- The NBOMes -

Metabolism and Detectability of *N*-2-Methoxybenzyl-Substituted Phenethylamines in Urine and Human Liver Preparations by Hyphenated Low and High Resolution 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 von Juni 2014 bis Juni 2018.

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“Ich liebe es,

wenn ein Plan funktioniert!”

George Peppard Jr. (1928 – 1994) als
John “Hannibal” Smith, The A-Team

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1. GENERAL PART

1.1. New Psychoactive Substances (NPS)

The use of mind-expanding substances is as old as humanity itself. Already thousands of years ago, human beings described the use of substances to talk to their deities during religious ceremonies.[1] Over the years, some well-known preparations were passed from generation to generation such as the brewery called “Ayahuasca”, which is still used today by indigenous people as traditional spiritual medicine in religious ceremonies.[1,2] However, in the mid of the twentieth century, some widespread psychedelic or entactogenic substances gained more and more importance also for common civilization. Prominent and well-known examples for these trends are substances or preparations such as cocaine, heroin, lysergic acid diethylamide (LSD), cannabis, and amphetamine or its analogs methamphetamine and 3,4-methylenedioxy-*N*-methyl-amphetamine (MDMA), better known as “Ecstasy”. Some of them were initially developed for medical use, but never were marketed or were taken from market after few years due to their adverse effects, especially addictive properties. These substances and preparations were also scheduled over the years and the trade became illegal. Over the time, a small number of slightly chemically modified analogs appeared on the drugs of abuse market to circumvent the existing laws.[3-8] However, at the beginning of the nineties, the book “PiHKAL: A Chemical Love Story” written by Alexander and Ann Shulgin and its availability via internet changed the situation on the drugs of abuse market and triggered the up-coming of a higher number of chemical related substances.[9] As chemist and pharmacologist, Alexander Shulgin synthesized a lot of compounds with widespread structure variability and administered them to himself.[9] The synthesis as well as the experiences and effects of the compounds were written down by Shulgin and his wife in their books “PiHKAL” and “TiHKAL” and served as references for many drug producers and consumers in the world.[9,10] Following these experiences and chemical structures, the phenomenon of the so-called NPS occurred in the end of the 2000s and lasts until today.[3-5] Based on known chemical substances and/or known drugs of abuse, a high number of new substances appeared on the market to circumvent the existing laws and they were sold usually by specialized shops in the internet as

"research chemicals", "legal highs", "plant food", "spice", or "bath salts" marked with "not for human consumption".[3-5] However, these substances did not undergo any clinical safety studies such as toxicokinetic, toxicodynamic, or toxicological studies.[3-5] Therefore, over the years, a lot of fatal or non-fatal poisonings were described in the literature with compounds from different structural classes.[3-5] Up-to-date, detection and identification of all new substances are one of the main challenges concerning the phenomenon of NPS for toxicological analyses.[5] Therefore, various drug monitoring systems and cooperations e.g. the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), the United Nation Organization on Drugs and Crime (UNODC), and the Swedish STRIDA project were founded to detect and to register up-coming NPS.[3,4,11,12] The annual reports of the EMCDDA and UNODC together with early warning systems help clinicians and laboratories to keep up-to-date.[3,4] According to the 2018 report of the EMCDDA, 670 NPS were identified in Europe from 2005 to end of 2017 with annually raising number.[4] The most frequently reported groups included synthetic cannabinoids, synthetic cathinones, phenethylamines, synthetic benzodiazepines, synthetic tryptamines, synthetic opioids, and piperazines.[3,4] However, according to the European Drug Report 2018, the number of new detected compounds declined since 2015.[4] This was most probably caused by better controlling, better preventing, or by new blanket bans or analogue-based legislation in several countries.[13,14] For example, such a law was introduced 2016 in Germany.[13,14] It defined several core structures for phenethylamines and synthetic cannabinoids with defined and common substituents.[13,14] Although the number of newly detected compounds declined, the challenges regarding the phenomenon NPS will attend the clinicians and laboratories also for the next few years.

1.2. Phenethylamine-Derived NPS

Among NPS, phenethylamine-derived substances play a central role.[4] A well-known group of phenethylamines is the so-called 2C-series, which emerged already in the early 2000s and were well described in the literature concerning pharmacology, metabolism, and misuse.[9,15-17] In their book "PiHKAL: A Chemical Love Story", Alexander and Ann Shulgin already described synthesis, effects, and suggested

dosage of some 2Cs.[9] Following these reports, compounds such as 4-bromo-2,5-dimethoxy-phenethylamine (2C-B), 4-chloro-2,5-dimethoxy-phenethylamine (2C-C), and 4-iodo-2,5-dimethoxy-phenethylamine (2C-I) were reported more and more in clinical or forensic toxicology.[15] The 2Cs were described as noradrenaline receptor agonists as well as serotonin receptor subtype 2A (5-HT_{2A}) agonists, which is also one target of LSD and linked to hallucinogenic effects.[15] Common dosages range from 6 to 150 mg depending on the compound and the administration route.[9,15] They were shown to be mainly metabolized by *O*-demethylation or oxidative deamination by monoamine oxidases.[15,18] Over the years, the most up-coming 2Cs were scheduled and new alternatives were synthesized by illicit drug producers.[15]

A new potent phenethylamine-based series appeared on the illicit drug market at the beginning of the 2010s.[3,4,16,17,19-22] The so-called NBOMes represented highly potent derivatives of the known 2Cs.[16,17,21,23] The substitution of the primary amine group of the 2C backbone with an *N*-2-methoxybenzyl (NBOMe) moiety increased the affinity to the 5-HT_{2A} receptors significantly.[23-26] They were originally synthesized as radiotracer for positron emission tomography due to their high affinity to the 5-HT_{2A} receptors. "[11C]Cimbi-5", the carbon-11 labeled derivative of 2-(4-iodo-2,5-dimethoxy)-*N*-(2-methoxybenzyl)-phenethylamine (25I-NBOMe) was found to be a promising tool for investigation of 5-HT_{2A} agonist binding in the living human brain.[26,27] The first authentic case after self-reported use of 25I-NBOMe, the NBOMe derivative of 2C-I, was published in 2013.[20] Thereafter, various other NBOMes appeared on the drug market such as 2-(4-bromo-2,5-dimethoxy)-*N*-(2-methoxybenzyl)-phenethylamine (25B-NBOMe) and 2-(4-chloro-2,5-dimethoxy)-*N*-(2-methoxybenzyl)-phenethylamine (25C-NBOMe), derivatives of 2C-B and 2C-C, respectively, and in the meantime, several fatal and non-fatal poisonings were reported.[16,17,21,26] Due to the significantly increased potency of the NBOMes compared to the corresponding 2Cs, poisonings are likely to happen.[16,17] Poisonings could be based on unknown intake of those compounds or due to wrong declaration e.g. as 2C or LSD.[16,17,28] Due to the high potency of the NBOMes, very low dosages, comparable to those of LSD in the µg ranges are consumed.[16,17] They are commonly administered sublingually on blotter paper, insufflated, or as nose sprays and sold under several street names such as "N-bombs", "Smiles", "Cimbi", "25B", 25C", or "25I".[16,17,21] They were suggested to lead to effects similar to LSD such as

hallucinations, euphoria, powerful visual and sensory effects, unusual body sensations, mystical experiences, empathic feelings, and alterations in consciousness.[17,21,22] The most often reported adverse effects of consumers coming to the clinic were agitation, tachycardia, hypertension, and seizures.[16,17,21] Furthermore, NBOMe intake is hard to detect due to the low dosages and presented a new challenge to analytical methods.[16,17] After few years, the most common NBOMes such as 25B-, 25C-, and 25I-NBOMe were scheduled in most countries.[16,17] In 2014, a new class of NBOMes was detected in a seizure from China by German custom authorities.[29] In contrast to the 2C-derived NBOMes with mescaline-like structures, these new detected NBOMes had an amphetamine backbone, with several substitutions at the aromatic ring system and/or the nitrogen.[29] The detected compounds were NBOMe derivatives of amphetamines such as 3,4-dimethoxy-amphetamine (3,4-DMA), 4-ethyl-amphetamine (4-EA), and 4-methyl-*N*-methyl-amphetamine (4-MMA).[29] However, no information about their pharmacological properties or potential effects were published yet.[29]

1.3. In vivo and In vitro Metabolism Studies

One important analytical challenge in clinical and forensic toxicology is the detection and identification of potentially administered or ingested substances or preparations. The hype of NPS demanded a lot of new challenges for analytical methods.[5-8] Besides low to ultra-low consumed dosages, the huge structure variability is one of the main issues. Analytical methods need to be sensitive and flexible enough to ensure the reliable and fast detection and identification of NPS to support clinical treatment or to elucidate the cause of fatalities. Usually, information about pharmacological and toxicological properties of up-coming NPS is missing because they were never tested or investigated in any studies prior to marketing.[5] Furthermore, nothing is known about the toxicity or the route of excretion. The only information about effects is often provided by non-scientific trip reports in online drug forums. To ensure that an intake of an NPS could be detected and identified in clinical or forensic toxicology, several studies need to be performed. Most screening procedures covering NPS were based on the detection in urine, which is the matrix of choice due to higher concentrations, longer detectability, and easier access and

collection compared to e.g. blood samples.[5] However, it is often unknown whether the NPS is excreted into urine unchanged or even only in form of metabolites.[5] Therefore, metabolism studies are necessary to elucidate the best analytical target in urine. Unfortunately, human authentic material is not available in most cases. Therefore, metabolism studies must be performed in vivo with mammalian models or in vitro to elucidate the metabolism of NPS. Standard study procedures described in the literature were based on the application of high dosages to capture as much metabolites as possible because it could never be excluded that e.g. a minor rat metabolite could be the major biotransformation product in humans.[5,30-33] This information could be useful to update the analytical methods to ensure that an intake of the ingested NPS could be possible in routine toxicological analyses. However, animal testing should be restricted due to ethical reasons and species differences concerning metabolism or excretion routes should be considered. Therefore, human in vitro models could be additionally used to confirm the metabolites found in the animal model or to detect different metabolite formation. There are several models to elucidate the human in vitro metabolism, namely pooled human liver microsomes (pHLM), pooled human liver cytosol (pHLC), or pooled human liver S9 fraction (pS9).[34-40] Furthermore, alternative models gained more and more importance in the last few years such as incubations with primary hepatocytes or with various cell lines.[35-37] The decision, which model should be used, depends on the aims of the respective study. In vitro studies with pHLM or pS9 are well-known, much cheaper, and easier to handle than incubations with cell lines or primary hepatocytes.[35] On the other hand, the more complex an in vitro model is, the more similar to in vivo are the results.[35] In the most cases, incubations with pHLM or pS9 are sufficient to identify the expected main human metabolites and to confirm the metabolites identified in the animal model.[35] This data could be used to update the routine toxicology screening procedures to identify the investigated compounds.

1.4. Cytochrome P450 (CYP) Enzyme Involvement and Kinetics

The cytochrome P450 (CYP) system is one of the main actors in the metabolism of xenobiotics in mammalian.[41-45] They are mainly expressed in the liver but also in the lung and the intestinal. They belong to the family of oxidoreductases and are

organized in various subfamilies.[41-45] CYPs are responsible for metabolism of endogenous but also for exogenous compounds.[41-45] Usually, CYPs make xenobiotics more hydrophilic mainly by oxidation or also by reduction prior to conjugation to ensure that the metabolites are not reabsorbed and thus excreted from the body. In most cases, these metabolic steps lead to detoxification of the compound but in rare cases also toxification could be observed. One prominent example for this is the biotransformation of paracetamol, which is metabolized to toxic metabolites in overdose patients. Furthermore, CYP enzymes could be subjected to genetic polymorphism or could be even inhibited or induced by other therapeutic drugs or NPS resulting in possible interactions.[41-43] Genetic polymorphisms could lead to poor metabolizers or ultra-rapid metabolizers resulting in varied excretion profiles of the affected compound. Furthermore, interactions between NPS and therapeutic drugs could influence both, the excretion route of the NPS or of the therapeutic drug leading to possible overdosing or poisoning. It is therefore also important to know for NPS, which CYP enzymes are responsible and involved in their metabolism. If only one CYP enzyme is involved, kinetic variations due to polymorphism or interactions are most likely to occur. Besides CYPs, also flavin-containing monooxygenases (FMOs) represents an important system for metabolism.[44]

After the initial monooxygenases activity screening, the kinetic properties of each involved isozyme were determined. The kinetic parameters such as the Michaelis-Menten constant K_m or V_{max} are helpful tools to elucidate whether the NPS is a relevant substrate for the respective CYP enzyme or not.[46-50] The K_m and V_{max} values can be determined using two different approaches.[46,47] On the one hand, the formation of a specific metabolite catalyzed by one CYP isozyme can be measured.[46,49] The used enzyme concentration and incubation time have to be in the linear range of metabolite formation to get valid results. On the other hand, the depletion of the parent compound can be measured.[46,49] Depletion rates at defined enzyme concentrations are calculated over a defined time range and plotted versus substrate concentration to determine the kinetic constants.[46] Based on these kinetic data, further predictions could be given for the expected human in vivo metabolism using the so-called relative activity factor (RAF) approach.[44,51,52] Using this approach,

a prediction of the contribution of each involved CYP isozyme to the hepatic net clearance could be done.[44,51,52]

1.5. (Nano)Liquid Chromatography-High Resolution Mass Spectrometry

Liquid chromatography (LC)-MS is one of the most important tools in analytical toxicology, besides GC-MS, because it is robust and reliable.[53-58] Many different approaches for the identification of metabolites in various matrices were described in the last years based on low or high resolution (HR) MS techniques.[53-58] HRMS allows the calculation of empirical molecular formulas of precursor masses as well as of the fragment ions and thus it is a helpful tool for metabolite identification and also for structure elucidation.[53-58] Mainly time of flight (TOF) or Orbitrap (OT) mass analyzers are used.[53-58] Recently, Helfer et al. developed an OT-based LC-HRMS screening method for routine drug testing.[59,60] This established approach was used as starting point to develop specific methods for the identification of metabolites of the studied NBOMes. Besides conventional LC methods, nanoLC coupled to HRMS was described in the past as useful tool for protein analyses but also described as applicable for metabolite identification of small molecules.[61-63]

1.6. Urine Screening Approaches

As already mentioned above, the phenomenon of NPS represents one of the main challenges concerning analytical toxicology in biosamples.[5-7] The occurrence of new chemical structures with slight modifications and the usually more potent compounds with resulting lower consumed dosages need to be addressed by sophisticated bioanalytical methods. However, some approaches for detection and identification of NPS in biosamples such as blood plasma or serum, urine, hairs, or even other matrices were published in the last years.[5-8] In general, the biosamples for detectability studies play a central role. For blood analyses, the parent compounds usually were suitable targets but with narrow detection window and lower concentrations compared to e.g. urine.[64] As already mentioned above, urine is the

sample of choice for screening methods. Based on metabolism studies, the metabolite-based reference libraries could be updated to perform comprehensive urine screenings.[64] Furthermore, the detection of metabolites in addition to the parent compound (if excreted) increases the reliability of results and confirms a suggested intake of a NPS. Therefore, comprehensive and broad phase I and phase II metabolite identification should be performed. In the present work, the detectability studies of the investigated compounds were performed in rat urine after application of a dose estimated based on reports of consumer in NPS forums or suggested dosages of internet shops in addition to data from scientific publications. Also human urine was investigated if available from case work.

2. AIMS AND SCOPES

The presented work aimed to investigate the metabolic fate of 25B-, 25C-, and 25I-NBOMe (upper part in Fig. 1), commonly consumed representatives of *N*-2-methoxybenzyl substituted potent hallucinogenic NPS and of 3,4-DMA-, 4-EA-, and 4-MMA-NBOMe (lower part in Fig .1), representatives of a newly discovered group of NBOMes. In addition, their detectability in standard urine screening approaches after commonly consumed low dosages was elucidated. The studies were performed in rat and human urine as well as in human liver preparations using hyphenated MS techniques. The extensive metabolized compounds should be furthermore analyzed by nanoLC-HRMS/MS to elucidate its power for metabolism studies. For all investigated compounds, the kinetic properties of CYP-mediated metabolism were determined using substrate depletion approach.

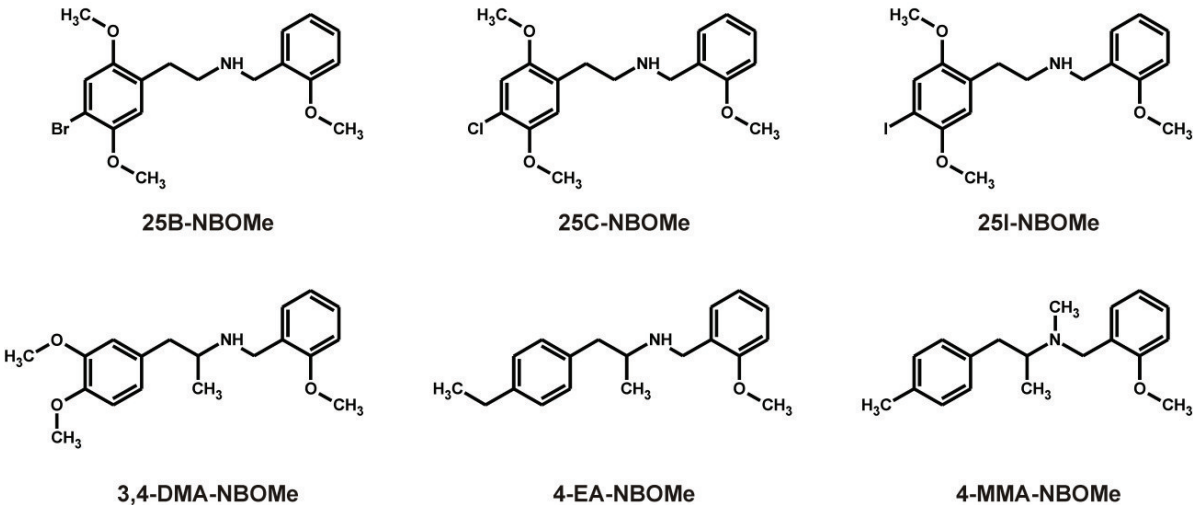

Fig. 1: Chemical structures of the studied NBOMes[65]

The following steps had to be conducted:

- Detection and identification of the phase I and II metabolites of 25B-, 25C-, 25I-, 3,4-DMA-, 4-EA-, and 4-MMA-NBOMe in rat urine after high dose administration by LC-HRMS/MS or nanoLC-HRMS/MS

- Detection, identification, and confirmation of the phase I and II metabolites of 25B- and 25I-NBOMe in human urine after unknown dosage by LC-HRMS/MS
- Detection, identification, and confirmation of the phase I and II metabolites in human liver preparations
- Investigations on the power of nanoLC-HRMS/MS for the detection and identification of metabolites exemplified for 3,4-DMA- and 4-MMA-NBOMe
- Investigations of the general monooxygenases involvement in the metabolism of NBOMes
- In vitro CYP kinetic studies using the substrate depletion approach and determination of the in vivo contribution to the hepatic net clearance
- Toxicological detectability in rat urine after low dose administration (and human urine for 25B- and 25I-NBOMe after unknown dosage) using standard urine screening approaches (SUSAs) by GC-MS, LC-MS^n, and LC-HRMS/MS[31,59,60,66-68]

3. PUBLICATIONS OF THE RESULTS

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

3.1. Studies on the metabolism and toxicological detection of the new psychoactive designer drug 2-(4-iodo-2,5-Dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine (25I-NBOMe) in human and rat urine using GC-MS, LC-MS^n, and LC-HR-MS/MS[69]

(DOI: 10.1007/s00216-015-8828-6)

3.2. Metabolic fate and detectability of the new psychoactive substances 2-(4-bromo-2,5-dimethoxyphenyl)-*N*-[(2-Methoxyphenyl)methyl]ethanamine (25B-NBOMe) and 2-(4-chloro-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine (25C-NBOMe) in human and rat urine by GC-MS, LC-MS^n, and LC-HR-MS/MS approaches[70]

(DOI: 10.1016/j.jpba.2016.11.040)

3.3. LC-high resolution-MS/MS for identification of 69 metabolites of the new psychoactive substance 1-(4-ethylphenyl-)-*N*-[(2-methoxyphenyl)methyl] propane-2-amine (4-EA-NBOMe) in rat urine and human liver S9 incubates and comparison of its screening power with further MS techniques[71]

(DOI: 10.1007/s00216-017-0526-0)

3.4. Nano liquid chromatography-high-resolution mass spectrometry for the identification of metabolites of the two new psychoactive substances *N*-(ortho-methoxybenzyl)-3,4-dimethoxyamphetamine and *N*-(ortho-methoxybenzyl)-4-methylmethamphetamine[72] (DOI: 10.1016/j.talanta.2018.05.064)

3.5. Human cytochrome P450 kinetic studies on six *N*-2-methoxybenzyl (NBOMe)-derived new psychoactive substances using the substrate depletion approach[65] (DOI: 10.1016/j.toxlet.2017.12.017)

4. DISCUSSION AND CONCLUSIONS

In the presented work, the metabolic fate in rats and humans of three common phenethylamine-derived NPS (25B-, 25C-, and 25I-NBOMe) and three amphetamine-derived NPS (3,4-DMA-, 4-EA-, and 4-MMA-NBOMe) of the NBOMe group was studied. Furthermore, their toxicological detectability in standard urine screening procedures was investigated. All compounds showed extensive metabolism in rats and humans. LC-HRMS/MS or nanoLC-HRMS/MS allowed the detection and tentative identification of numerous of phase I and II metabolites based on their HRMS/MS spectra. The fragmentation patterns were characteristic and comparable for all investigated NBOMes and also their metabolites. Based on their MS fragmentation pattern, the molecules could be broken down in several parts, the so-called 2C part for 25B-, 25C-, and 25I-NBOMe and their metabolites, the amphetamine part for 3,4-DMA-, 4-EA-, and 4-MMA-NBOMe and their metabolites as well as the characteristic NBOMe part representative for all studied compounds. Except for the *N*-demethoxybenzyl metabolites, the most abundant fragment ions were always formed by the NBOMe part and allowed confident identification together with the accurate masses of the protonated precursor molecules and the calculated molecular formulas. Furthermore, the fragment ion of *m/z* 121.0653 ($C_8H_9O^+$) coming from the NBOMe part represented a group indicating fragment ion for all NBOMe compounds and also for their main metabolites with metabolically unmodified NBOMe part.[73] In addition, the presented work described for the first time an observed rearrangement reaction discovered for the 2C-NBOMes based on the HRMS/MS fragmentation patterns.

The used nanoLC approach was applied for the detection and identification of metabolites of two amphetamine-derived NBOMes. Furthermore, the results concerning detectability were compared to those obtained by conventional LC. The suggested higher sensitivity of the nanoLC system could not be proven. However, it showed no disadvantages when compared to conventional LC systems with much lower eluent consumption and allowed the identification of numerous phase I and II metabolites of 3,4-DMA-NBOMe and 4-MMA-NBOMe.

Following the (nano)LCHRMS/MS data, several metabolic pathways could be predicted for the studied compounds. For 25B-, 25C-, and 25I-NBOMe, *O*-

demethylation could be proposed to be the main metabolic reaction in rats and humans degrading all three methoxy groups, even up to *tris*-demethylation. Furthermore, various aromatic hydroxylations, dehydration, and *N*-demethoxybenzylation could be found for the “classic” 2C-NBOMes. *N*-demethoxybenzylation lead to the respective well-known 2C compounds (2C-B, 2C-C, and 2C-I), which were further metabolized by *O*-demethylation, aromatic hydroxylation, and oxidative deamination what was in-line with published 2C studies.[15,32,33,74] Based on the main metabolic pathways, a lot of combinations could also be found leading to high number of identified phase I metabolites (35, 36, and 37). For 3,4-DMA-NBOMe, an amphetamine-derived NPS, also *O*-demethylation represented the main metabolic reaction together with aromatic hydroxylation. This was in-line with published metabolism data for 3,4-DMA.[75] In contrast, 4-EA- and 4-MMA-NBOMe were mainly metabolized by oxidation of the ethyl or methyl rest to the corresponding carboxylic acids. Also *O*-demethylation of the NBOMe part, aliphatic and aromatic hydroxylations as well as *N*-demethoxybenzylation could be observed. For 4-MMA-NBOMe, *N*-demethylation could additionally be found as one of the predominant pathways.

Concerning phase II metabolism, almost all common conjugation steps could be observed. The predominant phase II pathways were conjugation to glucuronic acid and/or sulfuric acid. For the 2C-NBOMes, also *O*-methylation and glutathione conjugation could be found in rats. *N*-Acetylation after previous *N*-demethoxybenzylation leading to the corresponding 2C was also found. Again being in-line with published data.[15,32,33] Almost all phase I metabolites of the amphetamine-derived NBOMes were excreted as conjugates with glucuronic acid and/or sulfuric acid. *N*-Acetylation after previous *N*-demethoxybenzylation could be observed for 3,4-DMA- and 4-EA-NBOMe in rats. In addition, for one of the main rat metabolites of 4-EA-NBOMe, even glycine conjugation could be found after previous oxidation of the ethyl side chain to a benzoic acid derivative.

With regards to the used metabolic models, no relevant species differences could be observed. Most phase I and II steps found in rat urine (or human urine if available) could be confirmed with the human in vitro tools. Differences in the detected number of metabolites could be explained by concentration issues and the collection time of rat urine of 24 hours. Nevertheless, for 4-EA-NBOMe, an important limitation of the

rat model could also be observed. Oxidation of the ethyl side chain to a benzoic acid derivative was found to be the main excretion product in rat urine, but could not be detected in the human in vitro model. This was in-line with published data about the different metabolism in rats and humans of ethyl benzene to benzoic acid describing that the loss of a C-atom was only observed in rats.[76-78] Furthermore, *N*-acetylation, *O*-methylation, as well as glutathione and glycine conjugation could only be detected in rat urine most probably due to concentration issues. For the 2C-NBOMes, pHLM were used to confirm in vitro phase I metabolites. However, for 25B- and 25I-NBOMe human urine was available to confirm the phase II metabolites. Again, no relevant species differences could be observed concerning metabolic pathways or metabolic patterns. For further studies on the human in vitro metabolism of amphetamine-derived NBOMes, incubations with pS9 instead of pHLM were performed, which was developed by Richter et al. and found to deliver better results and covered the formation of both, phase I and II metabolites.[34,35]

In addition to the identification of metabolites in urine and human liver preparations, the involvement of the ten most abundant human hepatic CYPs and FMO3 was tested. Incubations with recombinantly expressed isozymes revealed the main involvement of CYP2C19, CYP2D6, and CYP3A4 for all investigated NBOMes and in addition, CYP1A2 for 4-EA-NBOMe and CYP2B6 for 4-MMA-NBOMe. Furthermore, for these mainly involved isozymes, the kinetic constants (K_m and V_{max} values) were determined using the substrate depletion approach. The determination allowed the assessment of the human in vivo contribution of each CYP isozyme to the hepatic net clearance. All NBOMes were found to be good CYP substrates indicated by low K_m values in the nanomolar range particularly for CYP2C19 and CYP2D6. The calculation of the contribution to the hepatic net clearance based on the RAF approach suggested a main contribution of CYP2C19 and CYP2D6. Both isozymes are known to be subjected to genetic polymorphism resulting in possible interindividual variations in the metabolism. However, several CYP isozymes were involved and thus drug-drug or drug-food interactions are not likely.

The detectability studies were performed using rat urine collected over 24-h period after administration of an estimated consumer dosage. The established standard urine screening procedures using GC-MS, LC-MS^n, and LC-HRMS/MS after suitable wok-up were applied.[31,59,60,66-68] For all NBOMes, the parent compound was not

detected in the rat urine samples. In addition to the rat urine samples, human urine samples were available and tested for 25B- and 25I-NBOMe. In contrast to the rat urine samples, the parent compounds were detectable in the human urine samples. This could be explained by potential overdosing or poisoning. Furthermore, the time of intake and the consumed dosages were not known. However, the parent compounds were not found to be suitable targets for urinalysis. Based on the presented data, screening procedures need to include metabolites e.g. after *O*-demethylation or oxidation of the side chains. The applied GC-MS approach was not able to detect an intake of the studied 2C-NBOMes in the rat urine samples after low dose administrations most probably due to sensitivity issues. However, an intake of 25B- and 25I-NBOMe in the authentic human urine samples and in the rat urine samples after high dose administration suggested that in case of acute overdosing or poisoning an intake should also be detectable by GC-MS. In contrast, the amphetamine-derived NBOMes were detectable also with the GC-MS SUSA via their metabolites. Nevertheless, clinical and forensic screening procedures for NBOMes should be based on highly sensitive LC-MS devices to ensure reliable detection of the described compounds even after low dose or longer time since administration.

The results presented in this work showed that metabolism studies play a central role in analytical toxicology. NBOMe-derived NPS were extensively metabolized and the parent compounds were only detectable after assumed high dosages. Therefore, metabolism studies should be systematically performed to keep the screening methods up to date and to ensure reliable screening procedures. The information provided by this work helps to detect an intake of the studied compounds.

5. SUMMARY

The presented work describes the metabolic fate and detectability of phenethylamine-derived new psychoactive substances in rats and humans using various hyphenated low and high resolution mass spectrometry. All compounds showed extensive metabolism with series of phase I and II metabolites. Main metabolic pathways were *O*-demethylation, aromatic hydroxylation, and *N*-demethoxybenzylation for 25B-, 25C-, 25I-, and 3,4-DMA-NBOMe as well as oxidation of the side chain to the corresponding carboxylic acids, *O*-demethylation, and hydroxylations for 4-EA- and 4-MMA-NBOMe. The proposed main metabolic pathways were comparable in rats and humans. Initial monooxygenases activity screenings and CYP kinetic studies using substrate depletion approach revealed that CYP2C19 and CYP2D6 were the main human in vivo contributors to the hepatic net clearance. All NBOMes were only detectable via their metabolites with established standard urine screening approaches by GC-MS, LC-MS^n, and LC-HRMS/MS. Recommended analytical targets were the *O*-demethylated metabolites with or without conjugation to glucuronic acid for 25B-, 25C-, 25I-, and 3,4-DMA-NBOMe and the carboxylic acid metabolite with or without additional demethylation for 4-EA-NBOMe and 4-MMA-NBOMe. Furthermore, a nanoLC-HRMS/MS approach was developed for the metabolism studies of 3,4-DMA- and 4-MMA-NBOMe and its power was elucidated for metabolite detection and identification.

6. REFERENCES

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7. ABBREVIATIONS

2C-B	4-bromo-2,5-dimethoxy-phenethylamine
2C-C	4-chloro-2,5-dimethoxy-phenethylamine
2C-I	4-iodo-2,5-dimethoxy-phenethylamine
3,4-DMA	3,4-dimethoxy-amphetamine
4-EA	4-ethyl-amphetamine
4-MMA	4-methyl-*N*-methyl-amphetamine
3,4-DMA-NBOMe	*N*-(2-methoxybenzyl)-3,4-dimethoxyamphetamine
4-EA-NBOMe	*N*-(2-methoxybenzyl)-4-ethylamphetamine
4-MMA-NBOMe	*N*-(2-methoxybenzyl)-4-methyl-*N*-methyl-amphetamine
5-HT	serotonin
25B-NBOMe	2-(4-bromo-2,5-dimethoxy)-*N*-(2-methoxybenzyl)-phenethylamine
25C-NBOMe	2-(4-chloro-2,5-dimethoxy)-*N*-(2-methoxybenzyl)-phenethylamine
25I-NBOMe	2-(4-iodo-2,5-dimethoxy)-*N*-(2-methoxybenzyl)-phenethylamine
CYP	human cytochrome P450
e.g.	exempli gratia, for example
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
FMO	flavin-containing monooxygenases
GC	gas chromatography
HR	high resolution
K_m	Michaelis-Menten constant
LC	liquid chromatography
LSD	lysergic acid diethylamide

MDMA	3,4-methylenedioxy-*N*-methyl-amphetamine
MS	mass spectrometry
MS^n	multi stage mass spectrometry
MS/MS	tandem mass spectrometry
NBOMe	*N*-2-methoxybenzyl
NPS	new psychoactive substance(s)
OT	Orbitrap
pHLC	pooled human liver cytosol
pHLM	pooled human liver microsomes
pS9	pooled human liver S9 fraction
RAF	relative activity factor
SPE	solid-phase extraction
SUSA	standard urine screening approach
TOF	time of flight
UNODC	United Nations Office on Drugs and Crime
UP	urine precipitation
V_{max}	theoretical maximum reaction velocity that would occur at an infinite substrate concentration

8. ZUSAMMENFASSUNG

Die Dissertation beschreibt den Metabolismus und die Nachweisbarkeit von neuen psychoaktiven Substanzen des Phenethylamintyps in Ratten und Menschen mittels nieder- und hochauflösender Massenspektrometrie. Die Substanzen zeigten einen ausgeprägten Metabolismus mit vielen Phase I und II Metaboliten. Die Hauptstoffwechselschritte waren *O*-Demethylierung, aromatische Hydroxylierung und *N*-Demethoxybenzylierung für 25B-, 25C-, 25I- und 3,4-DMA-NBOMe, Oxidation der Seitenketten zu den entsprechenden Carbonsäuren sowie *O*-Demethylierung und Hydroxylierung für 4-EA- und 4-MMA-NBOMe. Die Hauptschritte waren in Ratten und Menschen vergleichbar. Kinetische Untersuchungen zu den am Metabolismus beteiligten CYP Enzymen zeigten, dass CYP2C19 und CYP2D6 hauptsächlich an der hepatischen Ausscheidung der Substanzen im Menschen beteiligt sind. Die in Urin durchgeführten Untersuchungen zur Nachweisbarkeit zeigten, dass die Substanzen nur durch ihre Metaboliten mittels etablierter Urinscreeningverfahren nachweisbar waren. Für 25B-, 25C-, 25I-, und 3,4-DMA-NBOMe wurden die jeweiligen *O*-demethylierten Metaboliten mit oder ohne Konjugation an Glucuronsäure und die jeweiligen Carbonsäure Metaboliten mit oder ohne zusätzliche Demethylierung für 4-EA- und 4-MMA-NBOMe als geeignete analytische Marker identifiziert. Des Weiteren wurde die Einsetzbarkeit eines neu entwickelten Verfahrens basierend auf nanoLC-HRMS/MS für die Identifizierung von Metaboliten getestet.