Stereochemistry of phase-1 metabolites of mephedrone determines their effectiveness as releasers at the serotonin transporter

Felix P. Mayer[a,1], Daniela Cintulova[b], Dorothea A. Pittrich[a], Laurin Wimmer[b], Dino Luethi[c], Marion Holy[a], Kathrin Jaentsch[a], Sonja Tischberger[d], Guenter Gmeiner[d], Marius C. Hoener[e], Matthias E. Liechti[c], Marko D. Mihovilovic[b], Harald H. Sitte[a,f]

[a] *Medical University of Vienna, Center for Physiology and Pharmacology, Institute of Pharmacology, Vienna, Austria*

[b] *Institute of Applied Synthetic Chemistry, Vienna University of Technology, Vienna, Austria*

[c] *University Hospital Basel and University of Basel, Division of Clinical Pharmacology and Toxicology, Department of Biomedicine, Basel, Switzerland*

[d] *Doping Control Laboratory, Seibersdorf Labor GmbH, Seibersdorf, Austria*

[e] *Neuroscience Research, pRED, Roche Innovation Center Basel, F. Hoffmann-La Roche Ltd, Basel, Switzerland*

[f] *Center for Addiction Research and Science - AddRess, Medical University Vienna, Waehringerstrasse 13A, 1090 Vienna, Austria*

[1] *Present Address: Department of Biomedical Science, Charles E. Schmidt College of Medicine, FL, USA*

Correspondence

Harald H Sitte, harald.sitte@meduniwien.ac.at

Medical University of Vienna, Center for Physiology and Pharmacology, Institute of Pharmacology, Waehringerstrasse 13a, A-1090 Vienna, Austria

Abstract

Mephedrone (4-methyl-*N*-methylcathinone) is a psychostimulant that promotes release of monoamines via the high affinity transporters for dopamine (DAT), norepinephrine (NET) and serotonin (SERT). Metabolic breakdown of mephedrone results in bioactive metabolites that act as substrate-type releasers at monoamine transporters and stereospecific metabolism of mephedrone has been reported. This study compared the effects of the enantiomers of the phase-1 metabolites nor-mephedrone, 4-hydroxytolyl-mephedrone (4-OH-mephedrone) and dihydro-mephedrone on (i) DAT, NET and SERT mediated substrate fluxes, (ii) determined their binding affinities towards a battery of monoamine receptors and (iii) examined the relative abundance of the enantiomers in human urine.

Each of the enantiomers tested inhibited uptake mediated by DAT, NET and SERT. No marked differences were detected at DAT and NET. However, at SERT, the *S*-enantiomers of nor-mephedrone and 4-OH-mephedrone were several times more potent than the corresponding *R*-enantiomers. Moreover, the *R*-enantiomers were ineffective as releasers at SERT. *S*-nor-mephedrone displayed moderate affinities towards human alpha$_{1A}$, human 5-HT$_{2A}$ and rat and mouse trace amine-associated receptor 1.

These results demonstrate that stereochemistry dictates the pharmacodynamics of the phase-1 metabolites of mephedrone at SERT, but not at DAT and NET, which manifests in marked differences in their relative potencies, i.e. DAT/SERT ratios. Chiral analysis of urine samples demonstrated that nor-mephedrone predominantly exists as the *S*-enantiomer. Given the asymmetric abundance of the enantiomers in biological samples, these findings may add to our understanding of the subjective effects of administered mephedrone, which indicate pronounced effects on the serotonergic system.

Keywords: New psychoactive substance, dopamine, serotonin, cathinones, mephedrone, stereoisomers

Abbreviations

5-HT, serotonin; CYP, Cytochrome P450 enzyme; DAT, dopamine transporter; MAT, monoamine transporter; MDMA, 3,4-methylenedioxymethamphetamine; MPP^+, 1-methyl-4-phenylpyridinium; NET norepinephrine transporter; NPS, new psychoactive substance; SERT, serotonin transporter.

1. Introduction

Mephedrone is a methcathinone analogue with stimulant properties. Although mephedrone was synthesized in 1929, first reports on its abuse appeared in 2007 (Kelly, 2011; Pedersen et al., 2013). Together with its congeners 3,4-methylenedioxypyrovalerone (MDPV) and 3,4-methylenedioxymethcathinone (methylone), mephedrone belongs to a series of cathinones that received major attention from the media as so-called "bath salts" (Spiller et al., 2011), thus creating public awareness of new psychoactive substances (NPS) that are introduced into global drug markets (Brandt et al., 2014). NPS provide synthetic and easily accessible alternatives to well-established and scheduled drugs (Baumann and Volkow, 2016). Psychostimulant-type NPS generally fall into the classes of phenethylamines or cathinones (Tyrkko et al., 2016). Some NPS became established within drug markets and abuse continues despite their ban whereas others appear only locally and/or temporarily (World Drug Report 2016). Mephedrone was banned in most western countries in 2010 to 2011 (Green et al., 2014). However, converging lines of evidence indicate persistent abuse of this compound (Archer et al., 2013; Archer et al., 2014; Castiglioni et al., 2015; Salomone et al., 2016; Styszko et al., 2016), indicating that mephedrone became a widely accepted drug for recreational consumption in some regions (Hockenhull et al., 2016; Ordak et al., 2018).

Pharmacologically, mephedrone exerts its effects by targeting the high-affinity monoamine transporters (MATs) for dopamine (DAT), norepinephrine (NET) and serotonin (5-HT, SERT), members of the solute carrier 6 family (Baumann et al., 2012; Eshleman et al., 2013; Hadlock et al., 2011; Martinez-Clemente et al., 2012; Simmler et al., 2013). DAT, NET and SERT retrieve monoamines from the extracellular space and terminate their activity at the pre- and postsynaptic receptors (Kristensen et al., 2011). Monoaminergic signalling is involved in various aspects of brain function, including motor function, motivation and reward. Thus, MATs are associated with a rich pharmacology and serve as molecular targets for clinically relevant (e.g. antidepressants) and recreationally ingested compounds (Sitte and Freissmuth, 2015; Torres et al., 2003). Generally, drugs that interfere with MAT-function are of two types: a) non-transported inhibitors, e.g. cocaine or antidepressants, which trap the transporter in a specific conformational state and arrest the transport cycle and b) substrate-type releasers. The latter are actively transported by MATs and promote reverse transport of cytosolic monoamines across the plasma membrane (Sitte and

Freissmuth, 2015). Mephedrone acts as a non-selective substrate-type releaser at DAT, NET and SERT (Baumann et al., 2012; Eshleman et al., 2013; Mayer et al., 2016; Pifl et al., 2015; Simmler et al., 2013) and thus induces transporter-mediated release of monoamines.

A variety of cathinones and phenethylamines contain a chiral carbon atom and exist as stereoisomers. Stereochemistry has a bearing on the neurochemistry and behavior associated with psychostimulants. For instance, racemic methcathinone was associated with neurotoxicity in dopaminergic neurons, whereas only *S*-methcathinone caused toxic effects on serotonergic neurons (Sparago et al., 1996). *S*-MDMA was found to be more potent than *R*-MDMA in inducing substrate-efflux at SERT (Rudnick and Wall, 1992) and both enantiomers differed in their endocrine and neurochemical effects (Murnane et al., 2010). Stereospecific effects have also been reported for mephedrone, both *in vitro* and *in vivo*. The *R*- and *S*-enantiomers of mephedrone are equipotent as releasers at DAT and NET. At SERT, however, *S*-mephedrone is 40 times more potent than *R*-mephedrone (Gregg et al., 2015). In the same study, only *R*-mephedrone resulted in conditioned place preference and *R*-mephedrone produced greater intracranial self-stimulation and sensitization to repetitive movements. In rats trained to self-administer the stereoisomers of mephedrone, *R*-mephedrone was associated with higher break points than *S*-mephedrone under progressive ratio access conditions (Philogene-Khalid et al., 2017b).

In contrast to the prototypical stimulant amphetamine ("speed"), mephedrone exerts additional entactogenic effects and drug users report that the subjective effects resemble those of 3,4-methylendioxymethylamphetamine (MDMA, "ecstasy") (Schifano et al., 2011). Simmler et al. (Simmler et al., 2013) concluded that the entactogenic effects of mephedrone arise from its ability to release serotonin in the brain *in vivo* to a greater extent when compared to dopamine (Baumann et al., 2012), resembling the effects of MDMA but contrasting those of amphetamine (Baumann et al., 2012; Kehr et al., 2011). Racemic mephedrone, however, does not favor SERT over DAT (Baumann, 2012, Eshleman, 2013, Hadlock, 2011). Enzymatic degradation of mephedrone gives rise to various phase-1 metabolites (Pedersen et al., 2013) (Figure 1). Our laboratory demonstrated that the phase-1 metabolites of mephedrone possess bioactive properties, thus might contribute to the actions of the parental compound. Systemic administration of the *N*-demethylated metabolite nor-mephedrone results in a profound increase in extracellular serotonin but has only blunted effects on extracellular dopamine in nucleus

accumbens (Mayer et al., 2016). Recently, stereospecific metabolism of mephedrone was reported (Castrignano et al., 2017). We therefore hypothesized that stereospecific pharmacokinetics and pharmacodynamics of the phase-1 metabolites may contribute to the overall effects of mephedrone. We performed *in vitro* assays to determine the effects of nor-mephedrone, 4-hydroxytolyl-mephedrone (4-OH-mephedrone) and dihydro-mephedrone in their enantiopure form on monoamine transporters and receptors. We have also analyzed human urine samples for the abundance of the stereoisomers of nor-mephedrone and 4-OH-mephedrone.

2. Methods (1948 words)

2.1. Cell Culture

Human embryonic 293 cells (HEK293 cells) were maintained at a subconfluent state in humidified atmosphere (37°C, 5% CO_2) in Dulbecco's Modified Eagle Medium (DMEM), containing 10 % heat-inactivated fetal calf serum (FCS), streptomycin (100 µg x 100 mL^{-1}) and penicillin (100 U x 100 mL^{-1}). Geneticin (50 µg x mL^{-1}) was added to select HEK293 cells stably expressing the human isoforms of DAT, NET and SERT as described in (Mayer et al., 2017). For uptake inhibition experiments cells expressing the desired transporter were seeded (40,000 cells per well in a final volume of 200 µL) onto poly-D-lysine (PDL) coated 96-well plates 24 hours prior to the experiment. The same procedure was applied for release experiments, with the only modification that PDL-coated glass-coverslips (diameter 5 mm) were placed into the 96-well plates (Mayer et al., 2017).

Cells used for membrane preparations for the radioligand binding assays were cultured and prepared as described previously (Luethi et al., 2018b). The cells were harvested by adding trypsin/ethylenediaminetetraacetic acid (EDTA), washed with ice-cold PBS, pelleted by centrifugation (1000 rpm for 5 minutes at 4°C), and frozen and stored at -80°C. The frozen pellets were then suspended in 20 mL (receptor binding) or 400 mL (transporter binding) HEPES-NaOH (20 mM, pH 7.4) containing 10 mM EDTA, and homogenized at 14,000 rpm for 20 seconds (receptor binding) or 10,000 rpm for 15 seconds (transporter binding). The homogenates were then centrifuged at 48,000 g and 4°C for 30 minutes and the supernatants were discarded. The pellets were then resuspended in 20 ml HEPES-NaOH (20 mM, pH 7.4) containing 0.1 mM EDTA and homogenized at 14,000 rpm for 20 seconds. Centrifugation and removal of the supernatant was repeated, before the final pellet was resuspended in HEPES-NaOH containing 0.1 mM EDTA and homogenized. The following transfected cell lines were used for the binding assays: HEK293 cells (5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C}, TAAR1, hDAT, hNET, and hSERT), Chinese hamster ovary (CHO) cells (α_{1A}) and Chinese hamster lung (CHL) cells (α_{2A}).

2.2. Uptake Inhibition Experiments

For uptake inhibition assays, cell culture medium was replaced with Krebs-HEPES buffer (KHB: 25 mM HEPES, 120 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, and 1.2 mM $MgSO_4$ and 5 mM D-glucose, pH adjusted to 7.3) (200 µL per well). Subsequently, the cells were incubated with increasing concentrations of the substance of interest. After 300 seconds, tritiated substrates (20 nM [^{3}H]MPP^+ for DAT and NET and 100 nM [^{3}H]5-HT for SERT) were added. After 180 (DAT and NET) or 60 seconds (SERT), the tritiated substrates were removed and the cells were washed with ice-cold KHB (200 µL per well). Finally, the cells were lysed with 1% sodium dodecyl sulphate (SDS, 100 µL per well) and the amount of tritium within the cells was determined by use of a beta-scintillation counter. Nonspecific uptake was determined in the presence of 10 µM mazindol (DAT, NET) or 10 µM paroxetine (SERT) and subtracted. Uptake in the absence of the substance of interest was defined as 100% uptake. The IC_{50} values were determined by non-linear regression fits (GraphPad Prism version 5.0; log(inhibitor) vs. response –variable slope four parameters (Y= Bottom + (Top-Bottom)/(1+10^((LogIC50-X)*HillSlope)); fitting method: Least squares (ordinary) fit without weighting to minimize the absolute distances squared) as described in (Mayer et al., 2017).

2.3. Release Experiments

Dynamic superfusion experiments were performed as previously described (Mayer et al., 2017; Sitte et al., 2000). Briefly, SERT expressing HEK293 cells were grown on PDL-coated glass coverslips and pre-loaded with [^{3}H]5-HT by exposing the cells to 0.4 µM [^{3}H]5-HT (in KHB) for 20 minutes at 37°C. Subsequently, the cells were placed in superfusion chambers with a volume of 200 µL and superfused with KHB at a flow rate of 0.7 mL per minute for 40 minutes to establish a stable baseline before the collection of 2 minute-fractions was started. After three basal fractions, the cells were superfused with KHB containing the substance of interest (0, 1, 3, 10, 30 and 100 µM) for five fractions. Finally, the cells were superfused with 1% SDS for three fractions to determine the total amount of radioactivity within the cells at the end of the experiment. The amount of tritium within the superfusates was determined with a beta-scintillation counter and the release of [^{3}H]5-HT was expressed as a fractional rate, i.e. the radioactivity present within each fraction was expressed as the percentage of the total radioactivity present at the beginning of that fraction (Sitte et al., 2000).

The superfusion apparatus has 12 individual chambers. Hence, each independent observation allowed for the monitoring of six different concentrations (each in duplicate). To monitor inter-experimental consistency, randomly selected concentrations were included in following experiments, giving rise to individual differences in sample sizes in Figure 3 and 4.

2.4. Receptor and Transporter Binding and Activation Assays

Radioligand binding assays were performed as described in detail for each receptor and transporter in (Luethi et al., 2018b). The membrane preparations that overexpressed the respective receptors or transporters (human genes, with the exception of rat and mouse genes for TAAR1) were incubated with radiolabelled ligands and ligand displacement by the compounds was measured. The difference between total binding (binding buffer alone) and nonspecific binding (in the presence of specific competitors) was determined to be specific binding. The radioligands and competitors are listed in Table 1.

IC_{50} values were derived from three concentration-effect curves using 10 different concentrations. The validity of this measurement is dependent on the amount of single data points (concentration-effect measurements) and the appropriate selection of sufficient points within the slope of the curve (>5 for each experiment). Thus, the amount and level of concentrations tested defines accurate IC_{50} values rather than the number of experiments (with potentially fewer concentrations or concentrations not within the slope). GraphPad was used to derive each IC_{50} value from the modeled curves using in total 30 data points by logistic regression. The Cheng-Prusoff equation was used to calculate K_i values. No observed affinity within a concentration range indicates that there was no binding at the highest tested concentration as well as no binding at 9 different lower concentrations.

Receptor/ Transporter	hTAAR1	rTAAR1	mTAAR1	h5-HT_{1A}	h5-HT_{2A}	h5-HT_{2C}
Radioligand	[^{3}H]RO5166017	[^{3}H]RO5166017	[^{3}H]-8-OH-DPAT	[^{3}H]Ketanserin	FLIPR assay	[^{3}H] Mesulergine
Non-specific binding	10 µM RO5166017	10 µM RO5166017	10 µM pindolol	10 µM spiperone	FLIPR assay	10 µM mianserin
Receptor/ Transporter	hα_{1A}	hα_{2A}	hDAT	hNET	hSERT	

Radioligand	[^{3}H]Prazosin	[^{3}H]Rauwolscine	[^{3}H]WIN35,428	*N*-methyl-[^{3}H]Nisoxetine	[^{3}H]Citalopram	
Non-specific binding	10 µM chlor-promazine	10 µM phentol-amine	10 µM indatraline	10 µM indatraline	10 µM indatraline	

Table 1: Radioligands and non-specific binding determinants used in receptor and transporter binding assays.

The activation potential at the serotonin 5-HT_{2B} receptor was assessed as described in (Luethi et al., 2018b). HEK293 cells expressing the human 5-HT_{2B} receptor were incubated in PDL-coated 96-well plates overnight. Thereafter, the growth medium was removed by snap inversion, 100 µl of calcium indicator Fluo-4 solution (Molecular Probes, Eugene, OR, USA) was added to each well and the plates were incubated for 45 minutes at 31°C. The Fluo-4 solution was then removed by snap inversion before 100 µL of Fluo-4 solution was added again for 45 minutes at 31°C. After washing the cells, 100 µL assay buffer was added. Finally, the plates were placed in a fluorescence imaging plate reader (FLIPR), 25 µL of the test drugs diluted in assay buffer was added online and the increase in fluorescence was measured. EC_{50} values were derived from concentration-response curves using nonlinear regression.

The activation potential at the human TAAR1 was assessed as described in (Luethi et al., 2018b). Recombinant HEK 293 cells expressing the human TAAR1 were harvested and pelleted by centrifugation at 900 rpm for 3 minutes at room temperature. The supernatant was removed and the cell pellet was resuspended in fresh culture medium. The cells were then plated into 96-well plates (100 µl per well, 80,000 cells per well) and incubated for 20 hours at 37°C. For the cAMP assay, the cell culture medium was removed, and 50 µl of PBS (without Ca^{2+} and Mg^{2+}) was added. Thereafter, PBS was removed by snap inversion and 90 µl of Krebs-Ringer bicarbonate buffer containing 1 mM IBMX was added. The plates were then incubated for 60 minutes at 37°C. All compounds were tested at a broad range of concentrations (300 pM – 30 µM) in duplicate and a standard curve (0.13 nM – 10 µM cAMP) was included on each plate. Additionally, a reference plate containing RO5256390, β-phenylethylamine and p-tyramine was included in each experiment. Typically, 30 µl of a compound solution, 30 µl of β-phenylethylamine (as maximal response) or a basal control was added, and the cells were incubated for 40 minutes at 37°C. Finally, the cells were lysed with 50 µl of detection mix solution containing Ru-cAMP Alexa700 anti-cAMP antibody and

lysis buffer for 120 minutes at room temperature under heavy shaking. Fluorescence was measured using a NanoScan (IOM reader; 456 nm excitation wavelength; 630 and 700 nm emission wavelengths). The FRET signal was calculated as the following: FRET (700 nM) – P × FRET (630 nM), where P = Ru (700 nM) / Ru (630 nM).
Receptor and transporter affinity data of a variety of previously published psychedelics and stimulants (Luethi et al., 2018a; Luethi et al., 2018b) verified that the material used was fully binding competent.

2.5. Enantiomeric profiling of nor- and 4-OH-mephedrone in human urine samples
After written consent, the urine sample was provided by the Vienna Police Department. The anonymized sample used in the present study tested positive for mephedrone and was obtained during roadside testing of drivers under the influence of drugs in the Vienna area. Prior to analysis of a human urine sample, an HPLC gradient was optimized for the chiral separation of nor-mephedrone and 4-OH-mephedrone. Since direct chiral chromatographic separation of enantiomeric pairs with mass spectrometry (MS) under compatible conditions was not successful, we employed a chiral derivatization protocol described previously (Mohr et al., 2012) to obtain the corresponding diastereoisomers in order to facilitate chromatographic separation. The single enantiomers of nor-mephedrone and 4-OH-mephedrone were treated with *S*-(-)-N-(trifluoroacetyl)pyrrolidine-2-carbonyl chloride (L-TPC) under basic conditions, thus yielding their corresponding diastereoisomeric amides. Under these conditions it is possible that partial racemization of the substrates may occur. We found that the racemization in the case of nor-mephedrone was negligible (approximately 1%), whereas in the case of 4-OH-mephedrone the *S*- enantiomer tended to slightly (12%) racemize towards the *R*-enantiomer (still enabling unequivocal assignment of stereochemistry when utilizing optically pure material). It is noteworthy, that partial racemization was also observed for free-base representatives of this compound series under physiological conditions. Hence, pure isomers were generally employed as hydrochloride salts displaying significantly higher stability of optical purity.
First, the MS behaviour of derivatized metabolites as reference standards was studied in order to find characteristic ionization and fragmentation patterns. Detection and identification of derivatized metabolites were accomplished by analysis by LC-MS/MS of directly diluted biological specimens. The urine sample was subjected to the derivatization protocol without prior glucuronidase and arylsulphatase work-up since

4-OH-mephedrone was found to decompose completely during this process. Metamfepramone was chosen as the internal standard due to its availability and structural similarity to the metabolites and due to the fact that it cannot be modified via derivatization conditions. Any urinary matrix effect on the calibration curve was evaluated by analyzing the standards spiked into human urine.

2.6. Data Acquisition and Analysis

Blinding: a code (three letters, three digits; e.g. LAU644 for *S*-4-OH-mephedrone) was assigned to the individual stereoisomers of the phase-1 metabolites. Experiments were performed with the identity of the metabolites being masked to the experimenter. Calculations were performed with Micosoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA). Analysis was performed with GraphPad 5.0 (GraphPad Software Inc., La Jolla, CA, USA). Efflux data were analyzed by two-way ANOVA (treatment x time) followed by Bonferroni's test. P values <0.05 were considered significant.

2.7. Materials

The phase-1 metabolites were synthesized in their enantiopure form. Detailed descriptions of the synthetic procedures and the chemical characterizations are given in the Supporting Information. [^{3}H]MPP$^+$ (80-85 μCi mmol^{-1}) was purchased from American Radiolabled Chemicals (St. Louis, MO, USA) and [^{3}H]5-HT (28.3 μCI mmol^{-1}), [^{3}H]8-OH-DPAT, [^{3}H]ketanserin, [^{3}H]mesulergine, [^{3}H]prazosin, [^{3}H]rauwolscine, N-methyl-[^{3}H]nisoxetine, [^{3}H]WIN35,428 and [^{3}H]citalopram were purchased from Perkin Elmer (Boston, MA, USA). [^{3}H]RO5166017 and RO5166017 were provided by F. Hoffmann-La Roche (Basel, Switzerland). All other chemicals were from Sigma-Aldrich (St. Louis, MO, USA).

3. Results (643 words)

3.1. The stereoisomers of phase-1 metabolites inhibit transporter-mediated uptake

To test whether each stereoisomer of the metabolites interacts with MATs, we performed uptake inhibition experiments. The stereoisomers of nor-, 4-OH and dihydro-mephedrone were fully efficacious inhibitors of DAT, NET and SERT mediated uptake (Figure 2). The stereoisomers of nor-mephedrone inhibited DAT and NET with comparable potencies in the low micromolar range, with IC_{50} values ranging from approximately 3 to 10 μM. At SERT, *R*-nor-mephedrone was 18 times less potent than *S*-nor-mephedrone, with corresponding IC_{50} values of 200.0 and 10.9 μM, respectively. The IC_{50} values of *S*- and *R*-4-OH-mephedrone to inhibit DAT-mediated uptake were within the same level of potency, i.e. 2.63 and 5.64 μM, respectively. For NET, the IC_{50} values were 9.53 and 18.04 μM for *S*- and *R*-4-OH-mephedrone, respectively. At SERT, *S*-4-OH-mephedrone (IC_{50} 32.1 μM) was a 19 times more potent inhibitor than *R*-4-OH-mephedrone (IC_{50} 625.4 μM). The four stereoisomers of dihydro-mephedrone displayed varying potencies as inhibitors at each MAT, with IC_{50} values spanning concentrations from 12 to 216 μM. The IC_{50} values and the corresponding NET/DAT and DAT/SERT ratios are summarized in Table 2.

3.2. The stereoisomers of nor- and 4-OH-mephedrone differentially affect SERT-mediated reverse transport

Nor-, 4-OH- and dihydro-mephedrone act as transportable substrates of MATs (Mayer et al., 2016). We performed release experiments to investigate whether the observed differences between the *R*- and *S*-enantiomers of nor- and 4-OH-mephedrone on SERT-mediated uptake also apply to reverse transport. HEK293 cells stably expressing hSERT were pre-loaded with [^{3}H]5-HT and superfused with buffer containing increasing concentrations of test drugs.

Application of two-way ANOVA (concentration x time) demonstrated that *S*- ($F_{5.51}$ = 26.71; $P < 0.0001$) and *R*-nor-mephedrone ($F_{5.38}$ = 6.14; $P = 0.0003$) significantly affected the release of tritiated substrate (Figures 3A and C). *Post hoc* tests revealed that 3, 10, 30 and 100 μM of *S*-nor-mephedrone significantly elevated reverse transport via SERT versus the vehicle control. For *R*-nor-mephedrone, significant effects were observed at 30 and 100 μM. Concentrations of *S*-nor-mephedrone exceeding 10 μM did not stimulate the release of tritiated 5-HT to a further extent but rather exhibited a trend back

towards baseline (Figure 3B). This finding is in agreement with earlier observations, demonstrating a biphasic effect of substrate type-releasers on SERT mediated reverse transport (Seidel et al., 2005). *S*-4-OH-mephedrone significantly affected outwardly-directed transport of [^{3}H]5-HT ($F_{5.63}$ = 44.74; P < 0.0001) (Figure 4A). *Post hoc* analysis demonstrated significant differences at concentrations of 10 µM or higher. No main effect was detected for *R*-4-OH-mephedrone ($F_{5.51}$ = 0.69; P = 0.63) (Figure 4B). Neither *R*-nor-mephedrone nor *S*-4-OH-mephedrone gave rise to bell-shaped concentration response curves (Figures 4C and D); this may indicate that the applied concentrations are non-saturating in our experimental system (Seidel et al., 2005).

3.3. Monoamine receptor and transporter binding profiles

Monoamine receptor binding profiles are summarized in Table 3 and Table 4. None of the tested metabolites bound to 5-HT_{1A} or 5-HT_{2C} receptors, nor did they activate the 5-HT_{2B} receptor. *S*-nor-mephedrone exhibited submicromolar affinities towards rat and mouse TAAR1 (K_i = 0.58 and 0.72 µM, respectively) and supramicromolar affinities towards human 5-HT_{2A} (K_i = 7.41 µM) and α_{1A} (K_i = 1.33 µM) receptors. 1*R*,2*R*-, 1*S*,2*S*- and 1*R*,2*S*-dihydro-mephedrone bound to rat TAAR1 (K_i = 3.71, 3.57 and 0.56 µM, respectively), but only 1*S*,2*S*-dihydro-mephedrone interacted with mouse TAAR1 (K_i = 3.85 µM). However, no human TAAR1 activity was observed in the concentration range investigated (EC_{50} > 30 µM). 1*S*,2*S*- and 1*R*,2*S*-dihydro-mephedrone interacted with 5-HT_{2A} receptors (K_i = 6.66 and 7.47 µM, respectively), whereas only 1*R*,2*S*-dihydro-mephedrone had affinity to the α_{2A} receptor (K_i = 1.85 µM). None of the test drugs bound to MATs with submicromolar affinities.

4. **Discussion**

The intention of the present study was to investigate the effects of selected phase-1 metabolites of mephedrone in their enantiopure form on monoamine receptors and transporters. Stereospecific pharmacology has been reported for various compounds that target MATs, including cathinone (Hutsell et al., 2016), methcathinone (Sparago et al., 1996), amphetamine (Sitte et al., 1998), MDMA (Rudnick and Wall, 1992) and mephedrone (Gregg et al., 2015; Philogene-Khalid et al., 2017a; Philogene-Khalid et al., 2017b; Vouga et al., 2015). In this study, we found that the stereochemistry affects the pharmacology of the phase-1 metabolites of mephedrone. Each of the stereoisomers tested inhibited MAT-mediated uptake in a concentration dependent manner. In the case of nor-mephedrone, both enantiomers inhibited DAT and NET with comparable potencies. At SERT, however, *S*-nor-mephedrone revealed a pronounced leftward-shift when compared to the *R*-stereoisomer. This observation is consistent with a recent publication (Hutsell et al., 2016), demonstrating that the *R*-enantiomer of nor-mephedrone is less potent than *S*-nor-mephedrone at SERT. Uptake inhibition assays with 4-OH-mephedrone revealed a potency loss from the *S*- to the *R*-enantiomer at all three MATs. Remarkably, this rightward-shift was again more pronounced at SERT (~19-fold) than at DAT and NET (~2-fold). The four stereoisomers of dihydro-mephedrone were associated with a more complex pharmacology. The differences in potency were again more pronounced at SERT (up to 10-fold) than at DAT and NET (~2-fold).

A recent study revealed that mephedrone undergoes stereospecific metabolism and the stereoisomers of nor- and 4-OH-mephedrone were detected in biological samples (Castrignano et al., 2017). Based on the differing potencies as inhibitors of SERT-mediated uptake, we sought to perform release experiments with the stereoisomers of nor- and 4-OH-mephedrone in SERT-expressing HEK293 cells. Remarkably, only the *S*-enantiomers of nor- and 4-OH-mephedrone acted as fully-fledged releasers at SERT. In the case of *R*-nor-mephedrone, blunted effects on SERT-mediated reverse transport were observed at 100 µM. *R*-4-OH-mephedrone failed to cause significant effects over the entire range of concentrations tested (1 to 100 µM). It is unlikely that this effect is solely attributable to the reduced potencies as uptake inhibitors: the IC_{50} of *R*-nor-mephedrone to inhibit SERT-mediated uptake (200 µM, which – following the Cheng-Prusoff equation – reflects its K_i-value) is only two times higher than the highest concentration applied in release-experiments. Hence, it can be assumed that a

considerable proportion of transporters will be occupied by *R*-nor-mephedrone at this concentration. According to Seidel and colleagues (Seidel et al., 2005), the maximal effect on reverse-transport elicited by amphetamine-like drugs is expected to occur at concentrations around the corresponding K_i-value of the tested drug. This explanation may not apply to *R*-4-OH-mephedrone (IC_{50} ~625 µM). However, due to limited drug availability, we did not test concentrations higher than 100 µM. We do not feel that increasing the concentration is necessary, as non-specific, i.e. non-SERT-related, effects may superimpose transporter-mediated tracer fluxes. Furthermore, considering the recreational doses of mephedrone (up to 90 mg nasally and 250 mg orally (Schifano et al., 2011)), it is more than unlikely that *R*-4-OH-mephedrone builds up at concentrations beyond the ones applied in our experiments. One possible explanation for the observed effects is that the three-dimensional difference between *S*- and *R*-enantiomers affects their interaction with the binding pocket, thus switching their activity from substrates to non-transportable inhibitors. Interestingly, Hutsell and colleagues (Hutsell et al., 2016) found that both enantiomers of 4-methylcathinone (nor-mephedrone), acted as fully efficacious releasers at SERT in rat brain synaptosomes. This discrepancy may be explained by the fact that we used non-neuronal HEK293 cells as the expression system or differences between the human and rat isoforms of SERT.

Binding of racemic mephedrone to α_{1A}, α_{2A}, 5-HT_{2A} and 5-HT_{2C} receptors has been reported (Luethi et al., 2018a; Simmler et al., 2013) and Philogene-Khalid and colleagues observed that *S*-mephedrone bound to 5-HT_{2A} and 5-HT_{2C} receptors without agonist activity (Philogene-Khalid et al., 2017a).

We found that *S*-nor-mephedrone bound to α_{1A} and 5-HT_{2A} receptors and mouse and rat TAAR1, albeit with moderate affinity towards 5-HT_{2A}. Agonistic activity at α_{1A} receptors is implicated in stimulant-induced behaviours, such as locomotor activity (Schmidt and Weinshenker, 2014). For instance, the α_1- and β-adrenergic receptor antagonist carvedilol reduced cardiostimulant and hyperthermic effects of MDMA in humans, but had no effect on subjective effects of the drug (Hysek et al., 2012). The same findings regarding carvedilol on mephedrone-induced hyperthermia were reported in rats (Zona et al., 2016). Activation of 5-HT_{2A}-receptors has previously been demonstrated for mephedrone (Luethi et al., 2018a) and hallucinogens (Rickli et al., 2016). This effect on 5-HT_{2A} receptors may partially contribute to hallucinogenic effects (Kraehenmann et al., 2017; Nichols, 2016) associated with mephedrone (Kasick et al., 2012; Schifano et al., 2011). Binding to TAAR1 exerts auto-inhibitory effects on monoamine releasing

neurons (Revel et al., 2011; Revel et al., 2012), which has been linked to neuroprotective effects (Di Cara et al., 2011; Lindemann et al., 2008; Revel et al., 2012). Interestingly, binding to rat TAAR1 has been reported for MDMA and mephedrone, with MDMA displaying higher affinities than mephedrone (Simmler et al., 2013). However, in contrast to MDMA, systemic administration of mephedrone did not deplete the 5-HT content in striatum and cortex (Baumann et al., 2012). This observation may be explained by different activities of both drugs at the vesicular monoamine transporter 2 (Cozzi et al., 1999; Pifl et al., 2015). However, this finding also indicates that the exact role of TAAR1 in psychostimulant action remains unclear (Sitte and Freissmuth, 2015). (1*S*,2*S*)-dihydro-mephedrone bound to mouse TAAR1, but not to rat TAAR1. In contrast, (1*R*,2*R*)-, (1*R*,2*S*)- and (1*S*,2*R*)-dihydro-mephedrone bound to rat TAAR1 but not to mouse TAAR1. (1*S*,2*S*)- and (1*R*,2*S*)-dihydro-mephedrone also bound to 5-HT_{2A}-receptors and the latter was also found to bind to α_{2A} receptors. Overall, the binding data suggest that some of the effects of mephedrone may be shaped – at least in part – by the activities of the metabolites at a subset of monoamine receptors.

In addition to our own *in vitro* studies, we performed a pilot chiral analysis of a urine sample obtained from a human mephedrone user (Figure 5). 4-OH-mephedrone was found to be problematic in our assays based on the partial racemization of *S*- and *R*-enantiomers under assay conditions, consequently affecting calibration curves and compound determination in the analysis. Given the limited stability of this metabolite and its lower abundance in the urine sample compared to nor-mephedrone, we refrained from drawing further conclusions for this metabolite.

In the case of nor-mephedrone we found that the *S*- enantiomer is almost 2 times more abundant in the human urine sample compared to its *R*- counterpart; hence, nor-mephedrone is predominantly excreted in its *S*-enantiomeric form. This novel finding is in excellent agreement with a recent report (Castrignano et al., 2017). Previously, we found that systemically administered nor-mephedrone exerts profound effects on extracellular serotonin, whereas the effects were less pronounced for dopamine (Mayer et al., 2016). According to drug user reports, the subjective effects of mephedrone resemble those of MDMA, which markedly affects the serotonergic system (Green et al., 2014; Schifano et al., 2011). Given the pharmacological profile of mephedrone, i.e. no selectivity for SERT (Baumann et al., 2012), it is tempting to speculate that some of the subjective effects are linked to *S*-nor-mephedrone. This interpretation is bolstered by the fact that *R*-mephedrone appears to be the predominant stereoisomer in human

samples and waste water (Castrignano et al., 2017). *R*-mephedrone shows greater selectivity for DAT over SERT than *S*-mephedrone. In line with this finding, only *R*-mephedrone induced conditioned place preference (CPP). Furthermore, although both enantiomers facilitated intracranial self-stimulation (ICSS), *R*-mephedrone did so to a greater extent (Gregg et al., 2015). ICSS and CPP positively correlate with the rewarding effects of psychostimulants, which appear to be defined by the relative DAT/SERT selectivity of drugs (Roberts et al., 1999). Considering the higher DAT/SERT ratio of *R*-mephedrone and its relative abundance in humans when compared to *S*-mephedrone, this feature provides a potential explanation for the re-dosing of mephedrone within sessions (Jones et al., 2016). The pharmacological profiles of *R*-mephedrone and *S*-mephedrone led Philogene-Khalid and colleagues to conclude that the rewarding effects of mephedrone are primarily due to the actions of *R*-mephedrone (Philogene-Khalid et al., 2017a). The same authors found that *S*-mephedrone reduces anxiety and depression-like effects in rats that were withdrawn from cocaine or MDPV. Possibly, due to the increased SERT/DAT ratio of *S*-mephedrone, administration of this drug adequately compensates for deficits in extracellular 5-HT, which are associated with cocaine withdrawal (Parsons et al., 1995, 1996). The increased SERT selectivity of *S*-nor-mephedrone may contribute to the entactogenic effects of mephedrone (Papaseit et al., 2017).

CYP2D6 has been demonstrated to be the main enzyme involved in the metabolism of mephedrone (Pedersen et al., 2013). Cytochrome P450 enzymes (CYPs) are abundantly expressed in the liver. However, it has been demonstrated that the expression of CYPs is not confined to the liver and various CYP isoforms have been detected in the nervous system (Ferguson and Tyndale, 2011; Miksys and Tyndale, 2002). CYP2D6 is expressed in numerous regions of the human brain (Siegle et al., 2001), including hippocampus and striatum (Chinta et al., 2002). Importantly, a recent *in vivo* study provided evidence that CYPs contribute to metabolic processes in the brain (Bromek et al., 2011). Hence, it is tempting to assume that the metabolites can be formed *in situ* and gain access to MATs and monoamine receptors, and shape the overall effects of mephedrone.

In addition, hepatically formed nor-mephedrone crosses the blood brain barrier, as evidenced by its effects on extracellular 5-HT and dopamine in the nucleus accumbens upon systemic administration. This might be attributed to its distribution coefficient (logD =1.29) (Mayer et al., 2016) as the lipid solubility and size of a substance correlates

with its likelihood to enter the brain (van Bree et al., 1988; Waterhouse, 2003) or transport processes that have yet to be determined.

The ability to induce reverse-transport via SERT is essential to the action of MDMA, since most of the subjective effects of MDMA were attenuated by co-administration of the SERT-inhibitor citalopram (Liechti et al., 2000). This observation further supports the hypothesis that *S*-nor-mephedrone contributes to the action of mephedrone as *R*-nor-mephedrone failed to induce release via human SERT. Aside from the effects at SERT, stereoselectivity at NET may also affect the pharmacology of mephedrone. Administration of the NET-inhibitor reboxetine not only reduced the effects of MDMA on heart rate and blood pressure, but also attenuated "drug-liking" and "good drug-effects" (Hysek et al., 2011). Olesti et al. (Olesti et al., 2017) recently published a study on the pharmacokinetics of mephedrone and its metabolites in human plasma and urine. In addition, this study quantified mephedrone and a subset of metabolites in rat prefrontal cortex after systemic administration of the parental substance.

4'carboxy-mephedrone (COOH-mephedrone, which is derived from 4-OH-mephedrone) and nor-mephedrone were the most abundant metabolites in human plasma with $AUC_{0\text{-}8h}$ levels of 113% and 46%, respectively, of those of mephedrone after ingestion of 150 mg of mephedrone (n=6 individuals). Other minor metabolites detected were *N*-succinyl-nor-mephedrone (SUCC-mephedrone) (4%) and dihydro-mephedrone (11%). The half-life of mephedrone was 2.2 h, compared to 4.5 h (nor-mephedrone), 8.2 h (SUCC-mephedrone) 5.7 h (DIHYDRO-mephedrone) and 1.9 h (COHH-mephedrone). Moreover, 1h after administration of mephedrone (30 mg/kg; i.p.), the concentration of nor-mephedrone in rat prefrontal cortex exceeded the concentration of the parental drug by 100%, with COOH-mephedrone and SUCC-mephedrone being detectable in small amounts. In the same study, the authors observed C_{max} values of 179 ± 29.3 and 54.4 ± 4.9 (ng/mL, ± SEM) for mephedrone and nor-mephedrone, respectively. Considering the moderate dose (150 mg) used in this study, the brain concentrations can be expected to be in the low micromolar range after the use of moderate to high doses. These findings strengthen the hypothesis that the effects of mephedrone may be partially shaped by its metabolites, with an emphasis on nor-mephedrone.

The limitations of this study are that we did not test conjugated metabolites for their activities at monoamine receptors and transporters and that limited information is available on the abundance of the metabolites in human brain.

Hence, further studies are warranted to unravel the pharmacokinetics of the metabolites in a time-resolved and stereospecific manner to clarify the overall contribution of the metabolites to the effects of mephedrone on neurochemistry and behavior.

Table 2: Effect of test drugs on monoamine transporter mediated uptake.

IC50 (µM) (95 % CI in brackets)					
	DAT	NET	SERT	NET/DAT ratio	DAT/SERT ratio
S-nor-mephedrone	8.72 (5.9 to 12.87)	3.84 (3.16 to 4.66)	10.88 (6.33 to 18.7)	2.27	1.25
R-nor-mephedrone	9.44 (6.51 to 13.68)	4.54 (3.44 to 5.99)	200.0 (106.8 to 374.6)	2,08	21.19
S-4-OH-mephedrone	2.63 (1.98 to 3.49)	9.53 (7.49 to 12.14)	32.05 (24.39 to 42.13)	0.28	12.19
R-4-OH-mephedrone	5.64 (3.68 to 8.64)	18.04 (11.76 to 27.67)	625.4 (478 to 818.3)	0.31	110.89
1*S*,2*R* Dihydro-mephedrone	12.67 (10.41 to 15.41)	22.66 (18.62 to 27.57)	65.55 (40.15 to 107)	0.56	5.17
1*R*,2*S* Dihydro-mephedrone	21.11 (17.21 to 25.88)	38.99 (33.45 to 45.44)	32.21 (26.72 to 38.84)	0.54	1.53
1*R*,2*R* Dihydro-mephedrone	14.12 (9.43 to 21.15)	39.05 (27.12 to 56.21)	302.3 (228.4 to 400.1)	0.36	21.41
1*S*,2*S* Dihydro-mephedrone	12.89 (10.12 to 16.44)	47.09 (27.83 to 79.67)	248.2 (220.8 to 279)	0.27	19.26

Data are shown as the mean (95% CI in parenthesis), obtained from non-linear regression fits as shown in Figure 2. NET/DAT ratio = 1/NET IC_{50} divided by 1/DAT IC_{50}. High value indicates greater NET-selectivity. DAT/SERT ratio = 1/DAT IC_{50} divided by 1/SERT IC_{50}. High value indicates greater DAT selectivity.

Table 3: Monoamine receptor binding profiles. Binding profiles were obtained as described in "materials and methods". K_i and EC_{50} values (µM) are given as mean and SD from 3 independent measurements.

	Human TAAR1	Rat TAAR1	Mouse TAAR1	$5HT_{1A}$	$5HT_{2A}$	$5HT_{2B}$	$5HT_{2C}$	α_{1A}	α_{2A}
	EC_{50} ± SD [µM]	K_i ± SD [µM]	K_i ± SD [µM]	K_i ± SD [µM]	K_i ± SD [µM]	EC_{50} ± SD [µM]	K_i ± SD [µM]	K_i ± SD [µM]	K_i ± SD [µM]
S-nor-mephedrone	>30	0.5873 ± 0.02	0.725 ± 0.026	> 17	7.41 ±0.305	>10	>5	1.33 ±0.051	>4.6
R-nor-mephedrone	n.d.	>5.19	>4.36	>17.59	>12.98	>10	>5.07	>2.24	>4.68
S-4-OH-mephedrone	n.d.	>5.19	>4.36	>17.59	>12.98	>10	>5.07	>2.24	>4.68
R-4-OH-mephedrone	n.d.	>5.19	>4.36	>17.59	>12.98	>10	>5.07	>2.24	>4.68
(1*R*, 2*R*)-dihydro-mephedrone	n.d.	3.71 ±0.53	>4.36	>17.59	>12.98	>10	>5.07	>2.24	>4.68
(1*S*,2*S*)-dihydro-mephedrone	n.d.	>5.19	3.85 ±0.39	>17.59	6.665 ±2.36	>10	>5.07	>2.24	>4.68
(1*S*,2*R*)-dihydro-mephedrone	n.d.	3.568±0.19	>4.36	>17.59	>12.98	>10	>5.07	>2.24	>4.68
(1*R*,2*S*)-dihydro-mephedrone	>30	0.558±0.049	>4.36	>17.59	7.476±2.126	>10	>5.07	>2.24	1.846±0.388

Table 4: Monoamine transporter binding profiles. Binding profiles were obtained as described in "materials and methods". K_i values [µM] are given as mean and SD from 3 independent measurements.

	DAT	NET	SERT
	K_i ± SD [µM]	K_i ± SD [µM]	K_i ± SD [µM]
S-nor-mephedrone	>8.52	>8.77	>7.42
R-nor-mephedrone	>8.52	>8.77	>7.42
S-4-OH-mephedrone	6.69 ± 1.54	>8.77	>7.42
R-4-OH-mephedrone	>8.52	>8.77	>7.42
(1*R*,2*R*)-dihydro-mephedrone	6.07 ± 0.11	>8.77	>7.42
(1*S*,2*S*)-dihydro-mephedrone	>8.52	>8.77	>7.42
(1*S*,2*R*)-dihydro-mephedrone	>8.52	>8.77	>7.42
(1*R*,2*S*)-dihydro-mephedrone	>8.52	>8.77	>7.42

Competing interests: H.H.S. has received honoraria for lectures and consulting from AbbVie, Aesca, Amgen, Astellas, AstraZeneca, Astropharma, Chiesi, Gebro, IMH, IIR, Janssen-Cilag, Lundbeck, MSD, Mundipharma, Pfizer, Ratiopharm, Roche, Sandoz, Sanofi-Aventis, Shire, Serumwerk Bernburg, Vertex (past 5 years). All other authors declare no conflicts of interest.

Author contributions: F.P.M., D.C., D.A.P., L.W., D.L., M.H., K.J., T.S., and M.C.H. performed experiments. F.P.M., G.M., M.C.H., M.E.L., M.D.M. and H.H.S. designed the experiments. F.P.M. and H.H.S. wrote the manuscript and received significant input from all other authors.

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Figure Legends

Figure 1: Chemical structures of the stereoisomers of the phase-1 metabolites of mephedrone.

Figure 2: Effects of the stereoisomers of nor-, 4-OH- and dihydro-mephedrone on transporter-mediated uptake into HEK293 cells. Uptake of [^{3}H]MPP$^+$ (DAT and NET expressing cells) and [^{3}H]5-HT (SERT expressing cells) was inhibited with increasing concentrations of the indicated test drugs. Data are shown as the mean and SEM obtained from 5 individual experiments, performed in triplicate.

Figure 3: *S*- and *R*-nor-mephedrone induced reverse transport via human SERT. HEK293 cells stably expressing hSERT were preloaded with tritiated 5-HT and superfused with buffer. After the collection of three 2-minute fractions, the test drugs were added at the indicated concentrations for five 2-minute fractions. (A) and (C) depict the effects of increasing concentrations of test drugs on the fractional release of [^{3}H]5-HT. (B) and (D) show the concentration response curves generated in (A) and (C). The Averaged efflux rate was defined as the mean of the last three rates (i.e. t=8 to 14 min) minus the mean of the first three rates (i.e. basal release; i.e. t=0 to 4 min). All data are mean and SEM. Φ, #, *, ∧ denote significant differences versus the time-matched control (0 μM) for 100, 30, 10 and 3 μM of test drug, respectively ($P < 0.05$, Bonferroni's). Numbers in parenthesis indicate independent observations performed in duplicate as described in materials and methods. *S*-nor-mephedrone: 0 μM (9), 1 μM (10), 3 μM (10), 10 μM (8), 30 μM (10), 100 μM (10). *R*-nor-mephedrone: 0 μM (5), 1 μM (6), 3 μM (7), 10 μM (11), 30 μM (8), 100 μM (7).

Figure 4: *S*- and *R*-4-OH-mephedrone induced reverse transport via human SERT. The experiment was performed as described in materials and methods and Figure 2. (A) and (C) depict the effects of increasing concentrations of test drugs on the fractional release of [^{3}H]5-HT. (B) and (D) show the concentration response curves generated in (A) and (C). The Averaged efflux rate was defined as the mean of the last three rates (i.e. t=8 to 14 min) minus the mean of the first three rates (i.e. basal release; i.e. t=0 to 4 min). All data are mean and SEM. Φ, #, * denote significant differences versus the time-matched control (0 μM) for 100, 30 and 10 μM of test drug, respectively ($P < 0.05$,

Bonferroni's). Numbers in parenthesis indicate independent observations performed in duplicates as described in materials and methods. *S*-4-OH-mephedrone: 0 µM (10), 1 µM (11), 3 µM (12), 10 µM (12), 30 µM (12), 100 µM (12). *R*-4-OH-mephedrone: 0 µM (8), 1 µM (10), 3 µM (10), 10 µM (9), 30 µM (10), 100 µM (10).

Figure 5: Chromatographic separation of derivatized *S*- and *R*-nor-mephedrone in human urine sample as pilot study. The urine sample was derivatized as described in the methods section and then analysed on a tandem-LC-MS/MS tandem system with an analytical HPLC column (Kinetex 1.7 µm C18 100 A 150 x 2.1 mm at 40°C using gradient conditions (35% acetonitrile to 100% over 26 minutes, followed by isocratic elution for 4 minutes)). The experiment was repeated twice, derivatizing 250 µL of the urine sample, yielding comparable results.