Metabolism of Tryptamine-derived New Psychoactive Substances and Development of Novel Screening Concepts Using Hyphenated Mass Spectrometry

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VORWORT

Die nachfolgende Arbeit entstand unter der Anleitung von Herrn Univ.-Prof. Dr. Dr. h.c. Hans H. Maurer in der Abteilung für Experimentelle und Klinische Toxikologie der Fachrichtung Experimentelle und Klinische Pharmakologie und Toxikologie der Universität des Saarlandes in Homburg (Saar) von Oktober 2013 bis September 2017.

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“Gift in den Händen eines Weisen ist ein Heilmittel, ein Heilmittel in den Händen des Toren ist Gift.”

Giacomo Casanova (1725 - 1798), Histoire de ma vie

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1. GENERAL PART

1.1. NEW PSYCHOACTIVE SUBSTANCES (NPS)

The phenomenon of new psychoactive substances (NPS) showed continuously increasing impact worldwide and represent a challenge for public health.[1] Such NPS are usually produced in bulk quantities by chemical and pharmaceutical companies e.g. in China or by clandestine laboratories either in Europe or elsewhere.[1] They are most commonly distributed by specialized shops in the web under terms such as “research chemical”, “bath salts”, “plant food”, or “spice”. Despite their free availability, a misuse with or without overdosing might lead to severe and even fatal poisonings.[1] After their appearance on the market and following misuse, they usually get controlled by international or national drug control laws with certain time offset. Then, the creation of new substances with similar chemical structures and effects appear, circumventing existing legislations making it a “cat and mouse game”.[2] The corresponding NPS with lack of legal enforcement are therefore also branded as “legal highs”. To keep tracking the flood of compounds, several drug monitoring systems such as the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) or the United Nation Organization on Drugs and Crime (UNODC) register new upcoming NPS. This information is shared with the community by Early Warning Systems or can be found in annual drug reports amongst other statistical information. According to the European Drug Report 2017 by EMCDDA, the number of new detected substances increased over the last years.[3] By the end of 2016, the appearance of more than 620 NPS could be monitored on the European drug market.[1] The number of 66 new detected substances during 2016, however, was

lower than in previous years.[1] This may lead to a positive trend, especially if it will sustain. However, the overall available NPS on the market still continue to grow despite slowing pace of new drug appearance.[1] Reasons for that trend might be the introduction of blanket bans or generic and analogue-based legislation in different countries. In Germany, for example, such a new legislation was introduced in late 2016, covering NPS that belong to groups of amphetamine-type stimulants including cathinones and synthetic cannabinoids.[4] Although this law was a huge step forward, it did not cover all groups of NPS, yet. One of these uncovered groups was that of synthetic tryptamines, whose members are still among the most commonly reported NPS besides synthetic cathinones, synthetic cannabinoids, synthetic benzodiazepines, phenethylamines, piperazines, and synthetic opioids.[1] This might lead to an increasing attention to this group in the following years.

1.2. Pharmacology and Misuse of Tryptamine-derived NPS

The psychoactive effect of synthetic as well as biogenic tryptamines are mainly based on the interaction with the serotonergic system. They could activate serotonin (5-HT) receptors, could increase the 5-HT release, and/or act as 5-HT reuptake inhibitors at the corresponding transporters causing hallucinogenic and stimulating effects.[5-8] Alexander and Ann Shulgin already described many synthetic tryptamines as potential entactogens in the book TiHKAL (Tryptamines I Have Known and Loved) in 1997.[5,9] Here, detailed syntheses of 55 tryptamine-derived compounds and qualitative or quantitative comments are given to the effects, their duration, and the suggested dosage after self-experiments.[9] Some of the described compounds appeared later on the drug market with reported misuse such as 5-methoxy-*N,N*-

diisopropyltryptamine (5-MeO-DIPT), also known under names such as “Foxy methoxy”,[10-13] or 5-methoxy-*N,N*-diallyltryptamine (5-MeO-DALT).[14] The latter belongs to the *N,N*-diallyltryptamine (DALT) derivatives, which represented an important group within the synthetic tryptamines.[15] 5-MeO-DALT and DALT itself were already described as hallucinogens in TiHKAL.[9] Receptor binding profiles with structure-affinity relationships of these compounds as well as four further ring-substituted derivatives were studied by Cozzi and Daley.[15] They concluded that ring-substituted derivatives, especially at 5-position, showed pharmacological potency[15] underlining again the probability that further DALT derivatives will once appear on the drug market as NPS.

1.3. URINE SCREENING PROCEDURES

For reliable drug testing, adherence monitoring, or detection of toxic compounds, several screening techniques are available.[16] Common screening procedures using, for example, immunoassays allow fast detection of particular drugs or limited drug classes. However, results have to be confirmed due to the risk of false positives, at least in forensic cases. Furthermore, most of the NPS are not covered by such screenings with the consequence of overlooking. Therefore, screening procedures based on mass spectrometry (MS) techniques with low or high resolution (HR) are widely used, providing reliable and confirmed screening results with sufficient sensitivity.[16-23] As further goal, a corresponding applied approach should allow comprehensive screening of a range of drugs and/or compounds. This could be achieved by analysis using full scan data, for example, with data dependent acquisition and library-based compound identification.[16] Here, a higher number of

targets could be addressed and retrospective data processing is possible after updates of the corresponding reference library. As most drug screening procedures were performed in urine due to usually longer detection window and higher analyte concentrations, the metabolites of corresponding drugs have to be covered, especially when the metabolized form is excreted exclusively.[24,25] For classic drugs, these metabolites are already investigated by the providing pharmaceutical companies. For NPS, however, the biotransformation is generally unknown. Thus, investigations on the NPS biotransformation by *in vitro* or *in vivo* studies are mandatory to identify their urinary excreted metabolites as screening targets.

1.4. NOVEL SCREENING CONCEPTS

1.4.1. DRIED MATRIX SPOTS

Beyond screening approaches using conventional sampling, workup, or analysis, there was an increasing interest in alternatives in recent years.[26-28] One of these alternatives were dried matrix spots (DMS). They represent a promising technique for microsampling as well as a strategy for sample collection and storage due to small sample volume requirements, high analyte stability, low transport and storage costs, and reduced infection risks.[29] For sampling, only a few microliters of a fluid are spotted, soaked, and dried on a filter paper card whereby mainly blood or urine were used in context of drug screening. So far, only limited numbers of targets were covered in corresponding dried blood spot (DBS) or dried urine spot (DUS) screening approaches with focus on particular drugs or drug classes.[30,31] However, a comprehensive DMS-based screening has not been described, yet.

1.4.2. PAPER SPRAY IONIZATION

Most mass spectrometric analysis were performed after an appropriate preparation of the sample and sufficient separation of the analytes using gas chromatography (GC) or liquid chromatography (LC). These steps resulted in high sensitivity and robustness, but also prolonged turnaround time. One promising technique, which might allow the reduction of workload and analysis time, is paper spray ionization (PSI). For this ambient ionization technique, a small amount of a fluid is spotted, soaked, and dried on a triangular piece of paper in analogy to DUS sampling. For PSI, however, complex matrices such as blood or urine might be directly analyzed without time and work-consuming preparation. In the last years, the potential and advantages of PSI coupled to MS with low or HR were shown in various publications.[32-37] In analogy to DUS screening approaches, however, published PSI approaches also covered only limited numbers of targets. Thus, a comprehensive PSI screening is still missing.

2. AIMS AND SCOPES

The aim of this dissertation was the elucidation of the biotransformation as well as the toxicological NPS detectability in urine of the tryptamine-derived DALT and its ring-substituted derivatives 5-MeO-DALT, 5-fluoro-DALT, 7-methyl-DALT, and 5,6-methylenedioxy-DALT. Their chemical structures are given in Figure 1. Furthermore, novel urine screening procedures for drug testing should be developed and validated by usage of DUS or PSI coupled to MS with low or HR. The approaches should then be compared to established procedures and techniques to show their possibilities and limitations.

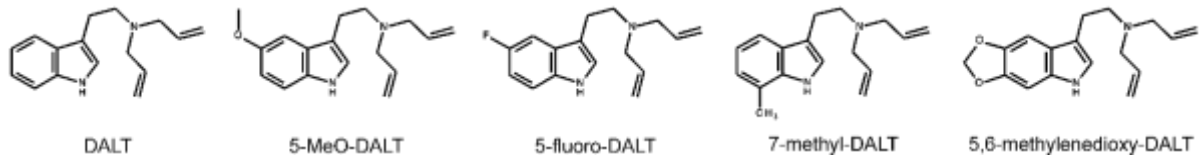

Figure 1. Chemical structure of the studied compounds *N,N*-diallyltryptamine (DALT), 5-methoxy-DALT (5-MeO-DALT), 5-fluoro-DALT, 7-methyl-DALT, and 5,6-methylenedioxy-DALT.

The following steps had to be conducted:

- Identification of the phase I and II metabolites of DALT, 5-MeO-DALT, 5-fluoro-DALT, 7-methyl-DALT, and 5,6-methylenedioxy-DALT in rat urine by LC-HR-tandem mass spectrometry (MS/MS)
- Confirmation of the metabolic phase I steps after incubations with pooled human liver microsomes (pHLM)

- Investigation of the general involvement of human cytochrome P450 (CYP) isoenzymes in the main metabolic steps
- Toxicological detectability in urine by standard urine screening approaches using GC-MS,[38,39] LC-multi stage mass spectrometry (MS^n),[16,40] and LC-HR-MS/MS[23,41] approaches
- Development of a DUS workup procedure for coupling to an established LC-MS^n approach[16,40] for metabolite-based comprehensive screening
- Validation of the new DUS approach according to international guidelines[42,43] using drugs of various drug classes
- Comparison to screening after a conventional sample preparation[16] by application to authentic human urine samples
- Transfer of the DUS workup to a broad LC-HR-MS/MS drug screening[23,41]
- Investigation of the screening power in comparison to established LC-MS^n,[16,40] LC-HR-MS/MS,[23,41] and GC-MS[38,39] techniques by application to authentic human urine samples and rat urine samples after drug administration
- Development of a suitable PSI setup and coupling to a HR-MS/MS system for developing a comprehensive urine screening for drug testing
- Validation of the new PSI-HR-MS/MS approach according to international guidelines[42,43] using drugs of various drug classes
- Comparably testing of authentic human urine samples with the new PSI-HR-MS approach or conventional LC-based approaches[23,41]

3. PUBLICATIONS OF THE RESULTS

The results of the studies were published in the following papers:

3.1. METABOLISM OF THE NEW PSYCHOACTIVE SUBSTANCES *N,N*-DIALLYLTRYPTAMINE (DALT) AND 5-METHOXY-DALT AND THEIR DETECTABILITY IN URINE BY GC-MS, LC-MS^N, AND LC-HR-MS-MS[44]

(DOI: 10.1007/s00216-015-8955-0)

3.2. Biotransformation and Detectability of the New Psychoactive Substances *N,N*-Diallyltryptamine (DALT) Derivatives 5-Fluoro-DALT, 7-Methyl-DALT, and 5,6-Methylenedioxy-DALT in Urine Using GC-MS, LC-MS^n, and LC-HR-MS/MS^{45}

(DOI: 10.1007/s00216-016-0117-5)

3.3. DRIED URINE SPOTS - A NOVEL SAMPLING TECHNIQUE FOR COMPREHENSIVE LC-MS^N DRUG SCREENING[46] (DOI: 10.1016/J.ACA.2017.05.033)

3.4. POWER OF ORBITRAP-BASED LC-HIGH RESOLUTION-MS/MS FOR COMPREHENSIVE DRUG TESTING IN URINE WITH OR WITHOUT CONJUGATE CLEAVAGE OR USING DRIED URINE SPOTS AFTER ON-SPOT CLEAVAGE IN COMPARISON TO ESTABLISHED LC-MS^N OR GC-MS PROCEDURES[47]

(DOI: 10.1002/DTA.2255)

3.5. Paper Spray Ionization Coupled to High Resolution Tandem Mass Spectrometry for Comprehensive Urine Drug Testing in Comparison to Liquid Chromatography-coupled Techniques after Urine Precipitation or Dried Urine Spot Workup[48]

(DOI: 10.1021/acs.analchem.7b03398)

4. DISCUSSION AND CONCLUSIONS

4.1. METABOLISM AND DETECTABILITY STUDIES OF THE INVESTIGATED TRYPTAMINE-DERIVED NPS

For the five studied tryptamines of the DALT group, an extensive metabolism was observed in rats. Considering the results obtained by incubations with pHLM, the same metabolites could be expected in humans. All in all, several aromatic and aliphatic hydroxylations, *N*-dealkylation, *N*-oxidation, and combinations thereof were proposed as the main metabolic pathways for all compounds as well as extensive glucuronidation or sulfation steps after phase I transformation. In addition, *O*-demethylation could be observed for 5-MeO-DALT, carboxylation after initial hydroxylation of the methyl group for 7-methyl-DALT, and *O*-demethylenation for 5,6-methylenedioxy-DALT. For the formation of the phase I metabolites, several CYP isoenzymes were responsible. The following CYPs were generally involved: CYP2D6 and CYP3A4 for all studied DALT derivatives; CYP1A2 and CYP2C19 for all derivatives except for DALT itself; CYP2C9 for 5-fluoro-DALT, 7-methyl-DALT and 5,6-methylenedioxy-DALT; CYP2B6 for 5-fluoro-DALT and 7-methyl-DALT; and CYP3A5 only for 7-methyl-DALT. LC-MS^n and LC-HR-MS/MS were suitable for monitoring consumption of all compounds after rat doses of 1 mg/kg body weight, respectively, which corresponded to a human dose of approximately 10 mg. The most abundant LC screening targets were as follows: for DALT a hydroxy-aryl metabolite and its glucuronide; for 5-MeO-DALT the *N,O-bis*-dealkyl metabolite and its glucuronide, for 5-fluoro-DALT one of two hydroxy-aryl metabolites and both corresponding glucuronides; for 7-methyl-DALT one *N*-deallyl-hydroxy-aryl, the

carboxy, and one dihydroxy-aryl metabolite; and for 5,6-methylenedioxy-DALT the demethylenyl metabolite, as well as its oxo metabolite and its glucuronide. In addition, a consumption of DALT should also be detectable by GC-MS via its main phase I metabolites. For the compounds 5-fluoro-DALT, 7-methyl-DALT, and 5,6-methylenedioxy-DALT, no data were available for drug dose or potency and thus the expected human users' dose could also be lower than 10 mg. Therefore, a 10-fold lower rat dose of 0.1 mg/kg body weight was additionally studied for these compounds to verify reliable detection. 5-fluro-DALT and 7-methyl-DALT could be detected by the LC-HR-MS/MS screening, 5-fluro-DALT in addition by the LC-MS^n approach. However, a reliable monitoring of 5,6-methylenedioxy-DALT could not be achieved after administration of such low doses. Here, an alternative sample preparation should be performed.

4.2. Dried Urine Spots as Alternative Sampling for LC-MS Urine Screening Approaches

The developed workup using DUS consisted of spotting on filter paper cards and enzymatic on-spot conjugate cleavage followed by liquid extraction (DUSglucE) and was coupled to LC-MS^n or LC-HR-MS/MS. For successful validation[42,43] of the workup, model compounds of several drug classes (antidepressants, benzodiazepines, cardiovascular drugs, neuroleptics, opioids, stimulants, etc.) were selected, covering a broad range of (physico-)chemical properties and chromatographic behaviors. By testing of the parameters recommended for qualitative approaches by LC-MS^n, the novel DUS workup showed results comparable to established urine precipitation (UP) with acetonitrile.[16] However, an

additional conjugate cleavage was necessary due to the basified extraction with low expected recoveries for glucuronides and sulfates. Hence, an on-spot deconjugation was included and tested with seven common phase II metabolites. The hydrolysis efficiencies were sufficient for most compounds and comparable to conventional cleavage procedures. It allowed reliable detection especially for drugs with high urinary glucuronide excretion. For applicability testing, 103 authentic human urine samples were analyzed as well as six rat urine samples after 0.1 mg/kg body weight substance administrations. The latter were tested to assess the sensitivity after DUSglucE for such low-dosed compounds with extensive metabolism and thus high target spreads. 5-Fluoro-DALT, 7-methyl-DALT, and 5,6-methylenedioxy-DALT were chosen for testing after rat administration as well as 2-(4-iodo-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine (25I-NBOMe), 25-bromo-NBOMe (25B-NBOMe), and 25-chloro-NBOMe (25C-NBOMe). For these compounds, extensive biotransformation and/or consumption in very low doses were described.[44,45,49,50] The DUSglucE was compared to an established workup by UP, which was performed without and with conjugate cleavage to assess its actual impact on the detectability. Furthermore, a comprehensive screening procedure by GC-MS[38] was additionally tested as this technique was the gold standard for drug screening for a long time.[18] The screening results were obtained after splitting the urine samples into four parts and worked up by (i) UP,[16,23] (ii) UP with conjugate cleavage (UglucP), or (iii) DUSglucE for LC-MS^n and LC-HR-MS/MS analysis. For GC-MS analysis, (iv) acid hydrolysis was performed, followed by liquid-liquid extraction, and acetylation (UHyAc).[38] For comparison of the different approaches and workups, the 770 detected drug intakes were set to 100%. In the LC-MS^n approach, the detected drug intakes ranged from 41-49%. In comparison, the LC-HR-MS/MS approach was able

to detect at least over one-third more drug intakes. In detail, 77% of the drug intakes were detected after DUSglucE, which was comparable to 80% after UP. After UglucP, overall impact of the deconjugation was shown to be +9%. The differences to DUSglucE should be caused mainly by the smaller urine volumes used. Moreover, the value of drug intakes identified by the GC-MS approach after UHyAc was 56%, lying between both LC-based screening approaches. Concerning the rat urine samples, an intake could not be reliably monitored in the LC-MS^n approach after the tested workups. Only the LC-HR-MS/MS approach was able to detect the compounds after UP and UglucP as well as after DUSglucE, what underlined the high sensitivity of the LC-HR-MS/MS system. In conclusion, DUS might be an alternative sampling technique for comprehensive metabolite-based drug testing or adherence monitoring showing best screening results after coupling to LC-HR-MS/MS.

4.3. New Paper Spray Ionization-HR-MS/MS Approach as Promising Alternative for Comprehensive Urine Drug Testing

The new PSI-HR-MS/MS approach was developed for reliable screening of a broad range of drugs of different drug classes and successfully validated.[42,43] For validation, again exemplary compounds were used which covered a broad range of chemical and physicochemical properties in analogy to the validation of the DUSglucE. The procedure showed high matrix effects for most drugs. Nevertheless, the limits of identification were acceptable for a broad urine screening and comparable with procedures using LC.[23] In addition, the international cut-off recommendations for the

tested drugs of abuse[51] were fulfilled. However, the detection of drugs in low concentrations and the risks of analyte carry-over marked limitations of the approach. Furthermore, it should be aware of false positive/negative results caused by mixed spectra due to the lack of analyte separation. For applicability testing, the identical authentic human urine samples were used as described for the DUSglucE study. This allowed a direct comparison of the results by the different approaches. In the new PSI-HR-MS/MS approach, 73% of all drug intakes were detectable. Among them, seven drug intakes could be detected only using the new approach, raising the total drug intakes to 777. Both LC-based approaches could detect more drugs with values of 88% after UglucP or 76% after DUSglucE. However, these discrepancies were mostly due to low urine concentrations of corresponding targets. In conclusion, these results showed that the PSI technology could become a promising alternative to conventional procedures. Nevertheless, limitations such as detection of drugs in low concentrations and risk of false positive or negative results caused by mixed spectra should be addressed in prospective studies.

5. SUMMARY

Biotransformation and detectability in urine were studied for the new psychoactive substances *N,N*-diallyltryptamine (DALT) and its derivatives 5-methoxy-DALT, 5-fluoro-DALT, 7-methyl-DALT, and 5,6-methylenedioxy-DALT. They all showed extensive metabolism catalyzed by several CYP isoenzymes. In addition, novel concepts for urine drug screening were investigated. For dried urine spot (DUS)-based screening, sufficient workup with on-spot conjugate cleavage was developed for a broad range of different drugs showing sufficient urine screening results of authentic human samples after LC-MS^n or LC-high resolution (HR)-MS/MS analysis, comparable with those obtained after established urine precipitation workup with or without conjugate cleavage. For paper spray ionization (PSI)-based screening, the PSI setup was modified and coupled to HR-MS/MS. Again, confirmation of drug intakes was comparable to LC-based screening approaches. However, the detection of drugs in low concentrations and the risk of false positive or negative results caused by mixed spectra set some limitations to this setup. In conclusion, the sampling by DUS and the direct urine analysis by PSI was suitable for comprehensive metabolite-based drug testing and adherence monitoring of different drugs and drug classes by coupling to LC-MS^n or LC-HR-MS/MS. They may become alternative strategies with the potential of reducing costs for sample collection and storage or workload.

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7. ABBREVIATIONS

25B-NBOMe	2-(4-bromo-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]-ethanamine
25C-NBOMe	2-(4-chloro-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]-ethanamine
25I-NBOMe	2-(4-iodo-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]-ethanamine
5-HT	serotonin
5-MeO-DALT	5-methoxy-*N,N*-diallyltryptamine
5-MeO-DIPT	5-methoxy-*N,N*-diisopropyltryptamine
CYP	human cytochrome P450
DALT	*N,N*-diallyltryptamine
DBS	dried blood spot
DMS	dried matrix spots
DUS	dried urine spot
DUSglucE	DUS spotting, enzymatic conjugate cleavage, and liquid extraction
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
GC	gas chromatography
HR	high resolution
LC	liquid chromatography

MS	mass spectrometry
MS/MS	tandem mass spectrometry
MS^n	multi stage mass spectrometry
NPS	new psychoactive substances
pHLM	pooled human liver microsomes
PSI	paper spray ionization
UglucP	urine precipitation with conjugate cleavage
UHyAc	acid hydrolysis, liquid-liquid extraction, and acetylation
UNODC	United Nation Organization on Drugs and Crime
UP	urine precipitation

8. ZUSAMMENFASSUNG

Für die neuen psychoaktiven Stoffe *N,N*-Diallyltryptamin (DALT), 5-Methoxy-DALT, 5-Fluor-DALT, 7-Methyl-DALT, und 5,6-Methylendioxy-DALT wurden Metabolismus und Detektierbarkeit im Urin untersucht. Alle Substanzen zeigten ausgeprägte Metabolisierung, katalysiert durch mehrere CYP Enzyme. Danach wurden neue Screeningkonzepte erforscht. Für das Konzept mit dried urine spots (DUS) wurde eine umfassende Aufarbeitung mit Konjugatspaltung entwickelt. Gekoppelt mit LC-MS^n oder LC-high resolution (HR)-MS/MS waren die Screeningergebnisse von authentischen Urinproben vergleichbar mit denen nach etablierter Urinpräzipitation mit oder ohne Konjugatspaltung. Das paper spray ionization (PSI)-basierte Screeningverfahren wurde mit HR-MS/MS gekoppelt und lieferte vergleichbare Ergebnisse wie LC-basierte Screeningverfahren. Allerdings stellten sich die Substanzdetektion in niedrigen Konzentrationen sowie das Risiko von falsch-positiven oder -negativen Resultaten durch überlagerte Spektren als Limitierungen dieses Verfahrens heraus. Zusammenfassend erwies sich die Probennahme per DUS und die direkte Urinanalyse mittels PSI gekoppelt mit LC-MS^n und/oder LC-HR-MS/MS als geeignet für umfassendes und Metaboliten-basiertes Drogenscreening oder Adhärenzkontrolle. Dies ermöglichte es verschiedenste Arzneistoff- und Drogenklassen zu erfassen, weshalb diese Techniken Alternativen werden können um die Kosten für Probennahme und -lagerung zu reduzieren, sowie den Arbeitsaufwand für die Probenaufarbeitung.