A pragmatic approach to detect SPICE-metabolites in urine with HPLC-MS/MS

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Abstract

Aim: A mayor problem making the detection of SPICE-Substances in urine so difficult is the high volatility of the current drug market. Metabolites of the main SPICE-compounds are hardly available and cause high costs. A pragmatic approach had to be found to counter the above mentioned difficulties in SPICE-Metabolite-Analyses. Methods: From the known substance-structure one can conclude the molecular weight of the monohydroxylated metabolite, known as Q1-mass for the LC/MS-MS detection. It does not matter where exactly the monohydroxylation takes place the molecular weight remains the same, as well as the m/z of the produced fragments. These fragments can be theoretically determined and conduce to the Q3 m/z. The urine sample preparation includes an incubation step with β-glucuronidase and a liquid/liquid extraction with 1-chlorbutane. The organic phase has to be evapourated and reconstituted and is now prepared for LC/MS-MS -Analyses. A reliable Immunoassay can be performed. Results: Analysed authentic urine samples containing the monohydroxylated metabolites of the commonly used SPICE-Substances AM-2201 and JWH-122 have shown the expected and theoretically determined fragmentation pattern in LC/MS-MS analyses. Subsequently performed immuno analyses showed 100% accordance to the LC/MS-MS findings. Conclusion: Given the fact that in authentic urine samples the theoretically determined fragments can be detected one can conclude that the presented pragmatic approach holds true for all metabolised SPICE-Substances. Therefore the laboratories are not dependent on commercially available metabolites to perform LC/MS-MS analyses to detect SPICE-metabolites in urine.

1. Introduction

For the past few years, a new type of drug has been flooding the European market: synthetic cannabinoids, better known as „SPICE"- substances [1]. They are easily obtained through the internet and not fully regulated by the German „Dangerous Drugs Act"(Betäubungsmittel Gesetz) [2]. The SPICE-market is extensively volatile and therefore a huge number of newly developed substances offer a wide choice for the SPICE-user every month. These preconditions form the main problems for the analysis:

1) The commonly detected metabolites in urine are scarcely obtainable through a standard-provider (and in some cases the main metabolites still remain unknown).
2) The variety of new substances makes it nearly impossible to keep up with the market conditions.
3) The purchase of all the reference material causes high costs and leads inevitably to non economical investments.

To find a solution for the above mentioned problems, a new pragmatic approach had to be found: Considering the fact, that many SPICE substances have a common chemical structure

(e.g. N-naphtylindoles), one can assume that their metabolites are similar, too and form a similar fragmentation pattern in MS/MS analyses (Fig. 1).

The Phase-I Monohydroxylated product can be hydroxylated in several different positions, most likely on the sidechain [4].

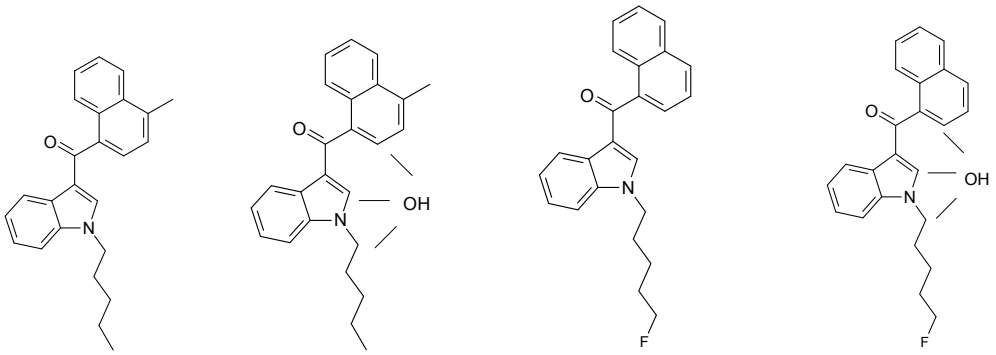

Fig. 1. JWH-122 and possible monohydroxymetabolites, AM-2201 and possible mono-hydroxymetabolites (from left to right).

To determine the possible fragments in the MS/MS pattern, it is irrelevant to know exactly where the hydroxylation occurs. The fragments will have the same mass, no matter where the main substance is hydroxylated. With help of this assumption, a theoretical fragment pattern of the monohydroxylated metabolites can be postulated [3]. This holds true for several related substances and therefore can be transferred to a number of SPICE-molecules with monohy-droxylated metabolites (Tab 1).

Tab. 1. Masses of several different SPICE-substances and their possible theoretically deter-mined monohydroxylated metabolites.

Analyte as Monohydroxymetabolite	Q1	Q3
JWH-018	358,2	155,1
JWH-073	344,2	155,1
JWH-250	352,2	149,1
1-Butyl-3-[-1-(4-Methylnaphtoyl)]-indole	358,2	169,1
JWH-081	388,2	185,1
AM-2201	376,2	155,1
JWH-122	372,2	169,1
RCS-4	338,2	135,1
JWH-210	386,2	183,1
JWH-200	401,2	155,1
1-Butyl-3-[-1-(4-Methoxybenzoyl)]-indole	324,2	135,1
WIN 55,212-2	443,2	155,1
AM 694	452,2	231
JWH-203	356,2	153,1
JWH-007	372,2	155,1
AM-1220	399,2	155,1
3-(-1-Adamantoyl)-1-pentylindole	366,2	163,1
1-(5-Fluorpentyl)-3-(1-(4-methylnaphtoyl))indole	390,2	169,1
3-[(2-Methoxyphenyl)acetyl]-1-((methylpiperidin-2-yl)methyl]indole	393,2	149,1
	344,2	155,1
JWH-015	336,2	133,1
JWH-251	395,2	135,1
WIN 48,098	392,2	189,1
JWH-398	392,2	149,1
RCS-8	436,2	233,1
JWH-387		

2. Material and Methods

2.1. Sample Preparation

500 µL urine are spiked with 20 µL Internal Standard (in this case either AM 2201 or MDPV-d8). The pH is adjusted to pH 5 with 100 µL phosphate buffer. The whole sample is treated with 25 µL glucuronidase/arylsufatase and incubated for 6 h at 37 °C. After the incubation period, 1 mL 1-chlorbutane is added to the sample for liquid/liquid extraction. The thoroughly mixed sample undergoes centrifugation, the supernatant is evaporated and resolved with a mixture of 75 µL acetonitrile/water/formic acid and is now ready LC/MS-MS analysis.

2.2. Immunoassay

In addition to the LC/MS-MS analyses, a homogenous immunoassay was evaluated, which is sensitive for a number of main substances as well as for monohydroxylated and carboxy metabolites (Specialty Diagnostix GmbH Passau, Tab. 2). The immunoassay was calibrated with the substance JWH-018 N-pentanoic acid and is designed for human use only.

Tab. 2. Cross reactivity with related drugs.

Analyte	[%] Cross reactivity
JWH-018 N-pentanoic acid	100
JWH-018 4-hydroxyindole	1,4
JWH-018 5-hydroxyindole	1,8
JWH-018 N-(5-hydroxypentyl)	125
JWH-018 N-(5-hydroxypentyl)-β-D-glucuronide	91
JWH-073 6-hydroxyindole	25
JWH-073 N-(4-hydroxybutyl)	132
JWH-073 N-butanoic acid	83
JWH-122 N-(4-hydroxypentyl)	13
AM-2201 6-hydroxyindole	26
AM-2201 N-(4-hydroxypentyl)	133
3-(1-naphtoyl)-1H-indole	16
Cannabipiperidiethanone	0.03
JWH-007	0.7
JWH-015	3
JWH-018	15
JWH-019	1.8
JWH-022	27
JWH-073	23
JWH-081	0.05
JWH-122	1.2
JWH-200	67
JWH-201	0.01
JWH-250	0.02
JWH-398	0.4
AM-1220	50
AM-2201	33
AM-2232	94
AM-2233	1.5

Tab. 3. Qualitative precision: 20 ng/mL cut-off intra-day-run (1 run, N = 40 reps. per level).

Within-run Precision	**Blank**	**Low Control**	**Cut-off Cal.**	**High Control**
Targets [ng/ml]	**0.0**	**10.0**	**20.0**	**30.0**
Mean (mAbs/min)	426	464	498	525
Total CV [%]	0.39	0.48	0.48	0.70
Mean (ng/ml) *	-0.01	10.46	19.93	27.51
Total CV [%] *	---	5.90	3.40	3.70
*Concentration data (in ng/ml) was calculated based on mAbs/min data sets				

Tab. 4. Qualitative precision: 20 ng/mL cut-off Inter-Day-Run (20 reps. per run, 2 runs a day over 4 days, being N = 160)

Total Precision	**Blank**	**Low Control**	**Cut-off Cal.**	**High Control**
Targets [ng/ml]	**0.0**	**10.0**	**20.0**	**30.0**
Mean (mAbs/min)	429	468	502	526
Total CV [%]	0.53	0.75	0.88	0.95
Mean (ng/ml) *	-0.03	10.68	19.87	26.50
Total CV [%] *	---	9.00	6.10	5.20
*Concentration data (in ng/ml) was calculated based on mAbs/min data sets				

Accuracy was tested by sixty-seven native urine samples which were analysed with the Immunanalysis Synthetic Cannabinoid Assay at 20 ng/mL on a Beckmann AU 400 analyzer and by LC/MS-MS.

Tab. 5. Comparison of the results gained by the immunoassay- and LC/MS-MS analyses.

20 ng/mL Qualitative		**LC/MS-MS**	
		+	-
Beckmann AU 400	+	30	0
	-	1*	36
*the false negative tested sample in the validation process contained JWH-250 which has a only a 1 % cross reactivity with the Immunoassay. [5]			

2.3. LC-MS/MS Parameters

For LC-MS/MS analysis the AB Sciex API 4000 QTrap was used employing a PFP column. With a flow rate of 1000 µL/min for the first 15 minutes of the run the following gradient was used employing 0.2 % ammonium formate, 0.2 % formic acid as solvent A and ACN as solvent B: 10 % B increase to 90 % B at 5.33 %/min, 90 % B for 5.0 min, decrease to 10 % B at 160 %/min and 10 % B for 2.5 min.The MRM- Transitions are listed in Tab. 1. Further MS-Parameters are for instance the Declustering-Potential (DP): 40 eV, the Collision-Energy-Spread (CES) of 20, 35 and 50 eV and the Cell-Exit-Potential (CXP):15 eV. The Scan rate was set to 4000 Da/s.

3. Results and Discussion

More than 1000 urine samples were tested with the above mentioned LC-MS/MS-method for monohydroxylated synthetic cannabinoids. Positive results were obtained in about 10% of total number of specimen. In the majority of the positive cases, hydroxybutyl and hydroxypentyl metabolites of the active compounds AM-2201 were detected, followed by hydroxypentyl metabolites of JWH-122, JWH-018, RCS-04 and JWH-210.

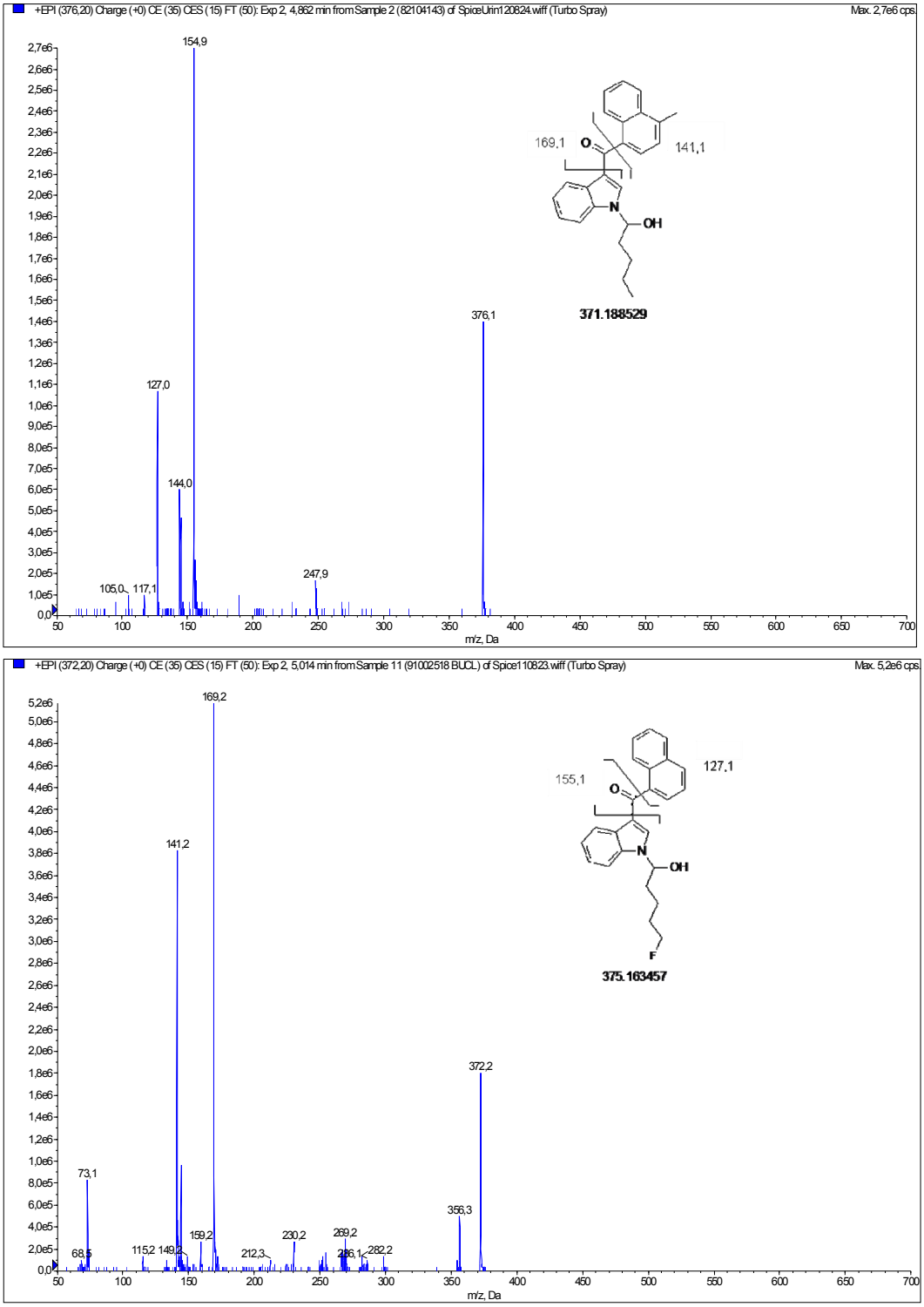

Fig. 2. Enhanced product ion scan of AM-2201 metabolite and JWH-122 metabolite in urine.

One can see in the chromatograms above (Fig. 2) that the main masses resulting from the fragmentation-process are the masses 169.2 and 141.2 for the monohydroxylated JWH-122 metabolite and the masses 154.9 and 127.0 for the monohydoxylated AM-2201 metabolite. These masses (169.2; 141.2; 154.9; 127) found in authentic urine are consistent with the expected calculated masses and proof the accuracy of the presented pragmatic approach. All of the subsequently performed immunoassays showed 100% accordance. No false positive results were observed.

Tab. 6. Analytical limits (DIN 32645).

Substance	LOD [ng/mL]	LOQ [ng/mL]
JWH-018	0.9	2.1
JWH-122	0.8	2.3
AM-2201	1.2	3.2

4. Conclusions

The three main problems presented in the introduction can be solved with this pragmatic approach:

1) The formerly unknown metabolites are provided by the consumer himself in his own urine sample.
2) The laboratory analyses can react fast enough to keep up with the current market.
3) There is no need to require the Standard solutions and therefore noneconomical expenses are unnecessary.

The additionally performed immunoassay showed an excellent performance resulting in 100 % sensitivity and specificity in this study.

5. References

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[4] Wohlfarth A, Scheidweiler KB, Chen X, Liu H, Huestis MA. Qualitative confirmation of 9 synthetic cannabinoids and 20 metabolites in human urine using LC-MS/MS and library search. Anal Chem 2013;85:3730-3738.

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