T-ReXing Synthetic Cannabinoid Receptor Agonists – Unveiling Metabolites Suitable for Urine Screening via UHPLC-timsTOF-MS and MetaboScape

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Introduction

Since the late 2000s, the consumption of synthetic cannabinoid receptor agonists (**SCRAs**), a subset of new psychoactive substances (NPS), has constantly increased in Europe.[1] SCRAs show similar effects to phytocannabinoids like Δ9-THC, but are substantially more potent and have unpredictable side effects, resulting in serve intoxications and fatalities. Since the parent substances of SCRAs are rarely detectable in **urine**, the elucidation of the **metabolism** has played a major role in **forensic toxicology** laboratories to keep ahead in this **game of cat-and-mouse**. The **challenge** is to implement biomarkers of SCRAs into mass spectral screening methods as quickly as possible following their first detection in drug seizures. As animal studies are time-consuming and ethically questionable, the use of pooled human liver microsomes (**pHLMs**) is an efficient and cost-effective alternative to generate potential consumption markers for screening human urine samples.

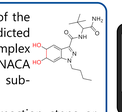
This study used a **Bruker timsTOF Pro 2**, **MetaboScape®** and its embedded **T-ReX®** (Time aligned Region complete eXtraction) algorithm for the software-assisted non-targeted identification of potential metabolites of three highly prevalent SCRAs. *In silico* metabolite prediction tools (BioTransfromer 3.0[2] (**BT**) and GLORYx[3] (**Gx**)) were used to predict unknown biomarkers since reference standards for novel SCRA metabolites are usually not commercially available. Hydrolyzed and non-hydrolyzed authentic forensic urine specimens found SCRA positive in routine analysis were re-acquired in positive PASEF (parallel accumulation serial fragmentation) and bbCID (broadband collusion induced dissociation) mode to select the most abundant metabolites *in vivo*.

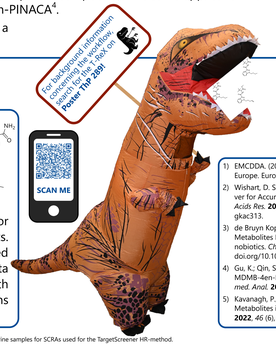
Conclusions

1) Qualitative findings from pHLM incubations and urine samples were mostly in good agreement.
2) MetaboScape was able to detect and annotate high abundant as well as compound specific metabolites.
3) The tentatively identified biomarkers were added to the UHPLC-qTOF screening method and can be used for retrospective evaluation of bbCID data and future screening of authentic urine samples with a subsequently developed TASQ-method.
4) The described approach is tremendously faster and less laborious compared to manual data evaluation following known metabolism patterns.
5) Enzymatic hydrolysis/cleavage of glucuronides for urine screening for SCRAs is suggested for increased sensitivity. Despite the fact that this extra step requires more time for sample preparation.
6) For MDMB-4en-PINACA only 4 metabolites (highest no. in selectable settings) were predicted *in silico* with the integrated BioTransformer 3.0. Different algorithms of BT (n=3) and GLORYx (n=1) with different number of biotransformation steps (n) may explain the dissimilarities.
7) ADB-BUTINACA and MDMB-BUTINACA share at least the hydrolysis metabolite (AB4/MB6). For interpretation at least one other compound-specific and high abundant metabolite is required (e.g. monohydroxylation of the parent compound). The same applies for MDMB-4en-PINACA sharing the ester hydrolysis metabolite (MP6) and further metabolites with ADB-4en-PINACA[4].
8) Adding a 4th dimension to SCRA metabolite screening allows for a higher confidence level in metabolite analysis.

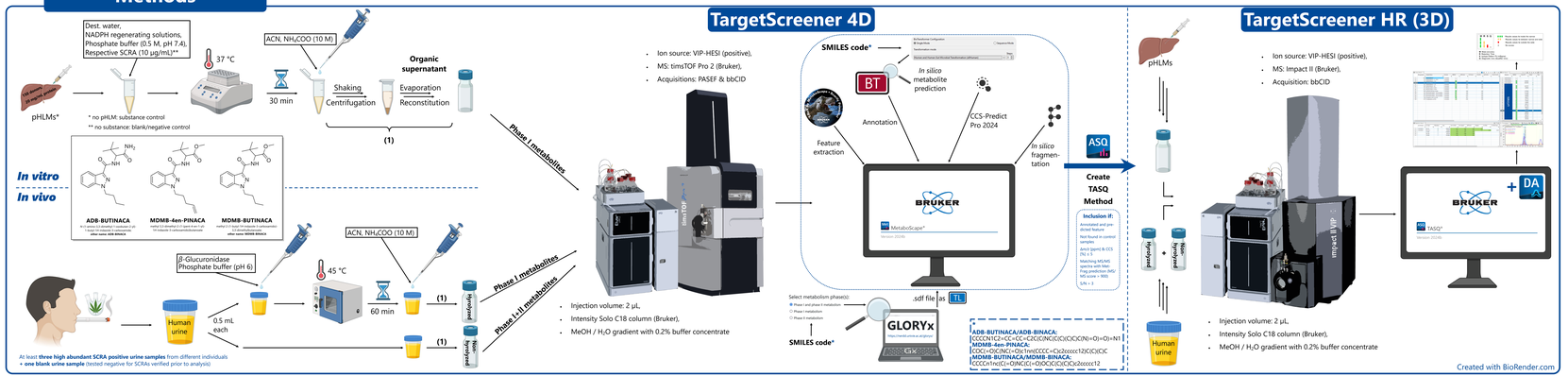
Limitations

1) Some of the main biomarkers for consumption of the respective SCRA already published were not predicted *in silico* probably because of multiple, complex reaction steps involved e.g. ADB-BUTINACA dihydrodiole (ADB-BUTINACA +2O+2H) and subsequent metabolites[5]
2) This issue could be solved by allowing more reaction steps or further manual reprocessing of *in silico* prediction results. Eventhough this would lead to an increase in annotated metabolites and consequenty in the time required for data processing in MetaboScape. With the occurrence of SCRAs with uncommon substituents like trimethylpropylsilyl (3TMS)-sidechains this might become especially challenging.





Methods



Results

ADB-BUTINACA

Formular Biotransformation	m/z calc. [M+H]+	MetID	Δm/z [ppm]	RT [min]	CCS$_{meas.}$ [Å²] [M+H]+	ΔCCS [%]	In vivo: Urines hydrolyzed	In vivo: Urines non-hydrolyzed	In vitro: pHLM	In silico: BT	In silico: Gx
Parent ($C_{19}H_{27}N_3O_2$)	331.2129	AB0	0.28	10.05	185.5	1.2	☒	☒	☑	☑	☑
		AB1	0.62	7.52	189.0	1.6	☑	☑	☑	☑	☑
Monohydroxylation (+O)	**347.2078**	**AB2**	**0.19**	**8.55**	**189.7**	**2.0**	☑	☑	☑	☑	☑
		AB3	0.35	9.22	188.6	1.4	☑	☑	☑	☑	☑
Amide hydrolysis (+O -N -H)	332.1969	AB4	0.51	9.90	185.6	1.6	☑	☑	☑	☑	☑
		AB5	-0.11	5.91	190.3	1.7	☑	☑	☑	☒	☒
Dihydroxylation (+2O)	363.2027	AB6	-1.50	8.07	194.0	3.1	☑	☑	☑	☒	☒
		AB7	-0.51	5.82	220.0	0.4	☒	☑	☑	☒	☒
Monohydroxylation + Glucuronidation (+O +C6H8O6)	523.2399	AB8	0.62	5.84	231.7	6.1	☒	☑	☑	☒	☒
		AB9	-0.82	6.10	233.1	6.7	☒	☑	☑	☒	☒
N-butanoic acid (+2O -2H)	361.1870	AB10	-0.34	7.06	186.1	-1.1	☑	☑	☑	☒	☑
Ketone formation* (+O -2H)	345.1919	A11	0.01	7.32	185.3	-0.0	☑	☑	☑	☑	☑

TASQ

Hydrolyzed MRSQ(C)	Urine	Non-hydrolyzed MRSQ(C)
AB2	AB1	AB2	AB1

*Presumably an aldehyde formation as common for many SCRAs oxidized from a N-4OH-butyl metabolite. However, monohydroxylation followed by dehydrogenation cannot be excluded.

Both metabolites could be detected in the hydrolyzed and non-hydrolyzed urine samples

For the screening method via the TargetScreener HR, the two most abundant metabolites **AB2** (monohydroxylation) and AB1 (monohydroxylation) based on the peak area were chosen.

MDMB-4en-PINACA

Formular Biotransformation	m/z calc. [M+H]+	MetID	Δm/z [ppm]	RT [min]	CCS$_{meas.}$ [Å²] [M+H]+	ΔCCS [%]	In vivo: Urines hydrolyzed	In vivo: Urines non-hydrolyzed	In vitro: pHLM	In silico: BT	In silico: Gx
Parent ($C_{20}H_{27}N_3O_3$)	358.2125	MP0	-1.73	11.63	190.8	0.4	☒	☒	☑	☑	☑
		MP1	-0.96	8.98	191.6	-0.6	☑	☑	☒	☑	☑
Monohydroxylation (+O)	374.2074	MP2	-0.92	9.53	193.7	0.2	☑	☒	☒	☑	☑
		MP3	0.17	10.42	194.7	1.0	☑	☒	☒	☑	☑
		MP4	1.24	11.03	192.7	-0.1	☒	☒	☑	☑	☑
		MP5*	-0.47	8.77	186.1	-0.1	☒	☒	☑	☑	☑
Ester hydrolysis (-CH2)	**344.1969**	**MP6**	**0.52**	**10.02**	**186.7**	**0.2**	☑	☑	☑	☑	☑
Dihydrodiol/double bond oxidation (+O2 -H2)	392.2180	MP7	-0.28	8.19	196.4	-0.1	☑	☒	☑	☑	☑
		MP8	-1.68	8.57	197.2	0.3	☑	☒	☑	☑	☑
N-dealkylation (-C4H8)	290.1499	MP9	0.59	8.43	167.9	-1.9	☒	☒	☑	☒	☑

TASQ

Hydrolyzed MRSQ(C)	Urine	Non-hydrolyzed MRSQ(C)
MP6	MP2	MP6	MP2

*Probably in source fragmentation of the respective phase II metabolites (two coeluting O-glucuronide conjugates but with different measured CCS values (210.6 and 224.3 Å²).

Only metabolite MP6 could be detected in the hydrolyzed and non-hydrolyzed urine samples.

For the screening method via the TargetScreener HR, the two most abundant metabolites **MP6** (ester hydrolysis) and MP2 (monohydroxylation) based on the peak area were chosen.

MDMB-BUTINACA

Formular Biotransformation	m/z calc. [M+H]+	MetID	Δm/z [ppm]	RT [min]	CCS$_{meas.}$ [Å²] [M+H]+	ΔCCS [%]	In vivo: Urines hydrolyzed	In vivo: Urines non-hydrolyzed	In vitro: pHLM	In silico: BT	In silico: Gx
Parent ($C_{19}H_{27}N_3O_3$)	346.2125	MB0	0.98	11.55	188.6	1.2	☒	☒	☑	☑	☑
		MB1	0.65	8.85	191.4	1.7	☒	☒	☑	☑	☑
Monohydroxylation (+O)	362.2074	MB2	1.38	9.64	192.5	1.7	☒	☒	☑	☑	☑
		MB3	0.89	10.44	193.2	2.1	☒	☒	☑	☑	☑
		MB4	1.46	10.93	191.4	1.1	☒	☒	☑	☑	☑
		MB5*	1.23	8.63	184.9	1.2	☒	☒	☑	☑	☑
Ester hydrolysis (-CH2)	**332.1969**	**MB6**	**0.62**	**9.91**	**185.3**	**1.4**	☑	☑	☑	☑	☑
Ester hydrolysis + monohydroxylation (-CH2 +O)	348.1918	MB7	-0.14	7.46	188.8	1.9	☑	☑	☑	☑	☑
Dihydroxylation (+2O)	378.2023	MB8	0.42	7.15	195.2	2.3	☒	☒	☑	☒	☒
Ester hydrolysis + dehydrogenation (-CH4)	330.1812	MB9	-0.01	9.43	182.4	0.6	☒	☒	☑	☒	☒
Ester hydrolysis + monohydroxylation + glucuronidation (-CH2 +O +C6H8O6)	524.2239	MB10	-0.23	6.30	217.6	-0.8	☒	☑	☒	☒	☑
Ketone formation (+O -2H)**	360.1918	MB11	1.20	8.94	188.8	0.1	☒	☑	☑	☒	☒
		MB12	-0.59	9.53	190.3	1.3	☑	☑	☑	☒	☒
Ester hydrolysis + ketone formation (+O -CH4)	346.1761	MB13	1.38	6.90	185.6	0.5	☑	☑	☑	☒	☒
N-dealkylation (-C4H8)	290.1499	MB14	1.81	8.45	167.9	-1.9	☒	☒	☑	☑	☑

TASQ

Urine	Hydrolyzed MRSQ(C)	Urine	Non-hydrolyzed MRSQ(C)
	MB6	MB7		MB6	MB7

*Probably in source fragmentation of the respective phase II metabolites (two coeluting O-glucuronide conjugates with different measured CCS values (225.6 and 231.4 Å²).
**A monohydroxylation with subsequent dehydrogenation, an aldehyde formation or a dihydroxylation with in source water loss could not be excluded.

The metabolites stated here are not substance specific ones. Both can be found (to a lesser extent) in urine samples positive for metabolites of ADB-BUTINACA.

Urine	Hydrolyzed MRSQ(C)	Urine	Non-hydrolyzed MRSQ(C)
	MB7		

In some urines, a compound specific metabolite (monohydroxylation) could be detected succesfully too.

Sources

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