytically. Matrix effects, the wide dynamic range of many potential targets, and isomeric forms often defy analysis by traditional methods. Adding ion mobility information, based on the three-dimensional structure of molecules, to high-resolution mass spectrometry (HRMS) has provided a critical new separatory dimension for complex sample analysis. The *in vitro* metabolites were confirmed and characterized using HRMS coupled to Trapped Ion Mobility Spectrometry (TIMS-HRMS). Enabling TIMS during the data acquisition generates a significantly enriched feature set as the starting point for data interpretation. TIMS definitively changes the realities of both targeted and untargeted MS screening and profiling applications.

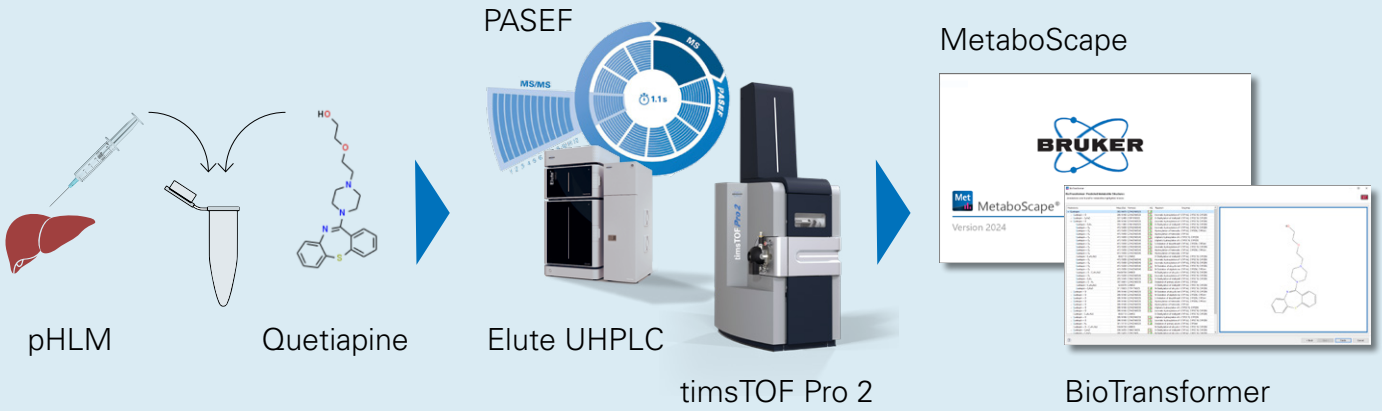
Software analysis of data

The experimental data can either be applied to conventional library search or to comparison with metabolite prediction based on biotransformation rules. The software MetaboScape® provides workflows for both routes. For the latter, the BioTransformer [1] software was used to predict the phase I metabolites of quetiapine. MetFrag [2] was then applied to predict the fragments of these biotransformation products. The obtained structural information was fed into the 'CCS-Predict' feature within the MetaboScape software package to generate theoretical CCS values of the metabolites and their fragments. All this theoretical information was compared with the experimental data for the pHLM assay to generate phase I metabolites from quetiapine.

[1] Djoumbou-Feunang et al.; Journal of Cheminformatics 2019, 11:2

[2] Wolf S., Schmidt S., Müller-Hannemann M., Neumann S. In silico fragmentation for computer assisted identification of metabolite mass spectra. BMC Bioinformatics 2010, 201011:148. doi: 10.1186/1471-2105-11-148

Experimental workflow



Phase I *in vitro* model

UHPLC-TIMS-QTOF-MS with PASEF

Metabolite prediction and identification

Figure 1. Experimental workflow including the phase I *in vitro* model for metabolite generation, data acquisition using UHPLC-TIMS-QTOF-MS and MetaboScape data processing.

Experimental

In vitro pHLM assay

Phase I quetiapine metabolites were produced using a standard protocol. The reaction mixture included quetiapine (1 mg/mL), Glc-6P and NADH (Solution A), Glc-6P-DH (Solution B), pHLM, phosphate buffer (0.5 M, pH 7.4), and distilled water was incubated for 30 min at 37 °C. After incubation, the reaction was quenched by adding 100 µL of ice-cooled acetonitrile, followed by 50 µL of ammonium formate (10 M). The mixture was then centrifuged at 13,200 rpm for 10 min. The organic layer was transferred to a vial and the solvent was evaporated under an N_2 stream. The dried sample was reconstituted in 25 µL LC mobile phase (50/50, A/B) for the mass spectrometric analysis. Blank pHLM samples were processed accordingly serving as negative controls.

LC-TIMS-HRMS

The system configuration and instrumental parameters of the timsTOF Pro 2 and Elute UHPLC (Bruker, Bremen, Germany) are shown in Table 1.

Table 1. Instrumental parameters of the combination of the Elute UHPLC and the timsTOF Pro 2.

LC	**Elute UHPLC**		
Column	Bruker Intensity Solo 1.8 C18-2, 2.1 x 100 mm, with precolumn		
Column oven temp.	40 °C		
Mobile phase	A: $H_2$0/MeOH (99:1) with 5 mM ammonium formate and 0.01 % formic acid B: MeOH with 5 mM ammonium formate and 0.01 % formic acid Injection of 2 µL sample volume		
Gradient	Time (min)	Flow (mL/min)	%B
	0.00	0.200	4.0
	0.10	0.200	4.0
	1.00	0.200	18.3
	2.50	0.223	50.0
	14.00	0.400	99.9
	16.00	0.480	99.9
	16.10	0.480	4.0
	19.00	0.480	4.0
	19.10	0.200	4.0
	20.00	0.200	4.0
MS	**timsTOF with PASEF**		
Ionization	VIP-HESI positive ion mode		
Mass range	20-1,300 *m/z*		
TIMS settings	$1/K_0$ 0.1 – 1.5 V·s/cm^2, ramp time 150 ms		
Nebulizer	3.0 bar		
Dry Gas	10.0 L/min		
Dry Temp	220 °C		
Sheath Gas Temp	470 °C		
Sheath Gas Flow	4.0 L/min		

Application of multiple workflows for metabolite annotation

The collected MS and MS/MS signals were processed within MetaboScape, including automated retention time alignment, T-ReX® 4D feature extraction, and mass and mobility recalibration. Known features were automatically annotated using a spectral library search against the MMHW library. BioTransformer-based metabolite predictions (with two steps allowed) were automatically compared in MetaboScape against the remaining features, including comparisons of experimentally determined CCS values and observed MS/MS fragmentation patterns against those predicted for each potential metabolite. The most probable structure of each was automatically annotated, considering all available characteristics. A plausibility control was as well performed in MetaboScape by checking peak shapes of extracted ion chromatograms and flagging of features with higher intensities in negative control samples.

Results

Benefits of TIMS in complex matrix analysis

Applying TIMS in addition to HRMS provides several benefits for the analysis of unknowns in complex matrices. First, it can be used to separate coeluting isobars and isomers, providing an additional separation to UHPLC and HRMS. Second, the TIMS ion trapping results in higher sensitivity and lower detection limits of the compounds of interest by background elimination. Finally, Collisional Cross Sections (CCS) as additional, orthogonal parameter to the MS information enhance the identification confidence.
The combination of the four different search criteria exact mass, isotope pattern, MS/MS fragmentation and CCS value is used to screen the data against the theoretical prediction of drug metabolites or against libraries. The instrument's VIP-HESI (Vacuum Insulated Probe-Heated ElectroSpray Ionization) source provides very high sensitivity and improved ionization efficiency.

Enhanced data analysis with MetaboScape software

With a new depth and breadth of data generated at a high acquisition speed, data analysis must be well-integrated and equally powerful to maximize the delivery of confident results. Bruker's MetaboScape is a unique and intuitive "all in one" software package for non-targeted workflows and automated annotation of unknown compounds, performing all key tasks in seconds. For the identification of known metabolites, spectral searches can be made directly within user-created libraries or within multiple commercial libraries, including the extensive MMHW HRMS library with 3,000 metabolite spectra in over 95 compound classification groups, the NIST 2020 HRMS library, the MetaboBASE® with MS/MS spectra of over 100,000 standards and 700,000 *in silico* generated MS/MS spectra as well as the Human Metabolome Database (HMDB).

Comprehensive metabolite annotation

For unknown signals which cannot be found in the libraries like unexpected metabolites or biotransformation products of new psychoactive substances (NPS), MetaboScape provides automatic annotations based on multiple analytical characteristics. Based on accurate mass and observed isotopic patterns, the most probable elemental compositions of the "unknown" feature can be automatically annotated. *In silico* biotransformation (BioTransformer) and *in silico* fragmentation (MetFrag) of desired features can be predicted in MetaboScape to extend metabolite annotation. Specific CCS values provided with the added separatory dimension of TIMS, based on the 3D shape of each parent molecule, serve as an independent, orthogonal identification criterion, and parent and fragment spectral data are automatically linked and aligned by their shared mobility values. Color-coded results within MetaboScape support rapid result review. Moreover, MetaboScape can provide statistical evaluations and feature alignment between groups or timepoints to visualize differentially regulated compounds.

Confidence in annotation with timsTOF Pro 2 instrument

The timsTOF Pro 2 delivered a high number of clean MS/MS spectra that could be annotated, including those from low abundance metabolites, by virtue of the unique ion trapping and focusing prior to mobility-based separation. Further, the predicted CCS values and fragmentation patterns supported confident annotation. The drug metabolites can be added to databases used for targeted screening, such as the TargetScreener 4D database.

Annotation results and pathways in MetaboScape

A total of 21 metabolites produced during incubation with pHLM could be annotated from a single UHPLC-timsTOF Pro 2 run (Fig. 1). Of these, 14 were found by the MMHW "known" spectral library (SL) search, while 19 metabolites were identified via the BioTransformer (BT) prediction workflow. Two metabolites were annotated exclusively by comparison against the established MMHW library. Seven were annotated exclusively by the BT workflow, and 12 metabolites were annotated by both methods, BT and library search (Figure 2).

Annotation results and pathways obtained by MetaboScape

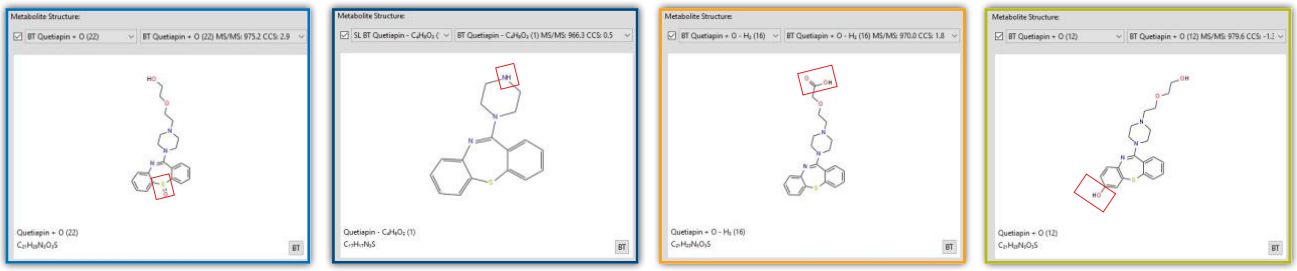
Feature							Spectral Library			BioTransformer		
Molecular formula	Annotation	theor. m/z	RT/ min	Δm/z/ ppm	mSigma	Rel. Int./ %	Name	MS/MS score	ΔCCS/ %	Name (Nr. of proposed structures)	MS/MS score	ΔCCS/ %
$C_{21}H_{25}N_3O_2S$	SL BT	384.1707	7.48	-0.33	16	31.57	Quetiapine	958	1.9	Quetiapine	959	1.9
$C_{17}H_{17}N_3S$	SL BT	296.1216	7.19	-1.335	26	15.11	Quetiapine-M (N-dealkyl-)	978	0.53	Quetiapine – $C_4H_9O_2$ (1)	966	0.53
$C_{17}H_{17}N_3OS$	BT	312.1165	9.73	-1.883	37	1.32	N/A	N/A	N/A	Quetiapine – C_4H_9O (9)	981	0.74
	SL BT		4.87	-0.686	6.0	0.22	Quetiapine-M (N-dealkyl-OH)	892	-0.91*	Quetiapine – C_4H_9O (9)	972	1.8
	SL BT		5.24	-0.06	26	0.93	Quetiapine-M (N-dealkyl-sulfoxide)	932	0.75	Quetiapine – C_4H_9O (9)	942	1.1
$C_{19}H_{19}N_3OS$	BT	338.1322	9.96	-0.996	2.3	0.02	N/A	N/A	N/A	Quetiapine – C_2H_5O (1)	867	1.5
$C_{16}H_{21}N_3OS$	BT	340.1478	7.35	0.209	3.5	12.91	N/A	N/A	N/A	Quetiapine – C_2H_3O (1)	954	2.0
$C_{19}H_{19}N_3O_2S$	SL BT	354.1271	7.94	-0.429	6.1	1.72	Quetiapine-M (N-CH2-COOH)	921	1.62	Quetiapine – C_2H_6 (12)	982	1.9
$C_{19}H_{21}N_3O_2S$	BT	356.1427	4.92	-0.556	9.0	0.19	N/A	N/A	N/A	Quetiapine – C_2H_4 (11)	1000	3.2
	SL BT		8.07	-0.459	6.6	0.94	Quetiapine-M (O-dealkyl-sulfoxide)	552	2.86	Quetiapine – C_2H_4 (11)	961	3.0
	SL BT		5.37	-0.163	13	0.3	Quetiapine-M (O-dealkyl-sulfoxide)	933	-1.3	Quetiapine – C_2H_4 (11)	965	-1.4
	SL BT						Quetiapine-M (O-dealkyl-sulfoxide)	957	2.5	Quetiapine – C_2H_4 (11)	976	2.6
$C_{19}H_{19}N_3O_3S$	SL	370.122	8.61	-0.822	18	2.68	Quetiapine-M (N-CH2-COOH-OH-piperazine)	842	-	N/A	N/A	N/A
	SL		5.7	-0.436	18	0.05	Quetiapine-M (N-CH2-COOH-sulfoxide)	894	-	N/A	N/A	N/A
$C_{21}H_{23}N_3O_3S$	BT	398.1533	7.82	-0.069	9.9	0.01	N/A	N/A	N/A	Quetiapine + O – H_2 (12)	983	1.9
$C_{21}H_{25}N_3O_3S$	SL BT	400.1680	5.56	-0.309	5.3	2.08	Quetiapine-M (HO-) isomer-1	918	0.40*	Quetiapine + O (11)	948	3.4
	SL BT		5.06	-0.02	15	17.5	Quetiapine-M (sulfoxide)	935	2.6	Quetiapine + O (11)	975	2.9
$C_{21}H_{25}N_3O_4S$	SL BT	416.1639	6.13	-0.405	15	5.91	Quetiapine-M (di-HO-)	664	-4.4*	Quetiapine + O_2 (58)	908	1.3
	BT		4.49	-0.129	13	0.09	N/A	N/A	N/A	Quetiapine + O_2 (57)	978	3.9
	BT		5.39	-0.122	16	0.2	N/A	N/A	N/A	Quetiapine + O_2 (58)	960	4.0
	SL BT		5.96	0.956	15	0.08	Quetiapine-M (di-HO-)	694	-1.6*	Quetiapine + O_2 (58)	864	3.8

*ambigous structure information in the spectral library

Figure 2. Quetiapine metabolites detected using UHPLC-timsTOF and MetaboScape.

Known metabolic pathways (Fig. 3) include: sulfoxidation (1), N-dealkylation (2), carboxylic acid formation on the ethoxyethanol sidechain (3) and 7-hydroxylation (4) [3].

(1) Sulfoxidation **(2) N-Dealkylation** **(3) Carboxylation** **(4) 7-Hydroxylation**



→ Rel. Int. 17.5 % → Rel. Int. 15.2 % → Rel. Int. 0.01 %

Figure 3. Examples for detectable metabolites from known metabolic pathways, including confident identification of metabolites of low relative abundance.

[3] R. C. Baselt, Disposition of Toxic Drugs and Chemicals in Man, 2017, 11th edition, p.1850

Conclusion

Revolutionizing metabolite monitoring

This AppNote represents a new way of getting the information of monitoring metabolites in urine and blood for the detection of both known and unknown psychoactive substances for toxicological screening and drug abstinence control. However, it requires sensitive detection as well as confident identification and annotation of multiple drugs and their metabolites, simultaneously.

Advanced annotation techniques

This application note demonstrates the utility of pooled Human Liver Microsomes to generate phase I metabolites of a model drug, and the annotation capacities for both known and previously unknown metabolites using the timsTOF Pro 2 system and MetaboScape software. Identification workflows includes both confidential library search and *in silico* biotransformation prediction. The experimental data comprises of a unique combination of accurate mass, isotope pattern, MS/MS fragmentation and determination of CCS values.

Efficient identification workflows

This workflow allows very fast annotation and review of drug metabolites in a single, multi-faceted analysis, with very good agreement with the expected metabolites from literature and libraries. The results can later be used in a targeted or non-targeted approach to track known and unknown psychoactive substances and to improve the overall picture of drug trafficking and consumption in clinical and forensic applications. All data can be used retrospectively and is suitable for reprocessing whenever new drugs or metabolites of interest are reported. Metabolites observed in a forensic urine or blood sample, potentially indicative for an unidentified NPS, can be linked to its parent through shared structural similarities, facilitating the NPS identification. The full *in silico* workflow provides results comparable to established *in vitro* assays but does not require any reference standards which are not commercially available in most instances.

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