Pharmacokinetics and Disposition of Prolonged-Release Ketamine Tablets for Treatment of Neuro-Psychiatric Diseases

Inauguraldissertation

zur

Erlangung des akademischen Grades

doctor rerum naturalium (Dr. rer. nat.)

an der Mathematisch-Naturwissenschaftlichen Fakultät

der

Ernst-Moritz-Arndt-Universität Greifswald

Vorgelegt von

Mahmoud Hasan

geboren am 19.08.1982

in Kairo. Ägypten

Greifswald, den 31/03/2017

Dekan: Prof. Dr. Werner Weitschies

1. Gutachter: Prof. Dr. Werner Siegmund

2. Gutachter: Prof. Dr. Martin Fromm

Tag des Promotionskolloquiums: 17-10-2017

Table of contents

1. Introduction

Treatment of the signs and symptoms of several frequently occurring chronic neurological diseases or mental disorders such as neuropathic pain, Parkinsonism, Alzheimer disease, dementia and depression is still challenging even in highly developed societies. On the other side, the prevalence of some of the diseases is constantly increasing during the last years and the same is true for the economic burden caused by them. For example, the World Health Organization (WHO) stated that there is currently no efficient treatment for dementia although 47.4 million people are suffering from this disease worldwide and in 2010, the total global societal costs of dementia were estimated to be 604 billion US$.

Moreover, the classical targets for drugs as the dopamine receptor for treatment of Parkinsonism, muscarinic receptor for influencing the fate of patients with dementia or opioid receptors and COX (Cyclooxygenase receptors) for relieving pain cannot furthermore be considered in the future development of promising new chemical entities. Hopeful exceptions might be receptors, which are involved in the cascade of the complex signal transmission mediated by the amino acid glutamate, particularly the N-methyl-D-aspartate (NMDA) receptor and the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor which in turn are involved in the pathogenesis of several diseases which makes them attractive as potential pharmacological targets.

The glutamate receptors play an important role in the function and cell survival/death of central neurons [1]. This dual action follows a hypothesis known as "Localization Hypothesis", where stimulation of extra synaptic receptors contributes to cell death. On the other hand, stimulation of synaptic NMDA receptors contributes to the health and longevity of the cell [1,2].

Initially, the nerve impulse or action potential is created by the entrance of sodium or calcium ions through special types of voltage-gated ion channels that open in response to an elevated membrane potential. Then, it is followed by efflux of potassium ions to regain the former state for the membrane. Once this impulse comes to the end of the presynaptic neuron, neu-

rotransmitters are released and move across the synaptic cleft to bind with glutamate receptors in the postsynaptic neuron.

The NMDA receptors are permeable to Ca^{2+} [3] but are blocked by Mg^{2+} ion [4]. The extrusion of Mg^{2+} ion requires excitatory postsynaptic current produced by AMPA receptor stimulation. Consequently, the voltage-dependent calcium channels (VDCCs) are opened for calcium influx and neurotrophins are released. Neurotrophins including BDNF (Brain Derived Neurotrophic Factor) [5] activates tropomyosin kinase B (TrkB) receptors. TrkB elevates signaling through activation of both extracellular signal related kinase (ERK) and protein kinase B (Akt) [6]. Furthermore, BDNF inhibits GSK-3 (glycogen synthasekinase 3). Both actions of BDNF lead to activation of mTOR (mammalian target of rapamycin) pathway that performs further protein translation synaptogenesis and causes various neuro-modulating effects e.g. antidepressant effect [7]. Additionally, there is evidence of elevated mTOR signaling in Alzheimer's disease [8].

mTOR forms complexes with other proteins to regulate different cellular processes. these complexes act as serine/threonine protein kinase, and control cell growth, survival, proliferation and protein synthesis [9]. mTOR activity is regulated by rapamycin, insulin, growth factors, phosphatidic acid and certain amino acids [10]. It plays a role as a sensor for cellular nutrient, oxygen, and energy levels [11]. Additionally, the mTOR pathway controls metabolism and its dysfunction is clear in many diseases including depression and cancer [12].

In regard with the relation of glutamate receptors and various CNS diseases, the NMDA receptor co-agonist D-serine (D-ser) was responsible for various types of neuropathology [13–16]. D-ser is generated by the enzyme serine racemase that is attenuated by inhibition of α7-nicotinic acetylcholine (α7-nACh) receptor located in the presynaptic neuron. D-ser is considered as a biomarker whose CNS concentration is elevated in some diseases as Alzheimer and Parkinson’s diseases while, on the other hand, it is decreased in schizophrenia [13].

Traditionally, one drug targets a distinct receptor for a certain indication. For example, dextromethorphan and morphine are indicated for analgesia; amantadine and levodopa for Parkinsonism; memantine and fluoxetine for depression.

One exception of this conception is ketamine (KET) which can exert by itself and via its primary and secondary metabolites very complex pharmacodynamical effects through targeting different receptors on the glutamate pathway. Consequently, it is not surprising for KET to have other neuro-modulating activity and could be promising for several diseases other than the common anesthetic use.

2. Rationales and objectives

Ketamine (KET) is a lipophilic, basic compound (log P = 2.2; pKa = 7.5). Thus, it is expected to have different degree of ionization depending on the surrounding medium. Consequently, its ionization degree could affect its absorption, distribution and elimination from the body as well as the sample preparation and the chromatographic separation required for its analytical quantification. Furthermore, KET is a chiral compound. It is present as *S*- and *R*-enantiomers. *S* (+) KET is more active as an NMDA receptor antagonist and is subjected to more metabolism than *R* (-) KET [17,18]. Moreover, the metabolites of KET are chiral as well and one of them is present as 12 isomers. Thus, stereo selectivity may have an impact on the pharmacokinetics and pharmacodynamics.

The hepatic and intestinal CYP2B6, CYP3A4 and CYP2C9 enzymes are responsible for the extensive metabolism of KET through two pathways as described in the following diagram (Figure 1): either demethylation to norketamine (n-KET) (the major metabolite) followed by either dehydrogenation to dehydronorketamine (DHNK) or hydroxylation to hydroxynorketamines (HNK) (black pathway). On the other hand, it may undergo hydroxylation to hydroxyketamine (HK) then demethylation to HNK (white pathway).

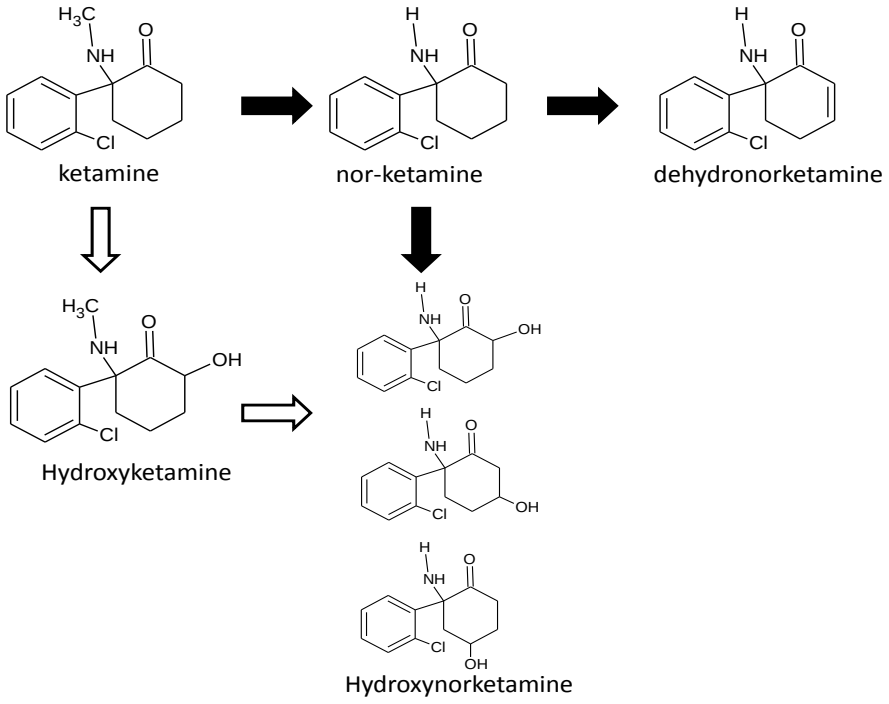


Figure 1: Metabolic pathways of ketamine

Rationales and objectives

The metabolic pattern of KET and the effect of this pattern on pharmacodynamics of KET are so interesting and make KET promising for wide range of indications. Where the parent drug and its major metabolite n-KET are strong antagonists of the NMDA receptor, (n-KET has 60 % efficacy of KET). The anesthetic [19] and analgesic [20] effects of KET seem to be related to the antagonism of the NMDA receptor (classical ketamine paradigm). According to this paradigm the other metabolites of ketamine are considered as pharmacologically inactive based on a study performed on rat [21]. However, the secondary metabolites for KET (hydroxylated norketamines) showed recently pharmacological activities as well [22]. The (2*S*,6*S*)-HNK was proved to be an inhibitor for α7-nACh receptor decreasing level of D-serine that is responsible for neurotoxicity of KET [23]. Additionally, (2*R*,6*R*)-HNK is responsible for the antidepressant activity as well as other neuro-modulating activity through AMPA receptor up regulation [22]. This leads to a new (Ketamine metabolite paradigm) that depends on both KET and its metabolites. This paradigm includes further receptors along the glutamate signal pathway, not only the classical NMDA as clarified in (Figure 2).

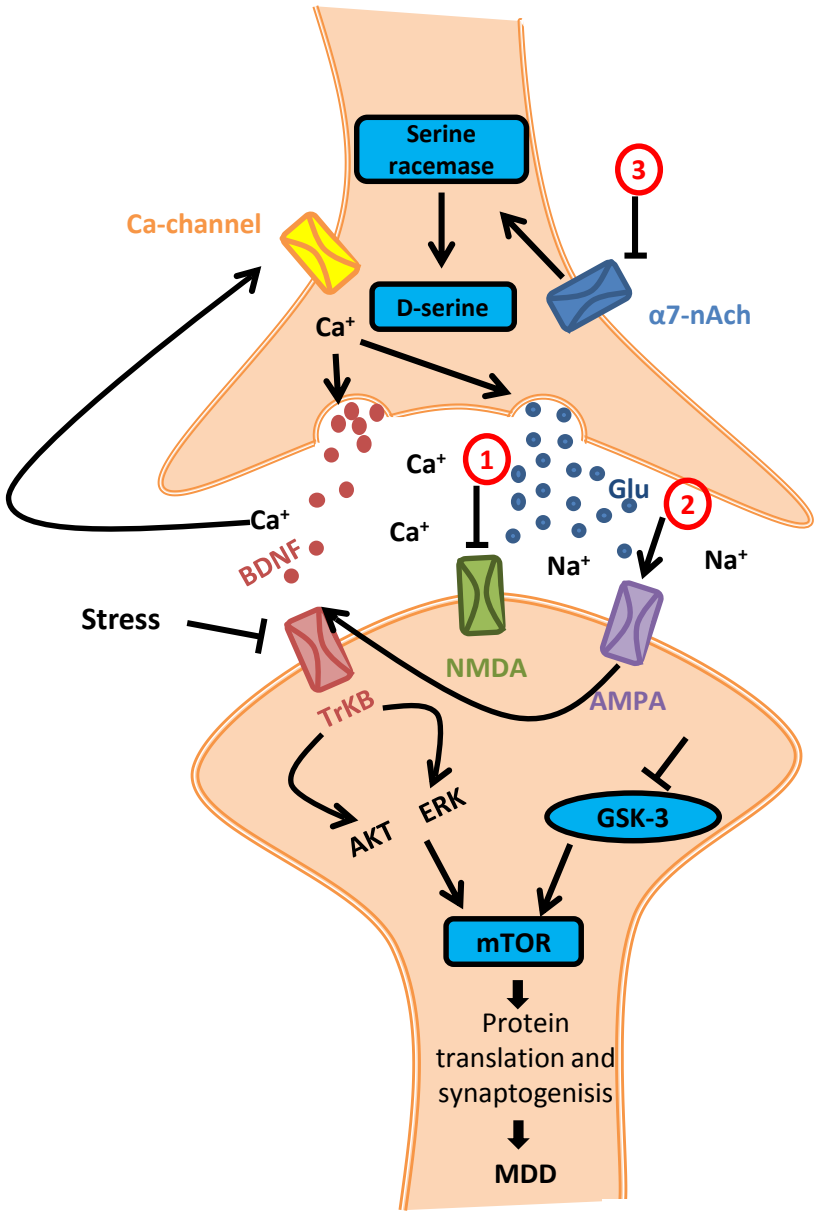


Figure 2: Pharmacodynamical sites of action of KET and its metabolites: (1) inhibition of the NMDA receptor (KET and n-KET) to induce analgesic and anesthetic effects; (2) stimulation of the AMPA receptor and TrkB receptor to induce neuroprotective effects by stimulation of AKT, ERK and the mTOR pathway inducing antidepressant activity; (3) antagonism of the α7-nACh receptor and decrease of the presynaptic release of D-serine that is co-agonist for NMDA receptors

Rationales and objectives

Nowadays, Ketamine (KET) is exclusively registered on the market as either intravenous (infusion) or intramuscular injections for anesthesia. However, these dosage forms are accepted when KET is used for the induction and maintenance of anesthesia. On the other side, when KET is intended to be used for chronic diseases e.g. pain, depression, Parkinson or dementia injection is not a favored administration route. Moreover, so far, known pharmacokinetic data for KET shows its wide distribution (5 l/kg) after intravenous administration (IV-KET), with high plasma exposure (more side effects) and short $T_{1/2}$ of ~ 5 h. Consequently, we are in need for an oral dosage form to be prescribed for these chronic diseases.

Currently, there is no registered oral dosage form for KET. However, in clinical trials for the investigation of the efficacy of oral KET, the marketed solution for injection/infusion (off-label use) or an extemporaneous preparation (eg. lozenge) representing an immediate release oral dosage forms (IR-KET) were used [24–26]. The pharmacokinetic parameters for this IR-KET differ from the IV-KET. IR-KET has higher metabolic ratio ($AUC_{n\text{-}KET/KET}$, 5) when compared to IV-KET injection (0.8) due to extensive first pass metabolism [27]. Thus, the oral dosage form in addition to the higher patient acceptance might be promising for wider range of CNS-diseases when compared to the injection due to the formation of more active metabolites.

Despite these advantages, IR-KET suffers from low bioavailability (25%), and high lipid solubility. Moreover, The $T_{1/2}$ (~ 5 h) is still short to fulfil the required whole day coverage needed for treating the aforementioned chronic diseases [27,28].

Therefore, our hypothesis was that the administration of KET as a prolonged release dosage form (PR-KET) instead of the IR-KET. PR-KET might have longer duration of action that makes it promising for treating the previously mentioned chronic diseases. Moreover, it might be able to generate more of the active metabolites (HNK and n-KET) with better safety profile. From the available knowledge, so far it can be assumed that PR-KET might have different metabolic pattern and pharmacokinetics as IR-KET and IV-KET.

These differences in pharmacokinetics might be due to the difference in expression of the biotransformation enzymes between the liver and intestine as well as along the intestine itself

[29]. Thus, the route of administration might have a substantial impact on the metabolic pattern of KET and consequently may affect its pharmacodynamics. In addition to drug metabolism, also membrane transporters, the expression pattern of which is markedly different in gut and liver, may contribute to the divergent pharmacokinetics that have been observed for KET after intravenous or oral administration. Moreover, one can further expect differences in the absorption of IR-KET and PR-KET due to the regio-selectivity (difference in expression) of the transporter protein abundance along the human intestine, especially for P-gp and OCT1/3[30]. These transporters are from the physicochemical point of view and from evidence provided by the literature expected to be involved in the transport of KET [31–33]. In this regard, P-gp was shown to be 3-fold higher expressed in the ileum than in both duodenum and colon. On the other hand, OCT3 is predominately expressed in the colon while the abundance of OCT1 is homogeneous along the length of the human intestine [34]. This feature of regio-selectivity is not only true for transporter proteins but was also described for metabolizing enzymes such as CYP3A4 [35]. Due to these anticipated changes in the pharmacokinetic profile of KET in dependence of its administered dosage form, the investigation of this aspect seems to be important but was not addressed yet.

From the physicochemical point of view, the ionization of KET depends on the medium. Thus, KET exists in the intestinal lumen (pH 6.5; log D = 2.65) mainly as a cationic moiety, where it is > 90% ionized. On the other hand, it is ~ 50% ionized in the systemic circulation (pH 7.4; log D = 3.12). Being ionized in the intestine, it is not plausible for oral KET to penetrate the intestinal membrane by passive diffusion rather it may need to be taken by transporters such as the organic cationic transporter (OCT1/3) and/or OCTP2B1 which are highly expressed in the intestine [36]. Additionally, cationic drugs like trospium, ranitidine and morphine that are taken up by OCT are effluxed back to gut lumen by apical P-gp. This might be also the case for KET [31–33]. Moreover, about 90% of IV-KET dose is expected to be eliminated via renal excretion, and this might be transporter dependent process. Renal transporters that are known to facilitate in tandem the urinary secretion of cationic compounds from through the renal tubule cells are OCT2 and MATE1/2k. Consequently, the in-

vestigation of the affinity of KET for these transporters is mandatory for better understanding of the difference in pharmacokinetics between these dosage forms as well as for predicting the probable drug-drug or drug-food interactions that might occur during KET oral absorption. Finally, in order to investigate the aforementioned hypothesis, it was the aim of this thesis to address the following objectives:

- Develop and validate sensitive and specific analytical methods for the quantification of KET and its metabolites (racemates and enantiomers) in serum, urine and feces.
- Evaluate the affinity of KET to drug transporter proteins in vitro.
- Perform and analyze a clinical study to evaluate the pharmacokinetic properties of KET and its metabolites (n-KET, DHNK, HK and HNK) in serum, feces and urine after intravenous and single-dose administration of a new prolonged-release tablet formulation of 10, 20, 40 and 80 mg KET (PR-KET).

3. Materials and methods

3.1. Chemicals

Table 1: Materials used and suppliers (manufacturers)

Material	Supplier
Isopropanol (LC–MS quality)	Carl Roth (Karlsruhe, Germany)
Acetonitrile (LC–MS quality)	Chromasolv®, Sigma–Aldrich, Taufkirchen, Germany
D4-Ketamine and D4-norketamine	Sigma–Aldrich (Taufkirchen, Germany)
Ketamine	Sigma-Aldrich (Taufkirchen, Germany)
Norketamine	Sigma-Aldrich (Taufkirchen, Germany)
Dehydronorketamine	Sigma-Aldrich (Taufkirchen, Germany)
2*R*,6*R*-HNK and 2*S*,6*S*-HNK	The National Center for Advancing Translational Sciences (Rockville, Maryland, USA)
Methyl *tert*-butyl ether	Merck (Darmstadt, Germany)
N-methyl-4-phenylpyridinium (MPP^+)	Sigma-Aldrich (Taufkirchen, Germany)
Bromosulfophthalein (BSP)	Sigma-Aldrich (Taufkirchen, Germany)
Tetraethylammonium (TEA)	Sigma-Aldrich (Taufkirchen, Germany)
Quinidine	Sigma-Aldrich (Taufkirchen, Germany)
Pyrimethamine	Sigma-Aldrich (Taufkirchen, Germany)
Verapamil	Sigma-Aldrich (Taufkirchen, Germany)
Rifampicin	Sigma-Aldrich (Taufkirchen, Germany)
Rhodamine-123 (Rh123)	Sigma-Aldrich (Taufkirchen, Germany)
Adenosinmonophosphate (AMP)	Sigma-Aldrich (Taufkirchen, Germany)
Adenosintriphosphate (ATP)	Sigma-Aldrich (Taufkirchen, Germany)
PSC-833	Novartis (Basel, Switzerland)
[3H]-BSP (10.2 Ci/mmol; 1 µCi/µl)	Hartmann Analytic (Braunschweig, Germany)
[3H]-TEA (55 Ci/mmol; 1 µCi/µl)	Hartmann Analytic (Braunschweig, Germany)
[3H]-MPP^+ (80 Ci/mmol; 1 µCi/µl)	Hartmann Analytic (Braunschweig, Germany)
[3H]-ketamine (80 Ci/mmol; 1 µCi/µl)	Hartmann Analytic (Braunschweig, Germany)

Deionized water (conductance: ≤0.055 µS/cm, pH 5.0–6.0) was generated with the Astacus® system (membrapure, Hennigsdorf, Germany). For the analytical methods, stock solutions were prepared in acetonitrile and stored at -20 °C. The working solutions were weekly fresh prepared by using a mixture of water and acetonitrile (50:50, v/v) to dilute the stock solutions and stored at 4 °C. All other chemicals were of analytical grade.

3.2. Analytical issues

3.2.1. Sample preparation

Samples kept at -80 °C were carefully thawed; 10 µl D4-ketamine and D4-norketamine solution as internal standard (final concentration 1 µg/ml) was added to 0.2 ml matrix, followed by the addition of 250 µL of sodium carbonate (1+1 dilution of a saturated solution). After mixing for 10 seconds, the samples were extracted by liquid-liquid extraction with 4 ml methyl *tert*-butyl ether under continuous shaking for 15 min at room temperature. After centrifugation for 5 min at 3.200 x g, the organic layer was separated and evaporated under a gentle nitrogen stream at 40 °C. The samples were finally reconstituted in 100 µl of an acetonitrile: water mixture (40:60, v/v) of which 15-35 µl were injected into the chromatographic system.

3.2.2. Separation and detection

The Agilent 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany) was coupled to the triple quadrupole mass spectrometer API4000 QTRAP via the patented electrospray ionization source Turbo V™; the LC-MS/MS system was controlled by the validated Analyst 1.6 software (AB Sciex, Darmstadt, Germany).

Achiral separation of KET and KET metabolites

Separation of racemic KET and its metabolites in serum, urine and fecal samples was done by isocratic elution using ammonium acetate buffer (5 mM; pH 3.8) / acetonitrile, 85:15, v/v) as mobile phase at a flow rate of 300 µl/min and the reverse phase column XTerra® MS (C18, 3.5 µm, 2.1 × 100 mm; Waters, Dublin, Ireland) temporized at 35 °C (injection volume: 15 µl) resulting in a chromatographic run time of 6 min.

Chiral separation of KET, n-KET and DHNK

The *R*- and *S*-enantiomers of KET, n-KET and DHNK were separated by gradient elution using ammonium acetate buffer (10 mM; pH 7.5) (A) and isopropanol (B) in the following manner: 0-13 min (97% A), 13.1-17 (80% A), 17.1-25 min (97% A) at a flow rate of 300 µl/min on the chiral column CHIRAL-AGP® (5 µm, 15 cm × 2 mm, Chiral Tech, West Chester, USA) temporized at 28 °C (injection volume: 20 µl). Using this elevated temperature increased the life span of the column substantially and performs better separation due to

decreased back pressure which in turn and increased the capability of the column to analyze more biological samples (up to 2000).

Chiral separation of 2*S*,6*S*- and 2*R*,6*R*-HNK

The HNK isomers were separated also by gradient elution with ammonium acetate buffer (5 mM; pH 9) (A) / isopropanol : acetonitrile (4:1) (B) as mobile phases in the following manner, 0-5 min (70% A), 45 min (60% A), 55 min (50% A), 55.1 – 60 min (70% A) at a flow rate of 350 µl/min using the chiral column Lux®-Amylose-2 (5 µm, 150 × 4.6 mm, Phenomenex, Aschaffenburg, Germany) temporized at 40 °C (injection volume: 35 µl).

The chromatographic flow was in each method directed to a 0.5 µm filter device (PEEK, Supelco, Taufkirchen, Germany) to avoid particulate contamination. The HPLC flow was connected to the mass spectrometer interface (Turbo V™ ionization source) operated in the positive ion mode. The following gas parameters have been used in all three methods: temperature, 400 °C; gas 1, 70 psi; gas 2, 40 psi (all nitrogen); voltage, 5500 V; collision-activated dissociation (CAD), 6 (arbitrary unit). The detailed mass spectrometry parameters are given in (Table 2). The Analyst 1.6 software was applied to evaluate the chromatograms using the internal standard method and peak-area ratios for calculation (quadratic regression, 1/x weighting).

3.2.3. Method validation

The validation of our method followed the recent EMA and FDA guidelines for bioanalytical method validation [37,38].

The method was investigated for selectivity in serum, urine and fecal homogenates by comparing six different batches of blank serum, urine and faecal samples containing KET, n-KET, DHNK, 2*S*,6*S*-HNK, 2*R*,6*R*-HNK and/or D4-KET, D4-n-KET.

The calibration curves (n=6) consisted in each case of a matrix sample with neither analyte nor internal standard representing the double blank, a matrix sample spiked with only the internal standard representing the blank and nine calibration values (0.1, 0.5, 1, 5, 10, 25, 50, 100, 250 ng/ml) in serum; nine calibration values (1, 5, 10, 25, 50, 100, 250, 500, 1000 ng/ml) in urine and feces for racemic KET, n-KET and DHNK; eight calibration values (0.5, 1,

5, 10, 25, 50, 100, 200 ng/ml) in serum; eight calibration values (1, 5, 10, 25, 50, 100, 250, 500 ng/ml) in urine for the enantiomers S-KET, *R*-KET, *S*-n-KET, *R*-n-KET, *S*-DHNK and *R*-DHNK. The calibration ranges for 2*S*,6*S*-HNK and 2*R*,6*R*-HNK were (1-200 ng/ml) in serum and urine.

Accuracy and precision were evaluated using quality control serum samples (QC) spiked with 0.25, 100 and 250 ng/ml racemic KET, n-KET and DHNK; 1.5, 100 and 200 ng/ml for *R*-/*S*-KET, *R*-/*S*-n-KET and *R*-/*S*-DHNK, while the quality control samples of racemic KET, n-KET and DHNK in urine and faeces contained 2.5, 500 and 1000 ng/ml and 2.5, 250 and 500 ng/ml in case of the enantiomers *R*-/ *S*-KET, *R*-/*S*-n-KET and *R*-/*S*-DHNK in urine but 3, 100 and 200 ng/ml for 2*S*,6*S*-HNK and 2*R*,6*R*-HNK in both, serum and urine. Inter-/between-day accuracy in terms of relative error and precision expressed as the respective coefficients of variation of the mean values was assessed by comparing the measured concentrations in six separately prepared QC sample sets measured on different days with the respective nominal concentrations. Intra-/within-day accuracy and precision was determined in the aforementioned manner but comparing data of six QC sample sets prepared and measured on one day.

To assess the recovery of the analytes from the biological samples, the following approach was used: blank matrix samples were spiked with appropriate amounts of analytes to reach the defined concentrations of QC samples (at each concentration and matrix six samples) and prepared and measured as described above; the resulting analytical signals (peak area) were compared to the analytical signals obtained from diluted stock solutions possessing the same analyte concentrations without any kind of extraction (100% values).

Matrix effects were studied by comparing the peak area ratios obtained from extracted blank serum samples which were afterwards spiked with the respective QC sample concentrations with the peak area ratios from samples obtained by diluting the stock solution (100% values).

Stability of KET, n-KET, DHNK and HNK in terms of bench-top, post-preparative and freeze-thaw stability in human serum samples was determined by using in each case six QC sample sets. Bench-top stability was tested after storing the samples at room temperature for 3 h

prior to extraction. Post-preparative stability was assessed by storing the prepared samples in the autosampler for 24 h at 4 °C. To assess freeze-and-thaw stability, the samples were thawed and frozen (-80 °C) up to three times prior to extraction. In each case, stability was assumed if the drug content after the given storage conditions was within the acceptable range of accuracy, i.e. ±15%.

3.2.4. Quality assurance within measurements

On each day of analysis, fresh calibration curves were prepared using the respective biological blank matrix as mentioned above. QC samples were prepared in the same matrix representing at least 10% of all analytical samples and were measured during the entire analytical run. To accept an analytical run at least 2 of 3 of all QC samples had to be within an accuracy range of ±15% of the nominal values as suggested by the FDA guideline [37, 38].

After the start of measurement of the clinical study, when the machine was out of order or more than 2/3 of the QCs were outside the accepted range mentioned before or some aberrant values were identified to attribute to processing errors, then all related samples were re-analysed (provided enough serum was still available).

When the initial measurement exceeded the highest calibration standard, a repeat analysis was mandatory after a suitable dilution with matrix, and the initial results were discarded from the concentration time list, but documented and stored (with comment) in the raw data list.

When a deviation from the theoretical level to be expected according to the individual concentration time profile was clear in a single sample, then this sample was re-checked through a repeat analysis (provided enough plasma was still available). The repeat analysis was in duplicate (provided enough material was available), and the median of all 3 measurements were reported. However, if only one re-analysis was possible, e.g. due to insufficient amount of sample, the following criteria had to be applied: If the second analysis was within ± 15% of the initial value, the first value was reported. While if the difference exceeded 30%, the result was not reportable. However, if the difference was between 15% and 30%, the respective mean value was reported.

3.3. Affinity of ketamine to drug transporter proteins

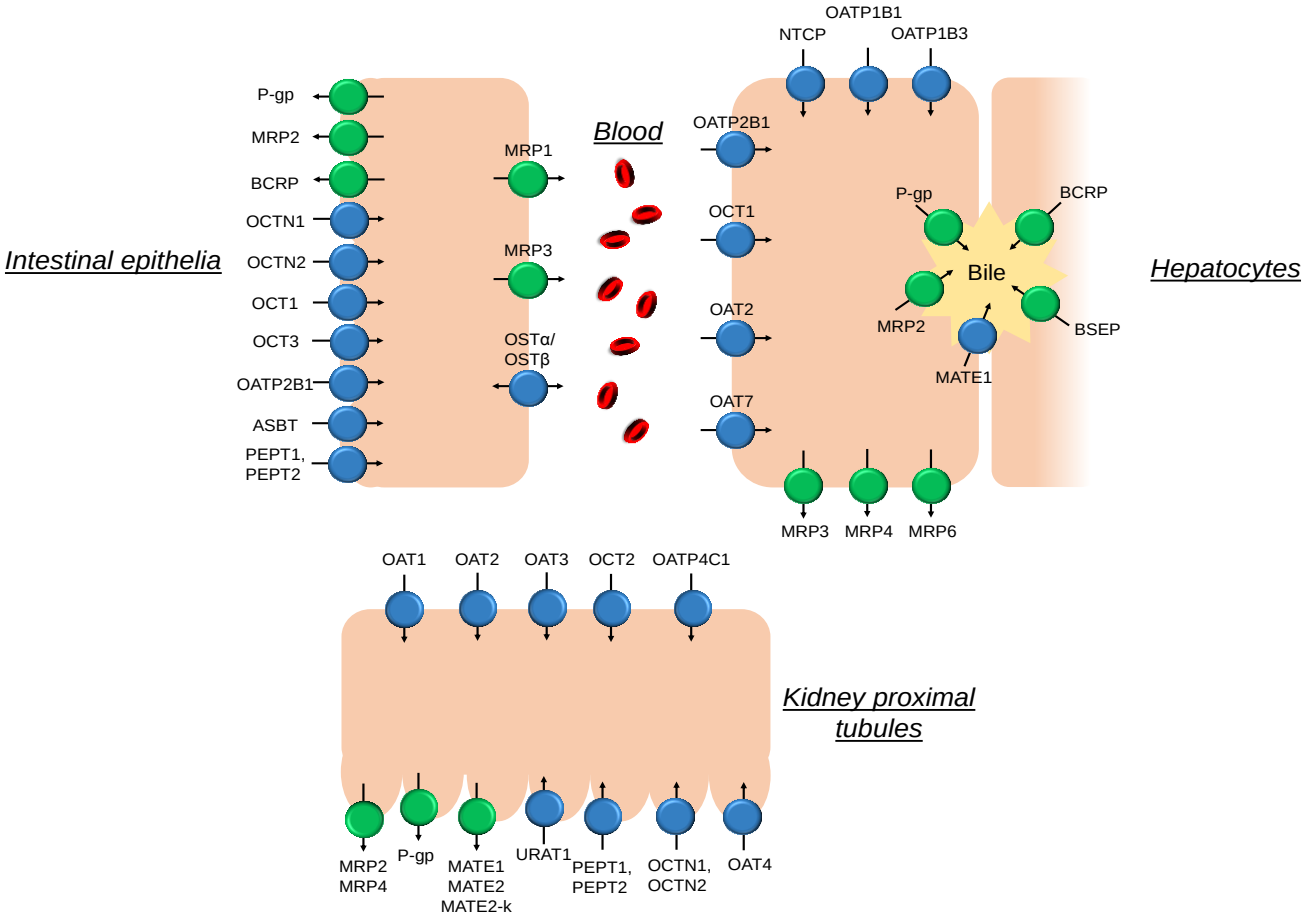


Figure 3: Diagram showing the distribution of body drug transporter; P-gp: P-glycoprotein; OCT1/3: organic cataionic transporter; OATP: organic anion-transporting polypeptide; MATE: multidrug and toxin extrusion protein; PEPT: peptide transporter; MRP: multidrug resistance associated protein; BCRP: breast cancer resistance; OCTN: organic cation/carnitine transporter; ASBT: apical sodium dependent bile salt transporter; OST: organic solute transporter; OAT: organic anion transporter; NTCP: Na+-taurocholate polypeptide; BSEP: bile salt exporting pump; URAT: urate anion exchanger

3.3.1. Cell culture

Cell lines

Stably transfected MDCKII or HEK293-cells overexpressing OATP2B1, OCT1, OCT2 and OCT3 and the respective vector-transfected control cells were generated as described previously [39–41]. MDCKII cells expressing MATE1 and MATE2k were purchased from Solvo Biotechnology (Szeged, Hungary).

In order to minimize the risk of contamination, all cell culture work was carried out under a sterile workbench, with the exception of the final tests. MDCKII were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, and 4 mmol L-glutamine. HEK293 cells were grown in minimal essential medium supplemented with 10% fetal bovine serum, 2 mmol L-glutamine, and 2 mmol nonessential amino acids, (PAN-Biotech, Aidenbach, Germany) at 37 °C, 95% humidity, and 5% CO2.

3.3.2. Cellular assays

MDCKII cells expressing OCT1, OCT2 or OCT3, cells were seeded in 24-well plates and incubated in full growth medium at an initial density of 100,000 cells/well for 2 days until cells reached a confluence of 90%. HEK293 cells expressing OATP2B1 were seeded in an initial density of 200,000 cells/well for 3 days. Experiments were performed as described previously [42]. Before each experiment, cells were washed once with 37 °C incubation buffer (142 mmol/l NaCl, 5 mmol/l KCl, 1 mmol/l K2HPO4, 1.2 mmol/l MgSO4, 1. mmol/l CaCl2, 5 mmol/l glucose, and 12.5 mmol/l HEPES; pH 6.5). After the respective experiment, cells were washed three times with ice-cold incubation buffer and lysed with 500 µl of room-temperature 0.5% Triton X-100 (Merck, Darmstadt, Germany) and 0.5% sodium deoxycholate (Sigma-Aldrich, Steinheim, Germany). One hundred microliters of cell suspension was mixed with 1 ml of scintillation cocktail (Rotiszintecoplus; Roth, Karlsruhe, Germany) and measured using liquid scintillation counting. Protein concentration was determined to quantify cell density after the experiments using the BCA assay according to the manufacturer's instructions (Pierce, Rockford, USA).

Time- and concentration-dependent assays

Uptake of KET was first measured in time dependent uptake assays for 10 – 300 seconds in OCT1, OCT2, OCT3, and OATP2B1 expressing cells or 600 seconds in MATE1 and MATE2k transfected cells using [^{3}H]-KET dissolved with unlabelled KET to reach a final concentration of 100 µmol/l. The Michaelis−Menten constant (K_m) and the maximal uptake rate (V_{max}) values for OCT1, OCT2, and OCT3 were calculated using 0−125 µmol of KET and an incubation time of 1 min. KET uptake rate was corrected to the respective transporter protein

expression, determined by using the ProteoExtract® Native Membrane Protein Extraction Kit (Merck, Darmstadt, Germany) according to the manufacturer's instructions and a validated LC-MS/MS method [43], the net intake in the concentration-dependent absorption assays was calculated by subtracting the amount of KET taken up in the control cell lines from the amount in the transfected cells.

Functionality assay

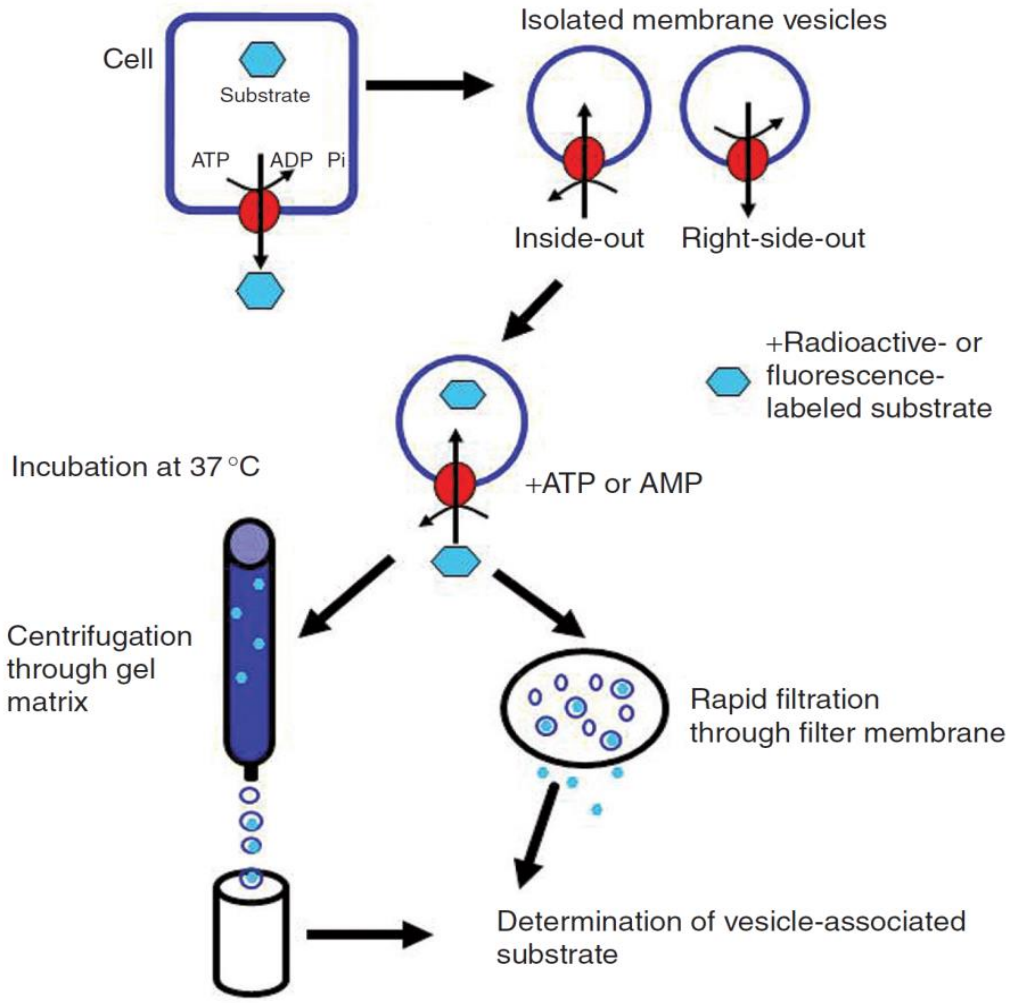
Functionality of OCT1, OCT2, and OCT3 cells were studied using [^{3}H]-MPP^+ dissolved with unlabelled MPP^+ to reach a final concentration of 10 µmol/l in presence or absence of 100 µmol/l verapamil.

Functionality of MATE1 and MATE2k expressing cells were verified using [^{3}H]-TEA dissolved with unlabelled TEA to reach a final concentration of 5 µmol/l in presence or absence of 100 µmol/l quinidine or 10 µmol/l pyrimethamine, respectively.

Functionality of OATP2B1 expressing cells was confirmed using [^{3}H]-BSP dissolved with unlabelled BSP in a final concentration of 1 µmol/l in presence or absence of 100 µmol/l rifampicin.

.

3.3.3. Lipovesicular assay



[44]

Figure 4: Lipovesicular assay for ketamine by P-gp, where cellular membrane is lysed, followed by uptake of the radiolabeled substrate by inside-out vesicles which are filtered and then the compound is detected by beta counter

In this work, commercially purchased vesicles from Sf9 cells with overexpressing effluent transporter (P-gp) were used. The principle of the experiments is an ATP-dependent transport of the substrate into the vesicles, which can then be quantified by a suitable method.

P-gp mediated transport of KET was investigated at pH 6.5 and 7.4 in inside-out lipovesicles purchased from Thermo Fisher Scientific (Darmstadt, Germany) as described previously [42]. The ATP-dependent transport 30 µg of total vesicle protein was incubated for 10 minutes at 37°C with [^{3}H]-KET, dissolved in tris-mannitol buffer (50 mmol/l tris, and 50 mmol/l mannitol, pH 6.5 or 7.4) with unlabelled KET to reach a final concentration of 10 µmol/l. Vesi-

cles were permeabilized with scintillation cocktail (Rotiszintecoplus; Roth, Karlsruhe, Germany) after rapid filtration using glass fiber filters (0.7 µM pore size; GE Healthcare, Freiburg, Germany), and uptake of KET in lipovesicles was measured by using a liquid scintillation beta counter (type 1409, LKB-Wallac, Turku, Finland). In control experiments, control vesicles (CV) which do not express P-gp were used or ATP was replaced by AMP.

Competition assay:

In the competition assay, P-gp vesicles were incubated with radiolabeled KET in presence and absence of 1 µmol/l of the known P-gp inhibitor PSC-833.

Functionality assay

The uptake of rhodamine (Rh123) in the presence or absence of the P-gp inhibitor PSC-833 (inhibition control) was therefore used to control the function of the vesicles in the presence of AMP (negative control) or ATP (positive control).

3.4. Clinical study in healthy subjects

3.4.1. Ethics and regulatory aspects

The study protocol complied with the requirements of the

- Declaration of Helsinki in its adopted version (Seoul, 2008),
- Note for Guidance on Good Clinical Practice (ICH Topic E6), CPMP/ICH/135/95, 1997,
- Note for Guidance on Structure and Content of Clinical Study Reports (ICH Topic E3), CPMP/ICH/137/95, 1996
- Note for Guidance on Statistical Principles for Clinical Trials (ICH Topic E9), CPMP/ICH/363//96, 1998
- § 2, 4, 10, 40, 41, 42, 84 Drug legislation (German Drug Act) BGBl I p. 2031,
- Regulation on the implementation of good clinical practice in the conduct of clinical trials on medicinal products for human use (GCP-Verordnung) BGBl I p.2081,
- Professional regulations for German physicians (105. Ärztetag, Rostock 2002),
- Standard Operating Procedures of the department of clinical pharmacology.

The quantitative determination of KET and metabolites as described above was done according to GLP-rules in our analytical laboratory with GLP-certificate (last inspection: 2014).

The study was initiated after approval by the Ethics Committee of the University Medicine Greifswald (identifier: MV01/14) and by the Federal Institute for Drugs and Medical Devices (BfArM). The study had been registered by the European Medicines Agency (identifier: EudraCT2014-000100-10) and www.ClinicalTrials.gov (identifier: NCT02494830).

3.4.2. Healthy subjects

The study was performed in 15 healthy German white subjects (10 males, 5 females; age 20-35 years; body mass index 19.4-27.6 kg/m^2). The subjects were enrolled after providing their written informed consent and after confirmation of good health by documenting each subject´s medical history, performing a physical examination and conducting routine clinical-chemical and hematological screenings. All subjects had negative results at time of screening for drugs, human immunodeficiency virus (HIV), hepatitis B virus and hepatitis C virus. Three subjects were smokers (<10 cigarettes/day) and two subjects did not drink any alcohol while thirteen subjects drank alcohol occasionally. None was on a special diet (e.g. vegetarian). The subjects did not take any medication with the exception of hormonal contraceptives (3 female subjects). The other females used an alternative, safe method of birth control; none had a positive pregnancy test at any screening time. Intake of grapefruit-containing food or beverages and poppy-seed containing products was not allowed from 14 days prior to, and during the study. Alcohol consumption was forbidden during the study.

3.4.3. Clinical study protocol

Design

The study was a single dose, open label, five-treatment, five-period, consecutive study with at least 7 days wash-out between the study periods. The subjects were hospitalized 12 h before, and up to 16 h after administration of the study medication.

The pharmacokinetics of KET and its metabolites were assessed under fasting conditions in the healthy subjects after oral administration of 10, 20, 40 and 80 mg ketamine hydrochloride in the prolonged release tablets (KET-PR) (DEVELCO PHARMA, Schopfheim, Germany) and after intravenous infusion of 5 mg ketamine hydrochloride (KET-IV) diluted in 240 ml 0.9 % saline within 30 min (Ratiopharm, Ulm, Germany).

KET-PR was administered using 240 ml tap water. Intake of beverages was allowed until 6 h before the planned administration of the study medication. After administration of the study medication, tap water was given up to 5 h afterwards standardized as described in the study protocol. Later on, the subjects were allowed to drink tap water as desired up to 2.5 l in 24 h. Intake of food and beverages were standardized during in-house confinement. The overnight fasting period before medications lasted 10 hours and the first meal after administration of the study medication was provided 5 h afterwards. Standard teatime and dinner were scheduled after 8 and 11 hours. The subjects had to eat the same individual amount of food in all study periods.

Blood sampling

Blood samples for pharmacokinetic analyses were collected from a cubital or forearm vein (after KET-IV from the contralateral arm) into 5.5 ml tubes (S-monovette® 5.5 ml, Z Sarstedt, Nümbrecht, Germany) by either an indwelling cannula or individual vein punctures. Blood was sampled before the beginning of the IV-KET- infusions and after 0.17, 0.33, 0.5 (end of infusion), 0.67, 0.83, 1, 1.5, 2, 3, 4, 6, 8, 12, 24 h. In case of PR-KET-, blood was collected before and 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6 ,7, 8, 12, 16, 24, 36, 48, 60 h after administration. After sampling and leaving the samples stand for at least 5 min, serum for quantification of KET and its metabolites was obtained by centrifugation at 2,000 x g at 4 °C (LABOFUGE-400 R, Heraeus Instruments, Hanau, Germany) for 10 min. The supernatant serum was transferred into two polypropylene tubes (Cryo.sTM, cryogenic vials, Greiner Bio-One, Frickenhausen, Germany); at least 1.0-1.5 ml into each one. Tubes were immediately placed on dry ice and afterwards into a freezer in upright position and were stored at least at - 80 °C until the quantitative drug assays of ketamine and its metabolites. The time span between blood sampling and freezing did not exceed 2 h.

Urine and fecal sampling

Urine was collected into 3 l plastic containers (Sarstedt, Nümbrecht, Germany) at room temperature. The volume per 24 h for up to 3 days was measured and two aliquots of about 5 ml were stored in two polypropylene tubes (Cryo.sTM, cryogenic vials, Greiner Bio-One, Frick-

enhausen, Germany) in upright position at a temperature of at least - 80 °C until the quantitative analysis. Feces were collected into plastic boxes (Vacumed, Tutow, Germany) for up to 5 days. A new box was used per defecation, which could be closed nearly odorless. Adequate aliquots were stored at least at - 80 °C until the quantitative drug assay.

3.4.4. Assessments of safety

Adverse events

Adverse events (AE) were detected both by a standardized questionnaire on tolerability and by querying the subjects at the scheduled times. Furthermore, the subjects were asked to report any AE spontaneously.

The intensity of an adverse event, the causal relationship of an adverse event with the investigational product(s) and outcome were classified as described in the respective standard operation procedures (SOPs) available in the clinical study unit of the department of clinical pharmacology and the study protocol of the study.

3.5. Biometrical evaluation

3.5.1. Quantitative evaluation of the in vitro transport

The OCT-mediated net uptake was obtained by subtracting the uptake in vector-transfected cells (control cell) from that in OCT1, OCT2, and OCT3-expressing cells. K_m and V_{max} were assessed using Prism 5.01 (GraphPad Software, San Diego, USA). The intrinsic clearance (Cl_{int}) was calculated by V_{max}/K_m. The same software was used for statistics.

3.5.2. Pharmacokinetic evaluation

Basic pharmacokinetic characteristics were evaluated by non-compartmental analysis. The maximum concentration (C_{max}) and the time of maximum concentration (T_{max}) were obtained directly from the measured concentration-time curves. The area under the concentrations-time curve (AUC) was calculated with the measured data points from the time of administration until the last quantifiable concentration (LLOQ) by the trapezoidal formula and extrapolated to T_∞ (AUC_∞) if concentration at T_{last} > lower limit of quantification (LLOQ). However, it is calculated only to 24 (i.v.) or 60 h (oral) if concentration at T_{last} < LLOQ ($AUC_{24\ or\ 60}$). Terminal

half-life ($T_{1/2}$) were calculated by $T_{1/2} = \ln 2/\lambda_z$. The terminal elimination rate constants (λ_z) were evaluated from the terminal slope by log-linear regression analysis.

Renal clearance (CL_R) after both routes of administration was derived from the cumulative excretion (A_e) of KET into urine over AUC of KET. Metabolic clearance (CL_M) was only assessed after intravenous administration of KET using the cumulative excretion of each metabolite, into urine and feces, respectively, over AUC of KET.

The absolute bioavailability (F) was calculated by $dose_{IV} / dose_{oral} \times AUC_{oral} / AUC_{IV}$. Volume of distribution and steady state (V_{ss}) was calculated as by dose × $AUMC/AUC^2$. The percentage of the metabolite (R_M) was calculated as follows $AUC_{metabolite} / AUC_{KET} + AUC_{metabolite} \times 100$

3.5.3. Statistical evaluation

Statistical evaluation was performed by accepted, model-independent appropriate methods for pharmacokinetic evaluation of serum concentration time curves of drugs and summary statistics.

Samples were presented as arithmetic means ± standard deviations (SD) and the non-parametric Wilcoxon test was used for statistical analyses as appropriate with $p \leq 0.05$ as the level of statistical significance using IBM SPSS, version 22,(IBM, New York,USA).

All subjects, who received at least one treatment, were included in the safety evaluation. Subjects who had completed the study according to the protocol (per-protocol population), were included into the statistical evaluation. Data of subjects, who had dropped out in one of the five periods or who had not been eligible for any reasons (missing or spurious results), were reported as far as available, but not included into descriptive statistics.

The statistical evaluation was performed according to the principles as described in the EMA-guidelines.

4. Results

4.1. Analytical issues

4.1.1. Separation and detection

In the positive ionization mode, the protonated molecule ($[M+H]^+$) for all compounds showed the highest analytical signals. Manual infusion was performed to identify the respective mass to charge transitions that were optimized in order to obtain maximum intensities (Table 2). MS/MS detection was performed in the positive multiple reaction monitoring (MRM) mode.

The deuterated form of both KET and n-KET (D4-KET and n-D4-KET) were used as internal standards as they have the benefit of possessing the identical chemical structure to KET and n-KET with distinct mass difference.

Racemic KET and n-KET were separated by isocratic elution with the reversed phase column resulting in short retention times of 3.45 min, 3.55 min and 3.85 min for DHNK, n-KET and KET, respectively (Figure 5). Thus, one analytical run could be finished after 6 minutes enabling a maximum sample throughput of about 200 samples per day.

S-KET was separated from *R*-KET in a stereoselective manner by gradient elution using CHIRAL-AGP column; *S*-KET appeared at 14.3 min while *R*-KET at 17.8; *S*-n-KET at 9.1 min while *R*-n-KET at 17.4 min; *S*-DHNK at 6.5 min while *R*-DHNK at 18.19 min (Figure 6). The analytical run was completed after 25 min.

Furthermore, 2S,6S-HNK and 2R,6R-HNK were successfully separated from each other and from the other isomers using Lux-Amylose-2 column, where 2S,6S-HNK was eluted at 10.5 min and 2R,6R-HNK at 11.93 min representing pair number 3 in (Figure 7) with more than one min of base line separation providing good selectivity. Moreover, the other pairs of HNK isomers appeared at 5.3, 8.6, 22.2, 36 and 39.9 min. However, due to the lack of reference compounds, we were not able to identify clearly the respective compounds.

Table 2: Mass spectrometry parameters for the detection of ketamine (KET), nor-ketamine (n-KET), D4-ketamine (D4-KET), D4-nor-ketamine (D4-n-KET), dehydronorketamine (DHNK), hydroxyketamine (HK) and hydroxynorketamine (HNK)

Compound	Q1 mass	Q3 mass	DP	EP	CE	CXP
KET	238.1	125.1	50	10	40	10
		163.0	50	10	31	10
		179.0	50	10	25	10
n-KET	224.1	125.1	50	10	35	10
		179.1	50	10	23	10
		207.1	50	10	17	10
D4-KET	242.2	129.3	50	10	35	10
		183.0	50	10	25	10
D4-n-KET	228.2	129.1	50	10	32	10
DHNK	221.9	204.8	40	10	25	10
		176.9	40	10	23	10
		141.9	40	10	35	10
HNK	240.5	125.0	60	10	40	10
		150.8	60	10	33	10
		177.0	60	10	25	10
		194.9	60	10	20	10
HK	254.0	125.0	60	10	45	10
		141.0	60	10	50	10
		151.0	60	10	33	10
		195.0	60	10	23	10

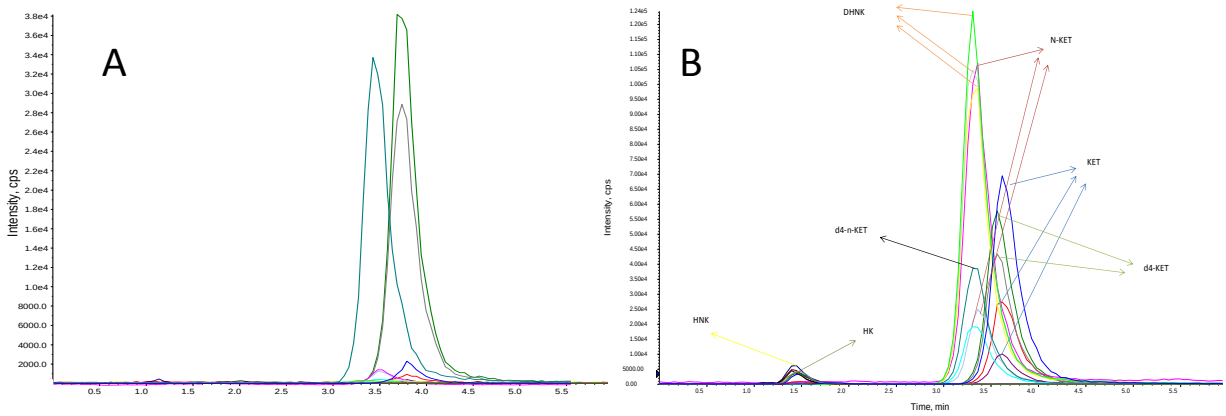


Figure 5: Total ion chromatograms of the achiral separation including a serum calibrator spiked with 0.25 ng/ml ketamine (KET), norketamine (n-KET) and dehydronorketamine (DHNK) (A), a urine sample of one volunteer from the clinical study (B)

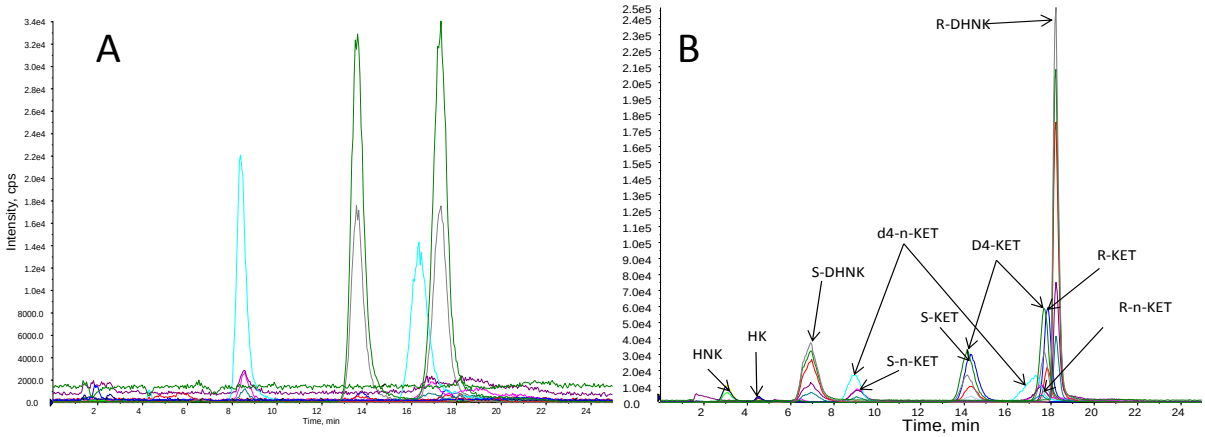


Figure 6: Total ion chromatograms of the chiral separation including a serum calibrator spiked with 1.5 ng/ml ketamine (KET), norketamine (n-KET) and dehydronorketemine (DHNK) (A) and a urine sample of one volunteer from the clinical study (B)

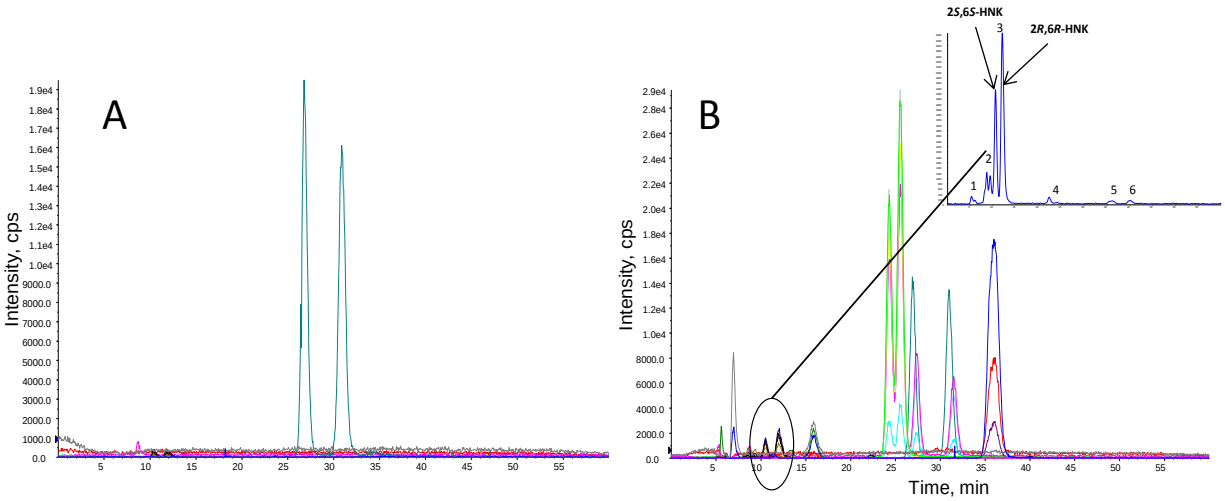


Figure 7: Total ion chromatograms of the enantioselective separation of the hydroxynorketamine diastereomers including serum calibrator spiked with 3 ng/ml 2S,6S- and 2R,6R-hydroxynorketamine mixture (HNK) (A) and a urine sample of one volunteer from the clinical study (B)

4.1.2. Method validation

We could demonstrate that the analytical methods were selective for the detection of KET and its metabolites in the investigated three matrices (urine, serum, and feces) as concluded from the absence of analytical signals in different batches of blank matrix and the lack of interferences between the analytes and the internal standards (Figure 5-7).

There was a quadratic relationship between the analyte concentration and the analytical signal for all methods in all matrices observed (each n=6, r =0.9983 or better), which was not due to the saturation of the mass spectrometry detector (analytical signals were markedly below its saturation level) but most likely caused by saturation of the extracting capacity of the extraction solvent. Consequently, the extraction yield was somewhat lower at the upper limit of quantification of the wide analytical range of 3 orders of magnitude. However, this quadratic relationship was reproducible in all biological matrices and enabled a reliable method validation.

Intra- / within-day as well as inter- / between-day accuracy and precision were in the accepted range as suggested by FDA / EMA guidelines on bioanalytical method validation (Tables 3, 4). Sample preparation was done by liquid-liquid extraction using methyl tert-butyl ether,

the recovery rates for the lower, medium and higher limit of quantifications were in the following ranges: KET, 54-60%; n-KET, 52-72%; DHNK, 51-61% and 64-67% for HNK.

No remarkable matrix effects could be assessed in serum, urine and feces as concluded from the absence of significant ion suppression or enhancement, i.e. signal intensity remained nearly unchanged compared to matrix free samples (Table 5). All compounds were found to be stable in serum at room temperature for at least 3 h; for at least 24 h when stored in the cooled autosampler at 4 °C as well as for three freeze–thaw cycles (Table 6).

Finally, our protocol was shown to fulfil the criteria of specificity, sensitivity, precision and accuracy in the quantification of KET and its metabolites in serum, urine and feces. We succeeded to increase the sensitivity by up to 10-fold higher than methods described previously using limited amount of matrix and introduce a new separation method for the HNK metabolites.

Table 3: Intra-day and inter-day accuracy and precision data for the validation range expressed as relative error (accuracy) or coefficients of variation (precision) of nominal and respective mean concentrations of ketamine (KET), nor-ketamine (n-KET) and dehydronorketamine (DHNK) in human serum, urine and feces as derived from six quality control sample sets prepared and measured one (intra-day) and different (inter-day) days

		Quality control	Concentration (ng/ml)	Precision (%)		Accuracy (%)	
				Intra-day	Inter-day	Intra-day	Inter-day
KET	serum	Q1	0.25	6.4	7.8	6.8	9.9
		Q2	100	5.2	4.9	-1.3	5.6
		Q3	250	0.9	6.3	-3.2	3.1
	urine	Q1	2.5	14.3	13.6	1.5	3.4
		Q2	500	10.0	7.9	6.8	0.4
		Q3	1000	4.3	13.3	-13.9	1.9
	feces	Q1	2.5	4.5	7.9	-2.6	5.6
		Q2	500	13.7	9.1	-9.0	-4.8
		Q3	1000	8.7	10.4	1.4	-1.0
n-KET	serum	Q1	0.25	6.3	3.1	9.7	6.7
		Q2	100	5.6	5.0	3.9	-3.6
		Q3	250	6.9	3.8	5.3	-3.9
	urine	Q1	2.5	10.3	7.1	-1.2	2.2
		Q2	500	0.4	6.6	-3.4	-2.0
		Q3	1000	7.9	9.0	-1.9	-1.2
	feces	Q1	2.5	6.6	6.2	0.5	8.2
		Q2	500	9.9	5.6	-6.8	-0.2
		Q3	1000	1.1	8.1	7.7	-3.2

Table 3 continued

DHNK	serum	Q1	0.25	4.2	8.0	-7.3	-2.5
		Q2	100	6.9	10.4	6.3	-12.7
		Q3	250	9.5	5.1	5.6	9.3
	urine	Q1	2.5	3.1	3.0	-13.0	-9.5
		Q2	500	14.5	7.4	-8.1	-2.7
		Q3	1000	6.9	12.2	-13.1	-7.7
	feces	Q1	2.5	2.0	12.0	-8.8	-4.8
		Q2	500	3.1	10.0	1.0	0.6
		Q3	1000	6.5	11.8	-4.6	-3.1

Table 4: Intra-day and inter-day accuracy and precision data for the validation range expressed as relative error (accuracy) or coefficients of variation (precision) of nominal and respective mean concentrations of S- and R-ketamine (S- and R-KET), S- and R-norketamine (S- and R-n-KET), S- and R-dehydronorketamine (S- and R-DHNK), and 2S,6S- and 2R,6R-hydroxynorketamine (2S,6S- and 2R,6R-HNK) in human serum and urine as derived from six quality control sample sets prepared and measured one (intra-day) and different (inter-day) days

		Quality control	**Concentration (ng/ml)**	**Precision (%)**		**Accuracy (%)**	
				Intra-day	**Inter-day**	**Intra-day**	**Inter-day**
S-KET	serum	Q1	1.5	6.3	14.2	0.6	7.8
		Q2	100	3.3	6.8	-1.2	-2.4
		Q3	200	5.6	6.6	-3.4	-1.4
	urine	Q1	2.5	12.7	8.9	-12.6	-5.6
		Q2	250	6.8	2.9	3.6	-4.6
		Q3	500	11.2	3.5	-7.2	-9.1
R- KET	serum	Q1	1.5	6.1	6.9	9.8	-3.0
		Q2	100	5.2	6.7	-3.6	-5.0
		Q3	200	6.2	9.0	-3.6	-5.9
	urine	Q1	2.5	4.4	9.5	6.1	4.3
		Q2	250	7.7	8.9	-4.3	-3.9
		Q3	500	9.9	3.2	-7.4	-9.4
S-n-KET	serum	Q1	1.5	5.6	6.7	6.3	-6.7
		Q2	100	4.3	3.9	9.5	4.2
		Q3	200	2.8	7.3	0.7	0.7
	urine	Q1	2.5	5.7	4.8	-7.2	-2.4
		Q2	250	5.2	5.1	0.7	1.8
		Q3	500	6.1	3.5	2.5	6.3

R-n-KET	serum	Q1	1.5	12.4	4.3	-3.1	-4.5
		Q2	100	4.6	5.8	8.5	6.4
		Q3	200	5.6	7.4	-5.3	0.7
	urine	Q1	2.5	7.0	6.5	10.7	5.3
		Q2	250	2.6	3.5	-0.3	1.3
		Q3	500	0.8	3.9	5.6	4.9
S-DHNK	serum	Q1	1.5	7.8	11.3	2.3	2.6
		Q2	100	10.2	9.1	3.7	10.7
		Q3	200	11.8	4.2	4.4	0.6
	urine	Q1	2.5	8.5	9.8	-8.1	-5.5
		Q2	250	4.7	4.2	2.9	2.5
		Q3	500	2.1	4.2	13.0	7.5
R-DHNK	serum	Q1	1.5	10.7	7.2	-6.1	1.1
		Q2	100	5.1	13.0	11.5	7.7
		Q3	200	9.2	10.6	-5.0	4.0
	urine	Q1	2.5	12.8	8.8	6.5	1.5
		Q2	250	6.1	7.0	-3.9	-1.8
		Q3	500	4.0	5.3	9.5	4.0
2S,6S-HNK	serum	Q1	3	13.7	14.0	0.1	-2.2
		Q2	100	4.8	8.4	1.4	-8.1
		Q3	200	13.1	14.3	14.9	5.1
	urine	Q1	3	11.2	9.5	14.3	11.9
		Q2	100	6.4	8.5	-0.8	-4.9
		Q3	200	10.3	14.8	-0.2	-8.2

Table 4 continued

2R,6R-HNK	serum	Q1	3	13.4	11.4	13.1	-6.3
		Q2	100	10.2	8.9	-2.1	-10.4
		Q3	200	9.8	13.1	12.1	2.9
	urine	Q1	3	18.7	10.4	3.6	6.0
		Q2	100	7.6	9.6	-0.1	-11.1
		Q3	200	12.7	4.6	3.9	-9.8

Table 5: Data on matrix effects of ketamine (KET), norketamine (n-KET) dehydronorketamine (DHNK) and hydroxynorketamine (HNK) as observed in quality control samples prepared in human serum, urine and feces. Data are given as mean from in percentage compared to diluted stock solutions

		Quality control	Concentration (ng/ml)	Matrix effects (%)
KET	serum	Q1	0.25	110.3
		Q2	100	103.4
		Q3	250	94.8
	urine	Q1	2.5	108.9
		Q2	500	99.8
		Q3	1000	96.3
	feces	Q1	2.5	87.7
		Q2	500	85.6
		Q3	1000	85.1
n-KET	serum	Q1	0.25	87.9
		Q2	100	101.7
		Q3	250	97.1
	urine	Q1	2.5	107.0
		Q2	500	93.7
		Q3	1000	89.5
	feces	Q1	2.5	85.0
		Q2	500	109.2
		Q3	1000	107.6
DHNK	serum	Q1	0.25	109.1
		Q2	100	94.5
		Q3	250	94.1
	urine	Q1	2.5	97.2
		Q2	500	94.8
		Q3	1000	96.8
	feces	Q1	2.5	102.4
		Q2	500	111.6
		Q3	1000	94.3
HNK	serum	Q1	3	93.5
		Q3	200	104.7
	urine	Q1	3	82.9
		Q3	200	89.1

Table 6: Data for short-term, rack and freeze-thaw stability of ketamine (KET), norketamine (n-KET), dehydronorketamine (DHNK) and hydroxynorketamine (HNK) as observed in quality control sample sets in human serum

	Quality control	Concentration (ng/ml)	Short-term stability (%)	Rack stability (%)	Freeze-thaw stability (%)		
					1st cycle	2nd cycle	3rd cycle
KET	Q1	0.25	100.3	99.5	112.6	101.9	98.5
	Q2	100	97.0	100.6	97.9	101.4	97.7
	Q3	250	93.7	100.3	105.5	100.7	102.8
n-KET	Q1	0.25	85.5	109.5	114.9	103.9	100.4
	Q2	100	102.9	103.7	107.0	104.7	100.9
	Q3	250	92.4	104.6	99.9	97.4	99.7
DHNK	Q1	0.25	103.6	99.6	99.5	107.5	100.6
	Q2	100	87.3	97.2	92.7	107.7	112.5
	Q3	250	91.9	97.2	94.7	99.3	92.2
HNK	Q1	3	93.8	112.6	91.4	101.3	96.9
	Q3	200	96.4	112.9	94.4	112.2	102.1

4.1.3. Quality assurance within study analysis

Inter-day accuracy in terms of relative error for: KET calibration function (n = 16) was in the range of - 1.5 to 2.8%, and that for quality controls (n = 52) was in the range of 0.1 to 6.2%; n-KET calibration function (n = 16) was in the range of - 1.8 to 2.9%, and that for quality controls (n = 52) was in the range of 2.4 to 8.7%. Accuracy for DHNK (n = 16) was in the range of - 4.4 to 6.7% and that for quality controls (n = 52) was in the range of -1.7 to 3.5%.

On the other hand, the inter-day precision in terms of relative standard deviation for KET calibration function (n = 16) was in the range of 0.4 to 10.0%, and that for quality controls (n = 52) was in the range of 6.5 to 11.5%; n-KET calibration function (n = 16) was in the range of 0.5 to 11.0%, and that for quality controls (n = 52) was in the range of 4.9 to 8.0%. DHNK calibration function (n=16) was in the range of 0.8-10.2%, and that for the quality controls (n=52) was in the range of 5.2 to 7.7%.

4.2. Affinity of ketamine to drug transporters

4.2.1. Uptake by OCT transporters and OATP2B1

KET was shown to be up-taken significantly in a time-dependent (100 µmol) (Figure 8-A) and concentration-dependent manner by OCT1-3 at pH 7.4 and pH 6.5 (Figure 9).

As expected from the physicochemical properties of KET, the affinity to the OCT transporters at pH 6.5 was several-fold higher than at pH 7.4. The respective K_m and V_{max} data of the cellular uptake were as follows: OCT1, 71.7 ± 31.1 µmol and 2.9 ± 0.64 µmol/mg x min; OCT2, 20.0 ± 21.1 µmol and 0.61 ± 0.22 µmol/mg x min; OCT3, 89.3 ± 49.1 µmol and 2.67 ± 0.75 µmol/mg x min, at pH = 6.5, respectively. The results observed at pH 7.4 were the following: OCT1, could not be calculated; OCT2, 61.7 ± 80.2 µmol and 1.42 ± 0.89 µmol/mg x min; OCT3, 146 ± 211 µmol and 1.82 ± 1.63 µmol/mg x min, respectively (Table 7).

OATP2B1 shows no relevance in KET uptake after incubation for up to 5 min (Figure 8-B).

The functionality of each cell line was verified by the established probe substrates, i.e. MPP (10 µmol) for OCTs, BSP (1 µmol) for OATP2B1 and by using the inhibitors verapamil (100 µmol) for OCTs, rifampicin (100 µmol) for OATP2B1 (Figure 10-A, 10-B).

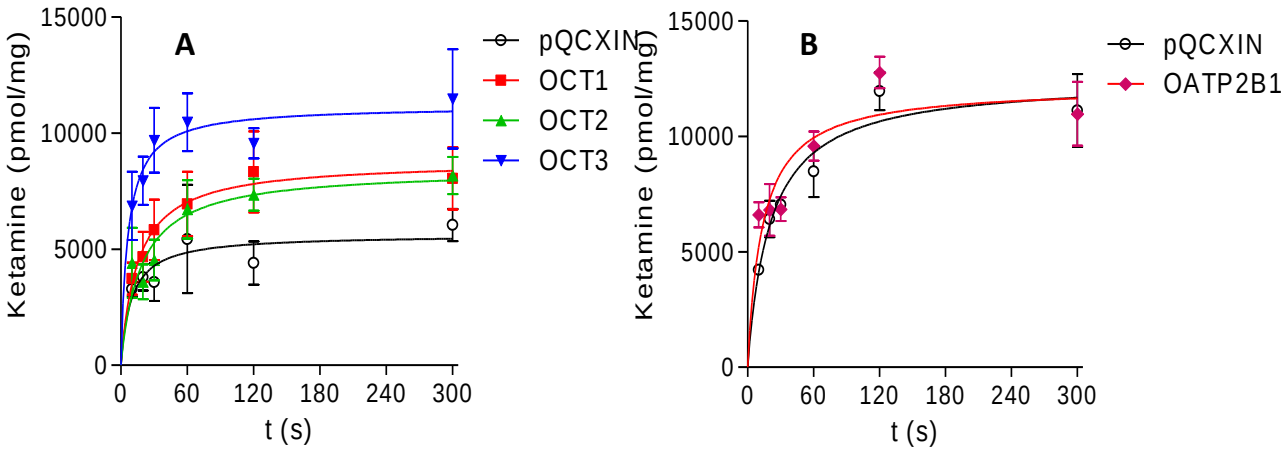


Figure 8: Time-dependent uptake of ketamine by transfected MDCKII cells stably expressing OCT1-3 (A) and HEK293 cells stably expressing OATP2B1 (B) or pQCXIN (control)

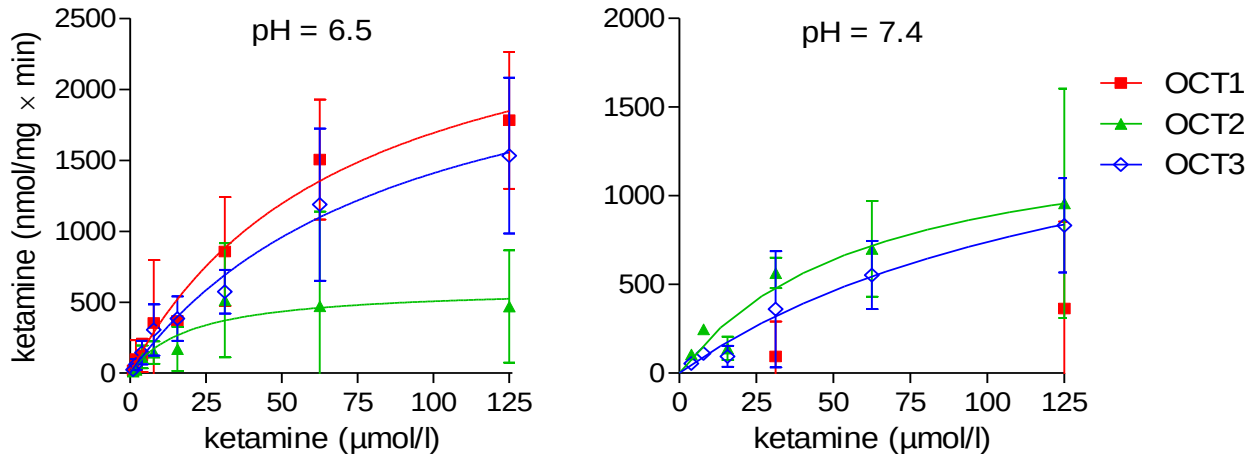


Figure 9: Concentration-dependent uptake curve for ketamine by OCT1-3 at pH 7.4 and pH 6.5

Table 7: Kinetic parameters for the transport of ketamine through MDCKII-cells transfected with human OCT1-3 at pH 7.4 and pH 6.5

	pH = 6.5			**pH = 7.4**		
	OCT1	**OCT2**	**OCT3**	**OCT1**	**OCT2**	**OCT3**
K_m (µmol/l)	71.7 ± 31.1	20.0 ± 21.1	89.3 ± 49.1	-	61.7 ± 80.2	146 ± 211
V_{max} (µmol/mg × min)	2.9 ± 0.64	0.61± 0.22	2.67 ± 0.75	-	1.42 ± 0.89	1.82 ± 1.63
Cl_{int} (ml/mg × min)	0.41 ± 0.20	0.30 ± 3.34	0.30 ± 0.18	-	0.31 ± 0.15	0.28 ± 0.23

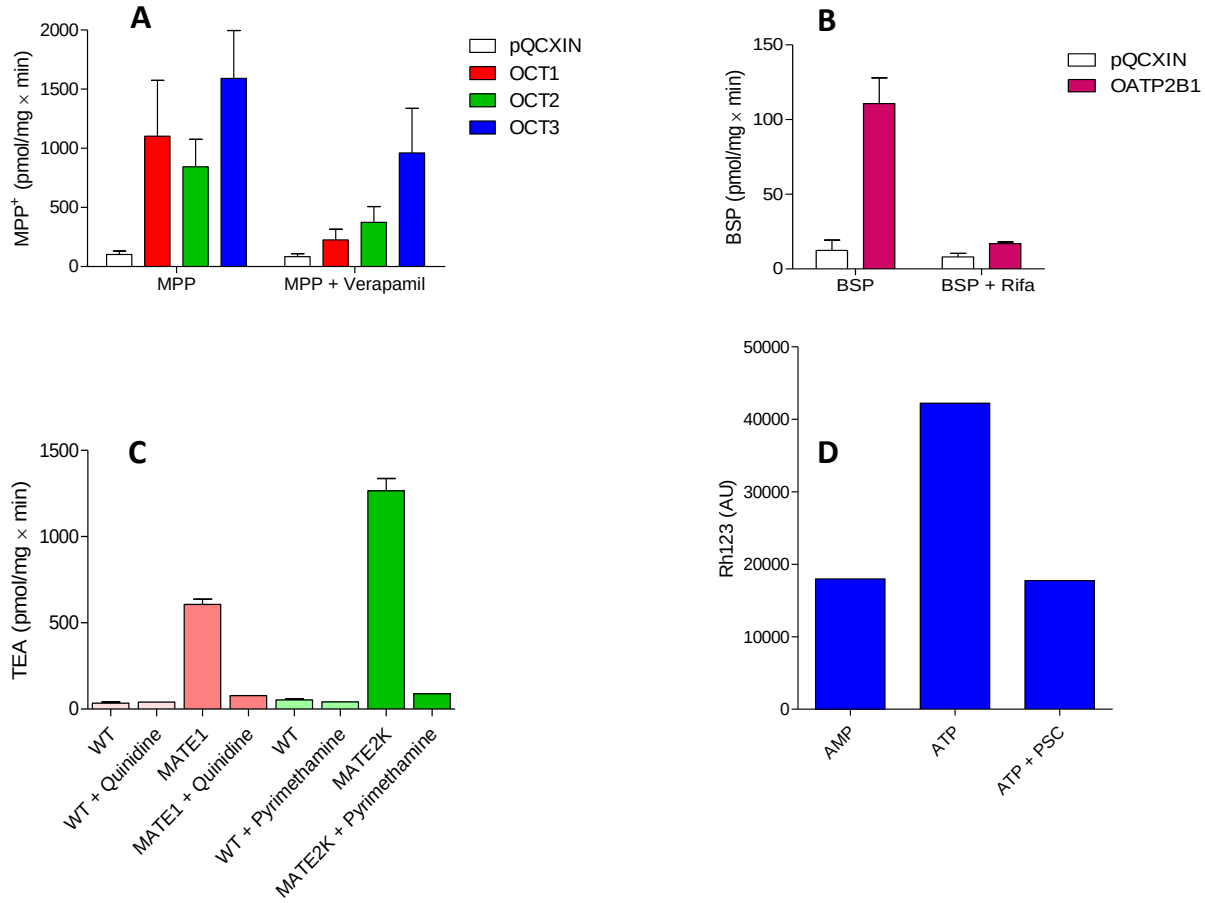


Figure 10: Functionality control for OCT1-3 transfected MDCKII cells (A), OATP2B1 transfected HEK293 cells (B), MATE1/2K transfected MDCKII cells (C) and P-gp-lipovesicles (D)

4.2.2. Efflux transport by P-glycoprotein

To study role of P-gp efflux, uptake of KET into P-gp-lipovesicles was studied at pH 6.5 and pH 7.4. Our experiments demonstrated that accumulation of KET (10 µmol) for up to 30 min at pH 7.4 in P-gp-lipovesicles was independent of ATP. On the other hand, at pH 6.5, it was found that KET has an affinity for P-gp and its accumulation in the vesicles was ATP-dependent (Figure 11). The uptake was decreased in presence of the P-gp inhibitor (PSC833), however, it had a low affinity as there was no saturation of the transporter for up to 2,000 µmol (Figure 12).

The functionally of the P-gp vesicle assays was verified using the P-gp probe substrate Rh123 and the inhibitor PSC833 (Figure 10-D).

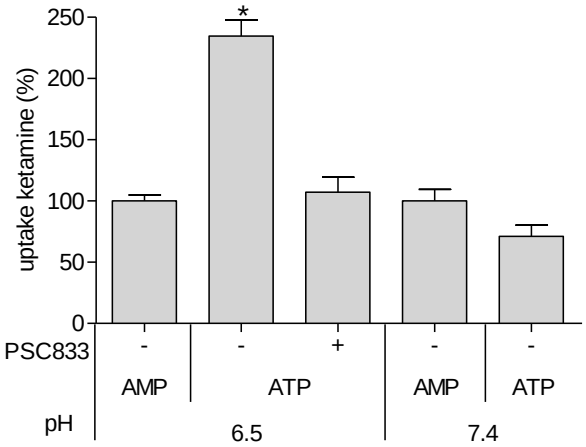


Figure 11: Accumulation of ketamine in P-gp-lipovesicles at pH 7.4 and pH 6.5, in presence and absence of the inhibitor PSC833 ($p<0.05$ (t-test); *vs. AMP)

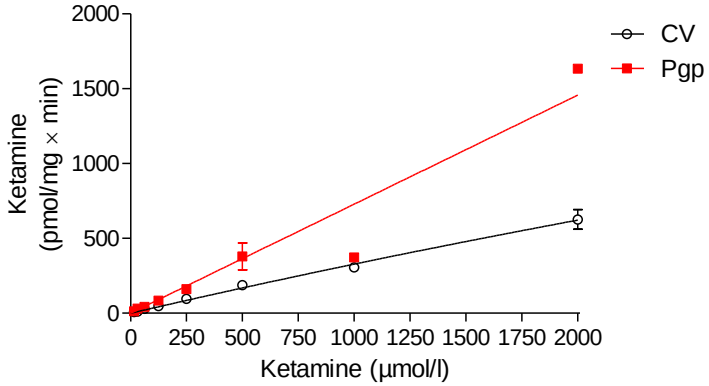


Figure 12: Concentration-dependent uptake of ketamine in P-gp-lipovesicles at pH 6.5

4.2.3. Transport by MATE1/2K

The relevance of MATE1/2K in the transport of KET was studied with MATE1/2K-transfected MDCKII-cells. Uptake screening experiments with up to 15 µmol KET did not show a significant difference between the control and transporter-transfected cells (Figure13).

The functionality of the cells was confirmed by using the probe compound TEA (5 µmol/l)) and the established inhibitors quinidine (100 µmol/l) for MATE1 and pyrimethamine (10 µmol/l) for MATE2K) (Figure10-C).

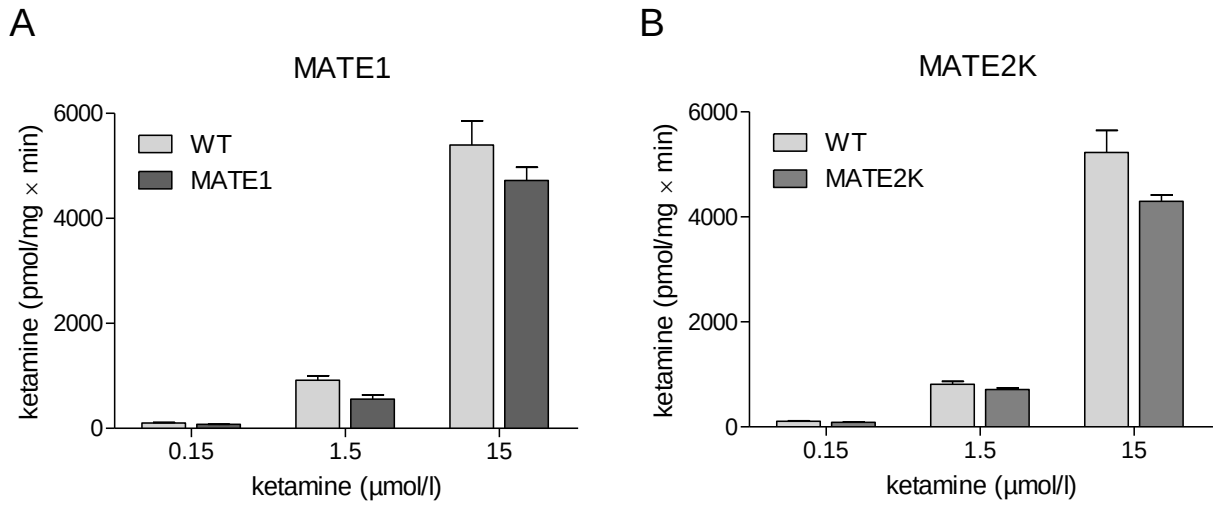


Figure 1: Uptake of ketamine in MATE1/MATE2K-transfected MDCKII-cells

4.3. Clinical study

4.3.1 Safety of ketamine

The number of adverse events (AE) occurred during the entire study was 26 AEs as follows; 10 AEs in treatment A, 7 AEs in treatment B, 1 AE in treatment C, 2 AEs in treatment D and 6 AEs in treatment E. 7 AEs were considered by the clinical investigators to be not or unlikely related. 4 AEs were considered to be possibly related to the study medication and 11 to be probably related. Dizziness (8 AEs), representing 40% treatment A and 14% treatment B; headache (4 AEs), representing 7% of all treatment; nausea, heaviness of head & sweating was recorded in only 7% of treatment B; tachycardia, in 7% of treatment A; weakness in 7% treatment B and palpitations (3 AEs) representing 7% of treatment A,B & E belonged to the

most frequent adverse events**.** No serious adverse events (SAE) and suspected unexpected serious adverse drug reactions (SUSAR) occurred. Study medication did not change systolic and diastolic blood pressure, heart rate, breathing rate and oxygen saturation in a clinically relevant manner from baseline.

4.3.2. Pharmacokinetics of racemic ketamine

Pharmacokinetics after intravenous infusion:

KET after intravenous infusion was widely distributed with V_{ss} of 7 l/kg. It was bi-exponentially eliminated with apparent terminal elimination half-life 5 h and renal clearance of 33 ml/min, where its dose was excreted as follows: 2% of the dose was excreted unchanged into the urine, 4% as HNK, 10% as DHNK, 2% as n-KET while the elimination via feces was negligibly low. Therefore, intestinal clearance could not be assessed for KET (Table 8).

The metabolic-ratios were as follows, $AUC_{n\text{-}KET/KET}$ 2; for $AUC_{DHNK/KET}$ 0.5; for $AUC_{HNK/KET}$ 0.3.

Pharmacokinetics of KET prolonged release tablets:

All pharmacokinetic characteristics of KET after oral administration as PR tablets were found to be highly variable.

After administration of KET-PR tablets, the absolute oral bioavailability of (±)-KET was fairly low (15%).There was also a bi-exponential elimination pattern of KET in serum visible after oral administration. The apparent terminal elimination half-life was 7 h and renal clearance of 70 ml/min, where 0.5% of the KET dose was excreted unchanged into the urine and 2% in the form of n-KET; 10% as DHNK and 3% as HNK. On the other hand, 1.5% was excreted unchanged in feces and 0.01% as n-KET (Table 8).

The metabolic-ratios of all metabolites over KET were markedly increased compared to intravenous ratios. Both $AUC_{n\text{-}KET\ /\ KET}$ and $AUC_{DHNK\ /\ KET}$ ratios decreases with increasing the dose from 10-80 mg. On the other hand, $AUC_{DHNK\ /\ n\text{-}KET}$ and $AUC_{HNK\ /\ n\text{-}KET}$ ratios were nearly constant with increasing doses (Figure 14). Moreover, it was clear that after oral administration the major circulating metabolite in plasma is n-KET, followed by HNK, DHNK and finally HK (Figure 15).

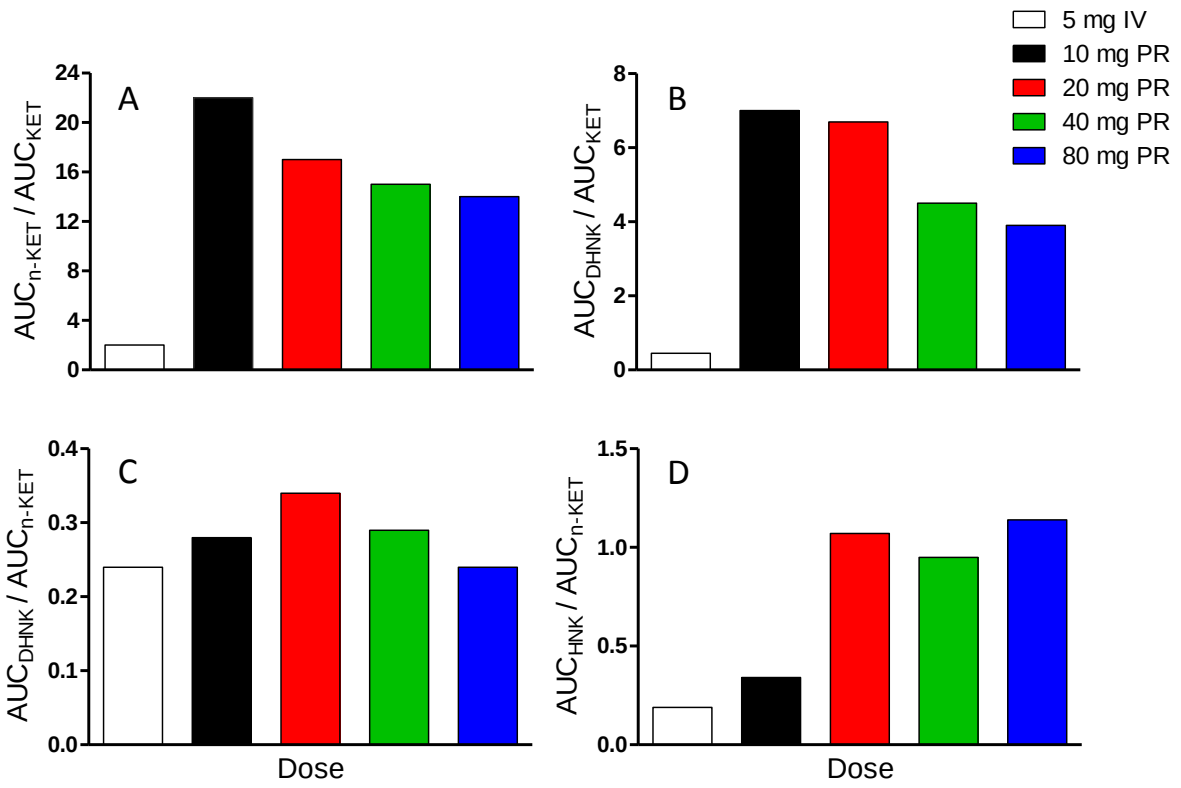


Figure 14: Diagram showing the difference between different doses in metabolic ratios: $AUC_{norketamine/ketamine}$ (AUC_{n-KET}/AUC_{KET}) (A), $AUC_{dehydronorketamine/ketamine}$ (AUC_{DHNK}/AUC_{KET}) (B), $AUC_{dehydronorketamine/norketamine}$ (AUC_{DHNK}/AUC_{n-KET}) (C) and $AUC_{hydroxynorketamine/norketamine}$ (AUC_{HNK}/AUC_{n-KET}) (D)

Table 8: Racemic pharmacokinetic characteristics after intravenous infusion (30 min) of 5 mg ketamine and oral administration of 10, 20, 40 and 80 mg ketamine prolonged release tablets (PR-KET)

		5 mg i.v.	10 mg p.o.	20 mg p.o.	40 mg p.o.	80 mg p.o.
AUC_{KET} [1]	ng×h/ml	52.3 ± 12.2	13.4 ± 13.3	33.5 ± 35.8	63.5 ± 42.6	124 ± 72.9
$AUC_{n\text{-}Ket}$/ AUC_{Ket}		1.79 ± 0.557	21.6 ± 14.2$^{\#}$	16.9 ± 8.58$^{\#}$*	14.9 ± 8.50$^{\#}$*	14.0 ± 6.50$^{\#}$*†
AUC_{HNK}/ AUC_{Ket}		0.334 ± 0.128	7.33 ± 7.05$^{\#}$	19.8 ± 16.4$^{\#}$*	14.4 ± 8.99$^{\#}$*	16.2 ± 7.93$^{\#}$*‡
AUC_{DHNK}/ AUC_{Ket}		0.446 ± 0.157	7.00 ± 6.19$^{\#}$	6.65 ± 5.52$^{\#}$	4.46 ± 3.19$^{\#}$*†	3.68 ± 2.11$^{\#}$*†
AUC_{DHNK}/ $AUC_{n\text{-}KET}$		0.24	0.28	0.34	0.29	0.24
AUC_{HNK}/ $AUC_{n\text{-}KET}$		0.19	0.34	1.07	0.95	1.14
C_{max}	ng/ml	29.9 ± 8.48	1.63 ± 1.33	3.70 ± 3.93	6.66 ± 4.25	11.8 ± 6.56
T_{max}	h	-	5.34 ± 1.18	5.70 ± 0.649	5.87 ± 0.915	6.27 ± 0.594*†
F	%	-	12.3 ± 10.7	15.3 ± 14.4*	14.9 ± 8.94*	14.6 ± 7.56*
V_{ss}		6.58 ± 3.07	-	-	-	-
$T_{½}$	h	5.89 ± 2.61	4.96 ± 1.25	6.74 ± 2.02*	7.21 ± 1.56$^{\#}$*	7.68 ± 1.43$^{\#}$*†
CL_R	ml/min	33.6 ± 36.5	78.9 ± 32.2$^{\#}$	70.2 ± 30.3$^{\#}$*	67.3 ± 28.0$^{\#}$	61.7 ± 23.3$^{\#}$*
CL_M	ml/min	268 ± 75.6	-	-	-	-
$CL_{M,NorKet}$	ml/min	34.6 ± 14.4	-	-	-	-
$CL_{M,HNK}$	ml/min	45.5 ± 8.65	-	-	-	-
$CL_{M,DHNK}$	ml/min	188 ± 68.1	-	-	-	-
$A_{e,urineKET}$	%	1.87 ± 1.43	0.448 ± 0.163$^{\#}$	0.483 ± 0.196$^{\#}$	0.524± 0.230$^{\#}$*	0.493 ± 0.208$^{\#}$
$A_{e,urine\ n\text{-}KET}$	%	1.87 ± 0.477	1.73 ± 0.364	1.76 ± 0.599	1.66 ± 0.454	1.60 ± 0.511$^{\#\dagger}$
$A_{e,urine,DNK}$	%	10.3 ± 2.20	10.4 ± 2.68	11.0 ± 2.86	9.08 ± 2.17†	9.09 ± 1.95$^{\#\dagger}$
$A_{e,urine,HNK}$	%	2.90 ± 0.916	2.65 ± 0.701	2.70 ± 1.01	2.74 ± 1.23	2.84 ± 1.19
$A_{e,fecesKET}$	%	0.003 ± 0.007	1.31 ± 1.18#	1.68 ± 2.01#	1.39 ± 1.16#	2.56 ± 2.21#*‡
$A_{e,feces,n\text{-}KET}$	%	0.043 ± 0.039	0.050 ± 0.040	0.048 ± 0.028	0.048 ± 0.045	0.049 ± 0.027

$p<0.05$ (Wilcoxontest); $^{\#}$vs. 5 mg i.v., *vs. 10 mg, †vs 20 mg, ‡vs. 40 mg p.o. [1]if T_{last} = 60 h (24 h for 5 mg iv) then $AUC_{0\text{-}\infty}$, else AUC0-60h (24h)

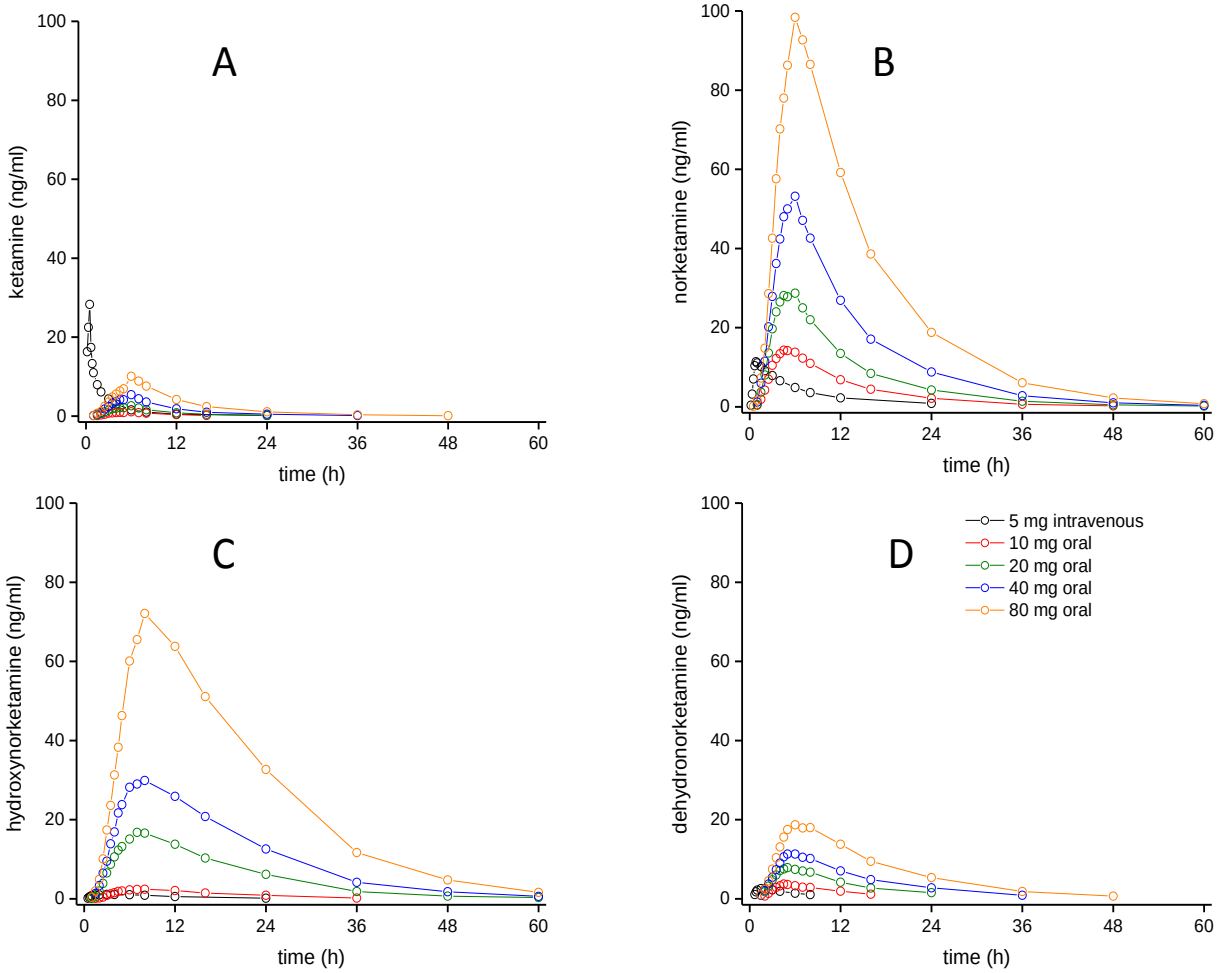


Figure 2: Geometric mean curves after administration of ketamine for: ketamine (A), norketamine (B), hydroxynorketamine (C) and dehydronorketamine (D)

4.3.3. Pharmacokinetics of ketamine enantiomers

Despite that, the AUC and C_{max} for *S*-KET are mostly lower than *R*-KET. However, there was an initial unexpected increase in *S*-n-KET AUC and C_{max} after 5 mg intravenous and 10 mg oral dose followed by the expected increase in *R*-n-KET values after 20, 40 and 80 mg oral dose (Table 9).On the other hand, both the AUC and C_{max} of *R*-DHNK are markedly higher than *S*-KET (Figure 16-C).The percentage of n-KET formation increased from ~ 60% (for intravenous doses) to ~ 90% (for oral doses) while that for DHNK increased from ~ 25% to 75% (Table 9).

Other pharmacokinetic parameters are very close to the racemic data with no significant differences between *R* and *S* forms.

Table 9: Chiral pharmacokinetic parameters of *S*- and *R*-ketamine, *S*- and *R*-norketamine and *S*- and *R*-dehydronorketamine after administration of 5 mg Ketamine intravenous and 10, 20, 40 and 80 mg ketamine prolonged release tablets (PR-KET)

		5 mg i.v.		10 mg p.o.		20 mg p.o.		40 mg p.o.		80 mg p.o.	
		S	R	S	R	S	R	S	R	S	R
AUC_{KET}	ng×h/ml	27.0 ± 5.70	28.5 ± 5.98	6.13 ± 3.74	7.67 ± 5.32	12.9 ± 9.88	18.3 ± 13.0	32.9 ± 18.8	42.3 ± 24.6	58.3 ± 31.6	72.1 ± 38.8
$AUC_{n\text{-}KET}$		44.0 ± 12.3	40.6 ± 12.2	82.5 ± 22.0	79.9 ± 24.2	176 ± 82.7	192 ± 89.4	303 ± 79.8	322 ± 83.4	581 ± 187	626 ± 216
AUC_{DHNK}		7.46 ± 2.68	11.4 ± 3.57	15.8 ± 6.71	28.5 ± 9.76	39.7 ± 14.1	72.9 ± 32.5	71.2 ± 9.55	100 ± 12.8	122 ± 22.9	199 ± 41.6
$R_{M,n\text{-}KET}$	%	61.3 ± 8.36	58.1 ± 9.05	93.4 ± 3.12$^{\#}$	91.3 ± 4.34$^{\#}$	93.1 ± 4.50$^{\#}$	91.0 ± 5.41$^{\#}$	90.8 ± 3.51$^{\#*\dagger}$	89.0 ± 4.21$^{\#*\dagger}$	91.2 ± 3.16$^{\#*\dagger}$	90.0 ± 3.41$^{\#\ddagger}$
$R_{M,DHNK}$	%	21.8 ± 7.33	28.7 ± 8.64	71.0 ± 17.8$^{\#}$	78.9 ± 12.5$^{\#}$	75.4 ± 15.5$^{\#}$	79.3 ± 12.6$^{\#}$	70.2 ± 12.6$^{\#}$	72.1 ± 11.3$^{\#*\dagger}$	69.1 ± 11.7$^{\#\dagger}$	74.5 ± 9.58$^{\#*}$
F	%	-	-	11.6 ± 7.08	13.5 ± 9.25	11.9 ± 8.65	16.1 ± 10.9*	15.4 ± 8.18*	18.5 ± 10.1*	13.5 ± 6.62	15.8 ± 7.76‡
V_{ss}		6.56 ± 2.18	5.64 ± 2.09	-	-	-	-	-	-	-	-
C_{max}	ng/ml	15.6 ± 4.51	16.5 ± 4.92	0.78 ± 0.434	0.95 ± 0.57	1.58 ± 1.12	1.99 ± 1.33	3.38 ± 2.00	4.19 ± 2.54	5.72 ± 3.11	6.95 ± 3.67
T_{max}	h	-	-	4.93 ± 1.32	5.14 ± 1.12	5.57 ± 0.78	5.57 ± 0.75	5.80 ± 0.94	5.87 ± 0.92*	6.10 ± 0.71*	6.13 ± 0.35*†
$T_{½}$	h	6.10 ± 3.09	5.22 ± 3.39	5.84 ± 3.18	6.02 ± 2.89	5.70 ± 2.21	7.61 ± 2.21	10.0 ± 3.90$^{\#*\dagger}$	11.1 ± 4.29$^{\#*\dagger}$	8.71 ± 2.10$^{\#*\dagger}$	10.3 ± 2.91$^{\#*\dagger}$
CL_R	l/min	0.03 ± 0.04	0.03 ± 0.03	0.07 ± 0.02$^{\#}$	0.07 ± 0.02$^{\#}$	0.08 ± 0.03$^{\#}$	0.06 ± 0.03$^{\#}$	0.06 ± 0.02$^{\#*}$	0.05 ± 0.02*	0.06 ± 0.02$^{\#*\dagger}$	0.06 ± 0.02$^{\#*}$
CL_M	l/min	0.21 ± 0.08	0.22 ± 0.09	-	-	-	-	-	-	-	-
$A_{e,urine,KET}$	mg	0.05 ± 0.04	0.05 ± 0.04	0.02 ± 0.01	0.03 ± 0.01	0.05 ± 0.02	0.06 ± 0.02	0.10 ± 0.05	0.12 ± 0.06	0.18 ± 0.07	0.22 ± 0.08
$A_{e,urine,n\text{-}KET}$	mg	0.23 ± 0.05	0.28 ± 0.06	0.48± 0.14	0.56 ± 0.13	1.06 ± 0.29	1.15 ± 0.29	1.56 ± 0.36	2.07 ± 0.54	3.25 ± 0.84	4.03 ± 0.78
$A_{e,urine,DHNK}$	mg	0.06 ± 0.02	0.05 ± 0.02	0.11 ± 0.02	0.09 ± 0.02	0.23 ± 0.08	0.19 ± 0.07	0.41 ± 0.11	0.35 ± 0.11	0.80 ± 0.24	0.71 ± 0.24

$p<0.05$ (Wilcoxontest); *vs. 10 mg, †vs 20 mg, ‡vs. 40 mg, $^{\#}$vs. 5 mg i.v.; bold = S vs. R

[1]if T_{last} = 60 h (24 h for 5 mg iv) then $AUC_{0\text{-}\infty}$, else $AUC_{0\text{-}60h\ (24h)}$

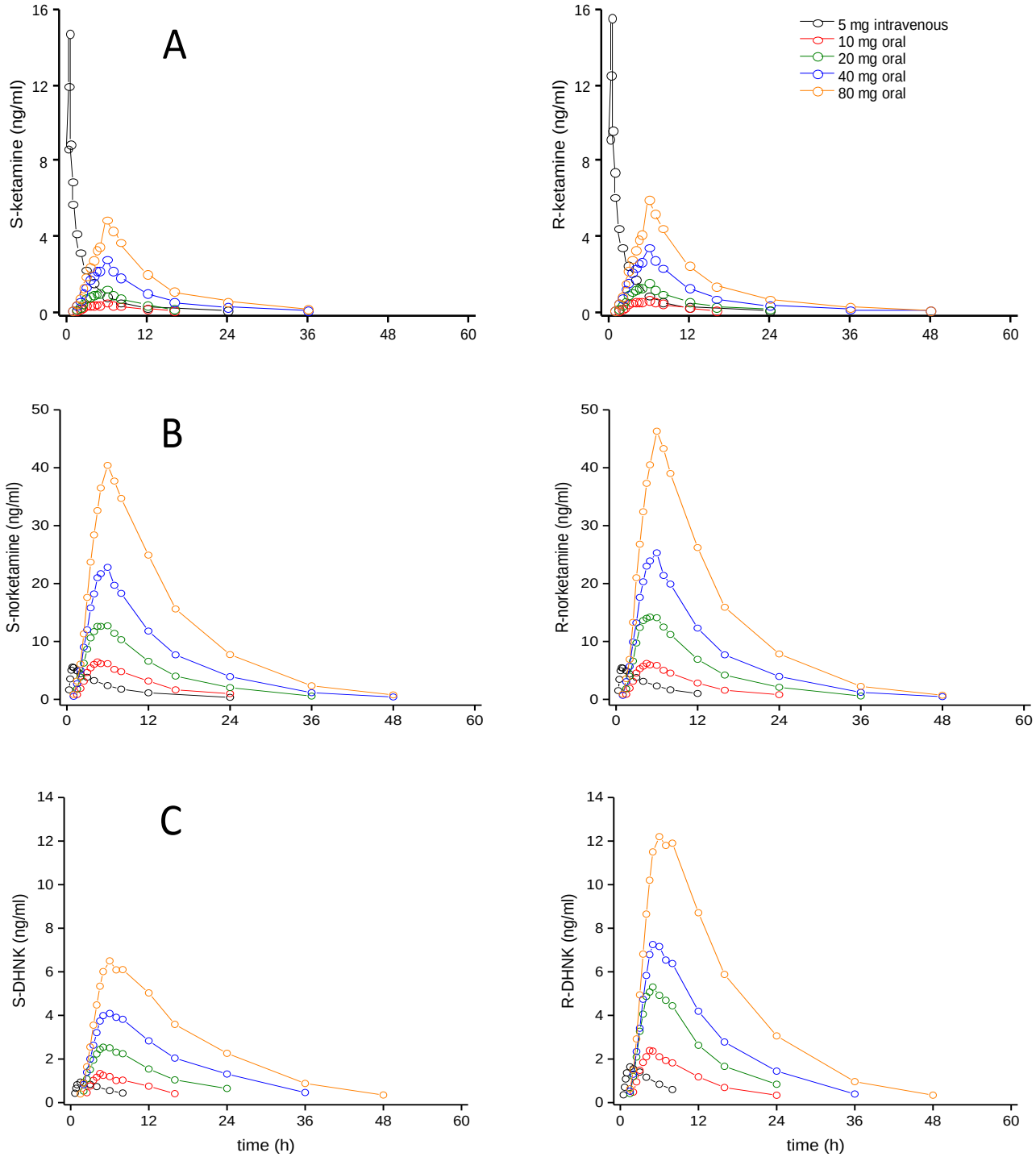


Figure 3: Geometric mean curves of *S* and *R* -ketamine (A), *S* and *R* -norketamine (B) and *S* and *R* -dehydronorketamine (DHNK) (C) after administration of ketamine

5. Discussion

5.1. Analytical issues

Within this PhD thesis, we developed and validated three LC-MS/MS methods to quantify KET and its metabolites in human serum, urine and feces. The first method enabled the quantification of racemic KET, n-KET and DHNK while the stereoselective determination of *S*-and *R*-KET; *S*-and *R*-n-KET; *S*-and *R*-DHNK was done by the second method in human serum and urine. Finally, we introduced an assay for the quantitative determination of the pharmacologically active isomers *2R,6R-HNK* and *2S,6S-HNK* (third method) in human serum and urine.

In so far published assays, the quantification of KET and n-KET in urine and plasma was done using HPLC-UV, gas chromatography or gas chromatography coupled with MS and capillary electrophoresis and reached maximum sensitivity of 10-50 ng/ml [45–59]. After application of LC-MS methods, the sensitivity became markedly better and reached LOQ of up to 1 ng/ml. However, the assays had to use a sample volume of 500 µl [60], while reducing the sample volume to 100 µl was associated with a loss of sensitivity (i.e. LOQ 5 ng/ml) [61].

In our protocol, we suggest an easy, fast and economic sample preparation from the three complex human matrices (serum, urine and feces) via liquid-liquid extraction with methyl *tert*-butyl ether after addition of 250 µl sodium carbonate to keep the pH alkaline for liberation of the free bases of KET and its metabolites. This new protocol enabled us to increase the sensitivity by up to 10-fold compared to previously published methods which is compulsory for our pharmacokinetic study in humans as we administrated low dose (5 mg KET) using only 200 µl matrix which is a compromise between sample volume and sensitivity.

This favourable feature of our method was most likely due to the following aspects: a) sufficient sample clean-up by the aforementioned liquid-liquid extraction, b) alkalization of the matrices in order to liberate the free bases, c) the use of deuterium-labelled internal standards and d) the appropriate chromatographic separation from the complex biological matrices (> 3 min).

Because *S*-KET is known to be the more potent pharmacological moiety of the drug and that the metabolism of KET appears to be stereoselective. Several chiral separation methods for KET have been published starting with HPLC-UV detection with LOQ = 40 ng/ml [62] and ending up with nowadays established LC-MS/MS methods with maximum sensitivity of 1-50 ng/ml which is at least 2-fold less sensitive than our method. In our method we applied a gradient elution and used a lower flow rate (300 µL) compared to previous publications (400-500 µL)[63,64]. Moreover, we succeeded in the chiral methods to have lower back pressure (i.e. long lifespan for the column) and better chromatographic separation between each enantiomeric pair (baseline separation of 1- 3 min difference in retention time) which appears to be superior in terms of peak integration and quantitative analysis.

It seems that the higher sensitivity as provided by our method is an essential prerequisite in order to characterize the serum concentration-time profiles especially for the chiral compounds in a sufficient manner.

Finally, the developed methods enabled the LC-MS/MS based sensitive and specific quantification of KET and its major metabolites n-KET, DHNK and HNK in human serum, urine and feces in a racemic and stereoselective manner. Moreover, we were are able for the first time to separate and quantify 2S,6S-HNK and 2R,6R-HNK in human serum and urine. As these metabolites were described to play a key role in the antidepressant activity of ketamine, this analytical feature may be of interest to analyze pharmacokinetic studies investigation for oral dosage forms as done in the clinical part of this thesis.

5.2. Affinity of ketamine to drug transporter proteins

5.2.1. Uptake by OCT transporters and OATP2B1

As previously discussed in rationales and objective section of this monograph, KET exists mainly as a cationic moiety (91% ionized) in the duodenum (pH = 6.5) which is the assumed site of absorption in the case of oral administration. As cations cannot penetrate the lipophilic cell membrane of the enterocytes by passive diffusion it may require intestinal uptake transport proteins. The expression of membrane transporters and the exact localization in the

enterocytes in human gut is controversial and still under discussion. Despite the previously accepted expression of OCT1 in the basolateral membrane [65–67], recently in 2013 Han et al suggested an expression of OCT1 in the apical layer [68]. Consequently, we investigated the relevance of OCT1-3 and OATP2B1 (both are expressed in the intestine) in the cellular uptake of KET by using transfected MDCKII and HEK293 cells overexpressing the respective human proteins.

KET was shown to be a substrate for OCT1-3 at pH 7.4 and 6.5 (Figure 9). This is in line with previously published data that KET is an inhibitor of the aforementioned transporters [69,70].

We performed this uptake study at pH 6.5 to mimic the situation in the intestinal lumen and at pH 7.4 to conclude on the situation in the systemic circulation. The so far clinically used oral doses of KET (~25 mg) are expected to result in $[I]_2$-value (intestinal concentration, dose / 240 ml) of about ~ 440 µmol/l, which makes an interaction with intestinal OCTs (K_m ~20-90 µmol/l) plausible as the $[I]_2$-value is several folds higher than the K_m. Additionally, the concentration of KET may be even higher than the previously mentioned value due to the initial rapid water absorption [71]. We would conclude from our results that the OCT-mediated cellular uptake is expected to be only of relevance in the human intestine, where OCT1 / 3 could be verified to be stably expressed [30,72], concentration of KET is higher than K_m of the transporters and where KET is predominately present as cation (91%).

In contrast to this, OCT-mediated uptake into renal tubule cells is not expected as the systemic pH 7.4 means low ionization of KET (~ 50%). Moreover, C_{max} of KET after ingestion of the usual oral dose of 25 mg is only 0.088 µmol/l, while the K_m for OCT2 is 61.7 µmol/l. Consequently, it could be concluded that KET renal elimination may be not a transporter dependent mechanism rather it follows the normal passive diffusion process.

On the other hand, for OATP2B1, which is expressed in intestine, liver and brain, we could not show any KET uptake at pH 6.5 and 7.4.

5.2.2. Efflux transport by P-gp

Incubation of KET (10 µmol) for up to 30 min with P-gp-lipovesicles resulted in vesicular accumulation of KET. This accumulation was independent from ATP (the known driving force for this transporter). This indicated that KET may not be a substrate for P-gp. This was also indirectly confirmed by an in-vitro experiment in which KET showed no inhibition of P-gp in ABCB1-transfected HEK293 cells as detected by flow cytometry [73]. Therefore according to these findings, P-gp might not be expected to contribute significantly to oral bioavailability, tissue (brain) distribution or renal excretion of KET. Despite that we should consider the lipovesicular assays limitation for lipophilic compounds that was already well established [74,75], which could end up in a false negative results due to the possible uptake of the lipophilic compound by passive diffusion independent from the membrane transporters, or the high unspecific binding to membranes for these compounds. Considering that we performed our study at pH=7.4, which is the assumed pH in intracellular compartments and the systemic circulation. The observed negative results might be due to the low ionization degree for KET at pH 7.4 and its high lipophilicity (log P 2.2).

Moreover, a recent mechanistic study revealed that P-gp substrates do not necessarily have to enter the cell and bind to P-gp from the intracellular space but may penetrate into the membrane from the outside and bind to P-gp in the intramembrane domain. The conformation switch of P-gp as initiated by intracellular binding and hydrolysis of ATP leads to extrusion of the respective substrate out of the cell membrane [76]. To acknowledge this hypothesis, we finally (re-)performed our vesicular uptake experiments at pH 6.5 and found that KET is a low affinity substrate of P-gp with no transporter saturation for up to 2,000 µmol. As already discussed for the OCT transporters, the relevance of P-gp seems to be limited to the human intestine due to the high concentration of the drug which may result in saturation of the efflux pump. However, a clinical drug-drug interaction study for oral KET with the P-gp and CYP3A4 inhibitor clarithromycin observed markedly increased AUC and C_{max} of KET about 3-fold while the terminal half-live was not significantly affected [77]. Interestingly, the exposure of the major metabolite n-KET was not changed suggesting that this interaction to

the opinion of the authors is not due to inhibition of intestinal metabolism but indicates a potential clinical relevance of P-gp in case of drug-drug interactions (e.g P-gp inhibition) at the intestinal absorption.

5.2.3. Transport by MATE1/2K

Uptake screening experiments with up to 15 µmol KET revealed no significant difference between the control and transporter-transfected cells (Figure13).

As KET is excreted predominately via the kidneys, we investigated MATE1/2K which works in many cases together with OCT transporters in order to facilitate a vectorial transport across the renal tubule cells. However, although KET was identified as an OCT substrate we could not verify a significant affinity to MATE1/2k. Taking also into account that under systemic pH of about 7.4 OCT transport of KET is not expected as discussed before [24], we conclude that the renal elimination seems not to be transporter dependent but rather a function of glomerular filtration and tubular reabsorption.

5.3. Pharmacokinetic properties of PR-KET

The classical KET paradigm that restricts the pharmacological activity of KET to inhibition of NMDA-receptors by the parent compound and n-KET should be revised because it does not provide a convincing explanation for the antidepressant effect of KET as shown recently in a clinical study performed in 67 patients with major depression. The results of this study show a higher level of *2R,6R*-HNK in plasma of patients under depression who responds to KET treatment when compared to patients who were non-responders [78]. These findings indicate the pharmacological activity of this metabolite. Moreover, this study stated that there was an inverse relationship between this ketamine metabolite and psychotomimetic or dissociative side effects, where higher levels of this metabolite were associated with lower side effects. In this regard, the *2R,6R*-HNK metabolite was proven recently to be responsible for the antidepressant activity as well as other neuro-modulating effects of KET but in a NMDA receptor independent mechanism, rather it acts through the activation of AMPA receptor [22]. Furthermore, it was published that the other isomer *2S,6S*-HNK is not a potent NMDA receptor antagonist when compared to either KET or n-KET; but it acts as a selective (α7-

nACh) receptor inhibitor which presents in the presynaptic terminal. The inhibition of the previous receptor promotes a decrease in the activity of serine racemase enzyme. This enzyme represents the source for endogenous D-ser[23]. This outcome indicates that this compound is also pharmacologically active. Consequently, these findings push us to rethink about the “classical Ketamine Paradigm”, and postulate a new “Ketamine metabolite paradigm” expecting KET to be promising for other indications like depression, neuropathic chronic pain, Alzheimer or Parkinson’s diseases, rather than being restricted to the usual anesthetic use. The aforementioned data support our hypothesis that PR-KET might be promising for the indicated chronic diseases. Our results demonstrate that PR-KET has a different metabolic pattern that affects both, the pharmacokinetics and pharmacodynamics of the drug. Consequently, PR-KET could even be considered as a completely new drug.

In line with the previously established knowledge, it was not surprising to find that our study medication was safe and well tolerated for up to 80 mg (1 mg/kg) PR-KET. This high dose represents an anesthetic dose if administrated intravenously. However, the absence of both the side effects as well as the narcotic effect of KET at this high dose emphasizes our hypothesis that we might be in front of a completely new drug that might be promising as an antidepressant due to the high throughput of the active HNK. Consequently, this difference in pharmacodynamics is based on the differences in pharmacokinetic properties of the new PR-KET when compared to either IV-KET or the IR-KET.

The PR-KET shows a T_{max} for KET in serum after ~ 6 h (median), thereby prolonging absorption of KET by ~ 4 h as compared to an IR-KET as reference with median T_{max} of 2 h (25 mg (±)-KET lozenges) [27]. This together with a half live of about 7 h makes PR-KET promising as an oral treatment for these chronic diseases.

The absolute oral bioavailability of PR-KET is low and remains constant between the 20 to 80 mg strength up to almost 14% (median). However, the bioavailability of 80 mg PR-KET is still lower than that of 25 mg IR-KET with a median 24% [27]. This decrease in bioavailability may be due to the fast absorption of water after gastric emptying that may lead to an elevated concentration of drug in the intestine after an IR-dosage form, this high concentration

could saturate the efflux transporters and the metabolizing enzymes or may be due to the regio-selectivity of these transporters and enzymes along the intestine [79].

It was clear from our data that n-KET is the major circulating metabolite in plasma followed by HNK, DHNK and HK (Figure 15) which differs from the results obtained by Moadel *et al* who mentioned HNK to be the major circulating metabolite followed by DHNK, n-KET and finally HK. This might be due to that the patient in this study where under complex regional pain syndrome, or the used of higher dose (40 mg IV) or the longer period of treatment (5 days) [64]. Additionally, the increase in the metabolic percentage (R_M) of both n-KET and DHNK for oral dosage form when compared to IV-KET clarifies the presystemic metabolism in the intestine (Table 9). In line with this data, both the metabolic ratio of $AUC_{n\text{-}KET/KET}$ and $AUC_{HNK/KET}$ increased from 2 and 0.3 for the IV-KET to 14 and 16 for the 80 mg PR-KET, respectively. These produced metabolites show better side effect profile and were pharmacologically active. Additionally, as expected the PR-KET $AUC_{n\text{-}KET/KET}$ (14) was several folds higher than either 25 mg sublingual ($AUC_{n\text{-}KET/KET,}$ 3) or 25 mg IR-KET ($AUC_{n\text{-}KET/KET}$, 5) measured in another study as IR-KET [27]. Indicating that our dosage form may be superior in metabolite formation and it might be promising as a new dosage form for chronic diseases especially depression with less side effects that was clearly proved by the safety and well tolerance of our study medication. The nearly constant metabolic ratios of $AUC_{DHNK/n\text{-}KET}$ and $AUC_{HNK/n\text{-}KET}$ and the decrease in the ratios of $AUC_{DHNK/KET}$ and $AUC_{n\text{-}KET/KET}$ over increasing dose indicate that the step of metabolism of KET to n-KET is a capacity limiting step and the enzymes or the hepatic uptake might be saturated (Figure 14).

The formation of the major metabolite n-KET remained constant within the dose range tested (10 to 80 mg), and there was a delay in its formation (~ 5-6 h) in comparison to the IR-KET (~ 1.5 h) [27].

The elimination of KET was delayed by the PR-KET which is noticed by the increase of $T_{1/2}$ from 5 h for the IR-KET to 7 h for the PR-KET.

The non-significant difference between either *S*- and *R*-KET or *S* and *R*-n-KET indicates that this metabolic step is not stereoselective. Our data is emphasized by the data of another

group which showed no significant difference between *R*-and *S*-forms of both KET and n-KET after injection of 20 mg or ingestion of 25 mg IR-tablets twice daily [80].On the other hand, the high difference between *R*- and *S*-DHNK clarify that this step might be stereoselective

.

6. Conclusions

Prolonged-release ketamine (PR-KET) undergoes “first-pass” metabolism, which generates substantially increased plasma exposure of downstream metabolites with potential neuro-modulating effects compared to ketamine after intravenous administration (IV-KET). Consequently, this patent dosage form could be promising for depression taking the benefit of modifying an already registered drug rather than introducing a new drug to the market that will need further years of clinical researches. Moreover, we succeeded for the first time to separate all the hydroxynorketamine (HNK) metabolites for KET in human serum and urine in a stereoselective manner. Additionally, we provide evidence that KET is a substrate for the uptake transporters OCT1-3 and the efflux transporter P-gp at pH 6.5 that could account for probable drug-drug interactions.

7. Summary

Introduction: Ketamine (KET) is widely used as anaesthetic drug. Beside its pronounced anaesthetic effects as caused by antagonism of NMDA receptors, ketamine also causes potent analgesia. Moreover, There are ample new evidences, firstly, that 2R,6R/2S,6S-enantiomers of hydroxynorketamine (HNK), exert neuro-modulating effects by AMPA-receptor activation and, secondly, that the plasma levels of norketamine (n-KET) after oral dosing are higher than after intravenous administration. From the physicochemical point of view ketamine is expected to be a substrate of drug transporters. Thus, it was the aim of this study to separate and quantify KET and its metabolites in human serum, urine and feces; investigate the role of transporter proteins in the intestinal absorption, distribution and elimination of ketamine; and evaluate pharmacokinetics and metabolism of a newly developed prolonged-release ketamine dosage form to confirm its suitability for chronic treatment of CNS-diseases (e.g. depression) according to the new “ketamine metabolite paradigm”.

Materials and methods: Quantification of ketamine was done by a LC-MS/MS-based quantification method on the QTRAP4000 instrument. Samples were extracted by methyl tert-butyl ether after addition of sodium carbonate to liberate the free base; Single transfected MDCKII cells overexpressing OCT1, OCT2, OCT3, and MATE1 or MATE2K, and HEK293 cells overexpressing OATP2B1 were used to study the cellular uptake of ketamine. Inside-out lipovesicles were used to determine the affinity of ketamine to the efflux transporter P-glycoprotein (P-gp). Uptake into cells or vesicles was determined by liquid scintillation counting. Functionality of all in vitro systems was assured by using in each case appropriate probe substrates; The dose-escalation study was performed in five consecutive periods (7 days washout) in 15 healthy subjects (5 females and 10 males. 20-35 years, BMI 19.4-27.6 kg/m2).

Results: We introduce for the first time the separation and quantification of the active metabolites 2R,6R/2S,6S-HNK; Ketamine was shown to be taken up significantly in a time- and concentration-dependent manner by OCT1-3. The affinity to OCT transporters at pH=6.5 was several fold higher than that at pH=7.4.), ketamine showed a significant but low affinity to P-gp. In contrast to this, we could not detect any transport of ketamine by MATE1 / 2K or

OACPT2B1; and PR-KET was safe and well tolerated with higher metabolites productivity, different pharmacokinetic properties and longer $T_{1/2}$ when compared to IV-KET or IR-KET.

Conclusion: the uptake transporters OCT1 & 3 and the efflux transporter P-gp may play a role in the intestinal absorption of the drug. On the other side, P-gp, MATE1 / 2K and OCT are not expected to contribute significantly to tissue (brain) distribution or renal excretion of ketamine; Moreover, the prolonged-release ketamine undergoes dose-dependent “first-pass” metabolism which generates substantially increased plasma exposure of downstream metabolites with potential neuro-modulating effects compared to ketamine after intravenous administration.

8. Reference

[1] G.E. Hardingham and H. Bading, The Yin and Yang of NMDA receptor signalling, Trends Neurosci., 26 (2003) 81-89.

[2] M.P. Parsons and L.A. Raymond, Extrasynaptic NMDA receptor involvement in central nervous system disorders, Neuron, 82 (2014) 279-293.

[3] J.M. Crowder, M.J. Croucher, H.F. Bradford, and J.F. Collins, Excitatory amino acid receptors and depolarization-induced Ca2+ influx into hippocampal slices, J. Neurochem., 48 (1987) 1917-1924.

[4] J.W. Johnson and P. Ascher, Voltage-dependent block by intracellular Mg2+ of N-methyl-D-aspartate-activated channels, Biophys. J., 57 (1990) 1085-1090.

[5] H. Jourdi, Y.T. Hsu, M. Zhou, Q. Qin, X. Bi, and M. Baudry, Positive AMPA receptor modulation rapidly stimulates BDNF release and increases dendritic mRNA translation, J. Neurosci., 29 (2009) 8688-8697.

[6] N. Li, B. Lee, R.J. Liu, M. Banasr, J.M. Dwyer, M. Iwata, X.Y. Li, G. Aghajanian, and R.S. Duman, mTOR-dependent synapse formation underlies the rapid antidepressant effects of NMDA antagonists, Science, 329 (2010) 959-964.

[7] J.M. Dwyer and R.S. Duman, Activation of mammalian target of rapamycin and synaptogenesis: role in the actions of rapid-acting antidepressants, Biol. Psychiatry, 73 (2013) 1189-1198.

[8] R.J. Griffin, A. Moloney, M. Kelliher, J.A. Johnston, R. Ravid, P. Dockery, R. O'Connor, and C. O'Neill, Activation of Akt/PKB, increased phosphorylation of Akt substrates and loss and altered distribution of Akt and PTEN are features of Alzheimer's disease pathology, J. Neurochem., 93 (2005) 105-117.

[9] J.O. Lipton and M. Sahin, The neurology of mTOR, Neuron, 84 (2014) 275-291.

[10] N. Hay and N. Sonenberg, Upstream and downstream of mTOR, Genes Dev., 18 (2004) 1926-1945.

[11] D.H. Kim, D.D. Sarbassov, S.M. Ali, J.E. King, R.R. Latek, H. Erdjument-Bromage, P. Tempst, and D.M. Sabatini, mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery, Cell, 110 (2002) 163-175.

[12] B.K. Kennedy and D.W. Lamming, The Mechanistic Target of Rapamycin: The Grand ConducTOR of Metabolism and Aging, Cell Metab, 23 (2016) 990-1003.

[13] J. Jiraskova-Vanickova, R. Ettrich, B. Vorlova, H.E. Hoffman, M. Lepsik, P. Jansa, and J. Konvalinka, Inhibition of human serine racemase, an emerging target for medicinal chemistry, Curr. Drug Targets., 12 (2011) 1037-1055.

[14] S.P. Cook, I. Galve-Roperh, P.A. Martinez del, and I. Rodriguez-Crespo, Direct calcium binding results in activation of brain serine racemase, J. Biol. Chem., 277 (2002) 27782-27792.

[15] H. Wolosker, E. Dumin, L. Balan, and V.N. Foltyn, D-amino acids in the brain: D-serine in neurotransmission and neurodegeneration, FEBS J., 275 (2008) 3514-3526.

[16] D. Rosenberg, S. Artoul, A.C. Segal, G. Kolodney, I. Radzishevsky, E. Dikopoltsev, V.N. Foltyn, R. Inoue, H. Mori, J.M. Billard, and H. Wolosker, Neuronal D-serine and glycine release via the Asc-1 transporter regulates NMDA receptor-dependent synaptic activity, J. Neurosci., 33 (2013) 3533-3544.

[17] M.P. Marietta, W.L. Way, N. Castagnoli, Jr., and A.J. Trevor, On the pharmacology of the ketamine enantiomorphs in the rat, J. Pharmacol. Exp. Ther., 202 (1977) 157-165.

[18] P.F. White, J. Schuttler, A. Shafer, D.R. Stanski, Y. Horai, and A.J. Trevor, Comparative pharmacology of the ketamine isomers. Studies in volunteers, Br. J. Anaesth., 57 (1985) 197-203.

[19] L.Y. Leung and T.A. Baillie, Comparative pharmacology in the rat of ketamine and its two principal metabolites, norketamine and (Z)-6-hydroxynorketamine, J. Med. Chem., 29 (1986) 2396-2399.

[20] K. Fisher, T.J. Coderre, and N.A. Hagen, Targeting the N-methyl-D-aspartate receptor for chronic pain management. Preclinical animal studies, recent clinical experience and future research directions, J. Pain Symptom. Manage., 20 (2000) 358-373.

[21] L.Y. Leung and T.A. Baillie, Comparative pharmacology in the rat of ketamine and its two principal metabolites, norketamine and (Z)-6-hydroxynorketamine, J. Med. Chem., 29 (1986) 2396-2399.

[22] P. Zanos, R. Moaddel, P.J. Morris, P. Georgiou, J. Fischell, G.I. Elmer, M. Alkondon, P. Yuan, H.J. Pribut, N.S. Singh, K.S. Dossou, Y. Fang, X.P. Huang, C.L. Mayo, I.W. Wainer, E.X. Albuquerque, S.M. Thompson, C.J. Thomas, C.A. Zarate, Jr., and T.D. Gould, NMDAR inhibition-independent antidepressant actions of ketamine metabolites, Nature, 533 (2016) 481-486.

[23] N.S. Singh, C.A. Zarate, Jr., R. Moaddel, M. Bernier, and I.W. Wainer, What is hydroxynorketamine and what can it bring to neurotherapeutics?, Expert. Rev. Neurother., 14 (2014) 1239-1242.

[24] C. Chong, S.A. Schug, M. Page-Sharp, B. Jenkins, and K.F. Ilett, Development of a sublingual/oral formulation of ketamine for use in neuropathic pain: Preliminary findings from a three-way randomized, crossover study, Clin. Drug Investig., 29 (2009) 317-324.

[25] Y. Yanagihara, M. Ohtani, S. Kariya, K. Uchino, T. Hiraishi, N. Ashizawa, T. Aoyama, Y. Yamamura, Y. Yamada, and T. Iga, Plasma concentration profiles of ketamine and norketamine after administration of various ketamine preparations to healthy Japanese volunteers, Biopharm. Drug Dispos., 24 (2003) 37-43.

[26] J.A. Clements, W.S. Nimmo, and I.S. Grant, Bioavailability, pharmacokinetics, and analgesic activity of ketamine in humans, J. Pharm. Sci., 71 (1982) 539-542.

[27] C. Chong, S.A. Schug, M. Page-Sharp, B. Jenkins, and K.F. Ilett, Development of a sublingual/oral formulation of ketamine for use in neuropathic pain: Preliminary findings from a three-way randomized, crossover study, Clin. Drug Investig., 29 (2009) 317-324.

[28] I.S. Grant, W.S. Nimmo, and J.A. Clements, Pharmacokinetics and analgesic effects of i.m. and oral ketamine, Br. J. Anaesth., 53 (1981) 805-810.

[29] K. Nakamura, M. Hirayama-Kurogi, S. Ito, T. Kuno, T. Yoneyama, W. Obuchi, T. Terasaki, and S. Ohtsuki, Large-scale multiplex absolute protein quantification of drug-

metabolizing enzymes and transporters in human intestine, liver, and kidney microsomes by SWATH-MS: Comparison with MRM/SRM and HR-MRM/PRM, Proteomics., 16 (2016) 2106-2117.

[30] M. Drozdzik, C. Groer, J. Penski, J. Lapczuk, M. Ostrowski, Y. Lai, B. Prasad, J.D. Unadkat, W. Siegmund, and S. Oswald, Protein abundance of clinically relevant multidrug transporters along the entire length of the human intestine, Mol. Pharm., 11 (2014) 3547-3555.

[31] N.M. Hagelberg, M.A. Peltoniemi, T.I. Saari, K.J. Kurkinen, K. Laine, P.J. Neuvonen, and K.T. Olkkola, Clarithromycin, a potent inhibitor of CYP3A, greatly increases exposure to oral S-ketamine, Eur. J. Pain, 14 (2010) 625-629.

[32] V. Massmann, B. Edemir, E. Schlatter, R. Al-Monajjed, S. Harrach, P. Klassen, S.K. Holle, A. Sindic, M. Dobrivojevic, H. Pavenstadt, and G. Ciarimboli, The organic cation transporter 3 (OCT3) as molecular target of psychotropic drugs: transport characteristics and acute regulation of cloned murine OCT3, Pflugers Arch., 466 (2014) 517-527.

[33] A. Amphoux, V. Vialou, E. Drescher, M. Bruss, C.C. Mannoury la, C. Rochat, M.J. Millan, B. Giros, H. Bonisch, and S. Gautron, Differential pharmacological in vitro properties of organic cation transporters and regional distribution in rat brain, Neuropharmacology, 50 (2006) 941-952.

[34] R. Moaddel, S.L. Venkata, M.J. Tanga, J.E. Bupp, C.E. Green, L. Iyer, A. Furimsky, M.E. Goldberg, M.C. Torjman, and I.W. Wainer, A parallel chiral-achiral liquid chromatographic method for the determination of the stereoisomers of ketamine and ketamine metabolites in the plasma and urine of patients with complex regional pain syndrome, Talanta, 82 (2010) 1892-1904.

[35] M.F. Paine, M. Khalighi, J.M. Fisher, D.D. Shen, K.L. Kunze, C.L. Marsh, J.D. Perkins, and K.E. Thummel, Characterization of interintestinal and intraintestinal variations in human CYP3A-dependent metabolism, J. Pharmacol. Exp. Ther., 283 (1997) 1552-1562.

[36] M. Drozdzik, C. Groer, J. Penski, J. Lapczuk, M. Ostrowski, Y. Lai, B. Prasad, J.D. Unadkat, W. Siegmund, and S. Oswald, Protein abundance of clinically relevant multidrug transporters along the entire length of the human intestine, Mol. Pharm., 11 (2014) 3547-3555.

[37] European Medicines Agency (EMA) and Committee for Medicinal Products for Human Use (CHMP). Guideline on bioanalytical method validation. FDA. 2013.

[38] Food and Drug Administration (FDA). Guidance for Industry - Bioanalytical Method Validation. FDA. 2013.

[39] M. Grube, S. Ameling, M. Noutsias, K. Kock, I. Triebel, K. Bonitz, K. Meissner, G. Jedlitschky, L.R. Herda, M. Reinthaler, M. Rohde, W. Hoffmann, U. Kuhl, H.P. Schultheiss, U. Volker, S.B. Felix, K. Klingel, R. Kandolf, and H.K. Kroemer, Selective regulation of cardiac organic cation transporter novel type 2 (OCTN2) in dilated cardiomyopathy, Am. J. Pathol., 178 (2011) 2547-2559.

[40] W. Siegmund, C. Modess, E. Scheuch, K. Methling, M. Keiser, A. Nassif, D. Rosskopf, P.J. Bednarski, J. Borlak, and B. Terhaag, Metabolic activation and analgesic effect of

flupirtine in healthy subjects, influence of the polymorphic NAT2, UGT1A1 and GSTP1, Br. J. Clin. Pharmacol., 79 (2015) 501-513.

[41] M. Leonhardt, M. Keiser, S. Oswald, J. Kuhn, J. Jia, M. Grube, H.K. Kroemer, W. Siegmund, and W. Weitschies, Hepatic uptake of the magnetic resonance imaging contrast agent Gd-EOB-DTPA: role of human organic anion transporters, Drug Metab Dispos., 38 (2010) 1024-1028.

[42] M. Bexten, S. Oswald, M. Grube, J. Jia, T. Graf, U. Zimmermann, K. Rodewald, O. Zolk, U. Schwantes, W. Siegmund, and M. Keiser, Expression of drug transporters and drug metabolizing enzymes in the bladder urothelium in man and affinity of the bladder spasmolytic trospium chloride to transporters likely involved in its pharmacokinetics, Mol. Pharm., 12 (2015) 171-178.

[43] C. Groer, S. Bruck, Y. Lai, A. Paulick, A. Busemann, C.D. Heidecke, W. Siegmund, and S. Oswald, LC-MS/MS-based quantification of clinically relevant intestinal uptake and efflux transporter proteins, J. Pharm. Biomed. Anal., 85 (2013) 253-261.

[44] K.L. Brouwer, D. Keppler, K.A. Hoffmaster, D.A. Bow, Y. Cheng, Y. Lai, J.E. Palm, B. Stieger, and R. Evers, In vitro methods to support transporter evaluation in drug discovery and development, Clin. Pharmacol. Ther., 94 (2013) 95-112.

[45] P. Delatour, P. Jaussaud, D. Courtot, and D. Fau, Enantioselective N-demethylation of ketamine in the horse, J. Vet. Pharmacol. Ther., 14 (1991) 209-212.

[46] G. Geisslinger, S. Menzel-Soglowek, H.D. Kamp, and K. Brune, Stereoselective high-performance liquid chromatographic determination of the enantiomers of ketamine and norketamine in plasma, J. Chromatogr., 568 (1991) 165-176.

[47] J.O. Svensson and L.L. Gustafsson, Determination of ketamine and norketamine enantiomers in plasma by solid-phase extraction and high-performance liquid chromatography, J. Chromatogr. B Biomed. Appl., 678 (1996) 373-376.

[48] G. Geisslinger, W. Hering, P. Thomann, R. Knoll, H.D. Kamp, and K. Brune, Pharmacokinetics and pharmacodynamics of ketamine enantiomers in surgical patients using a stereoselective analytical method, Br. J. Anaesth., 70 (1993) 666-671.

[49] P. Hartvig, J. Valtysson, K.J. Lindner, J. Kristensen, R. Karlsten, L.L. Gustafsson, J. Persson, J.O. Svensson, I. Oye, G. Antoni, and ., Central nervous system effects of subdissociative doses of (S)-ketamine are related to plasma and brain concentrations measured with positron emission tomography in healthy volunteers, Clin. Pharmacol. Ther., 58 (1995) 165-173.

[50] Y. Yanagihara, M. Ohtani, S. Kariya, K. Uchino, T. Aoyama, Y. Yamamura, and T. Iga, Stereoselective high-performance liquid chromatographic determination of ketamine and its active metabolite, norketamine, in human plasma, J. Chromatogr. B Biomed. Sci. Appl., 746 (2000) 227-231.

[51] T. Chang and A.J. Glazko, A gas chromatographic assay for ketamine in human plasma, Anesthesiology, 36 (1972) 401-404.

[52] J. Wieber, R. Gugler, J.H. Hengstmann, and H.J. Dengler, Pharmacokinetics of ketamine in man, Anaesthesist, 24 (1975) 260-263.

[53] J.N. Davisson, Rapid gas chromatographic analysis of plasma levels of ketamine and major metabolites employing either nitrogen selective or mass spectroscopic detection, J. Chromatogr., 146 (1978) 344-349.

[54] M.L. Williams, D.E. Mager, H. Parenteau, G. Gudi, T.S. Tracy, M. Mulheran, and I.W. Wainer, Effects of protein calorie malnutrition on the pharmacokinetics of ketamine in rats, Drug Metab Dispos., 32 (2004) 786-793.

[55] K. Lian, P. Zhang, L. Niu, S. Bi, S. Liu, L. Jiang, and W. Kang, A novel derivatization approach for determination of ketamine in urine and plasma by gas chromatography-mass spectrometry, J. Chromatogr. A, 1264 (2012) 104-109.

[56] R. Theurillat, M. Knobloch, O. Levionnois, P. Larenza, M. Mevissen, and W. Thormann, Characterization of the stereoselective biotransformation of ketamine to norketamine via determination of their enantiomers in equine plasma by capillary electrophoresis, Electrophoresis, 26 (2005) 3942-3951.

[57] R. Theurillat, M. Knobloch, A. Schmitz, P.G. Lassahn, M. Mevissen, and W. Thormann, Enantioselective analysis of ketamine and its metabolites in equine plasma and urine by CE with multiple isomer sulfated beta-CD, Electrophoresis, 28 (2007) 2748-2757.

[58] A. Schmitz, R. Theurillat, P.G. Lassahn, M. Mevissen, and W. Thormann, CE provides evidence of the stereoselective hydroxylation of norketamine in equines, Electrophoresis, 30 (2009) 2912-2921.

[59] R. Theurillat, M.P. Larenza, K. Feige, R. Bettschart-Wolfensberger, and W. Thormann, Development of a method for analysis of ketamine and norketamine enantiomers in equine brain and cerebrospinal fluid by capillary electrophoresis, Electrophoresis, 35 (2014) 2863-2869.

[60] M.E. Rodriguez Rosas, S. Patel, and I.W. Wainer, Determination of the enantiomers of ketamine and norketamine in human plasma by enantioselective liquid chromatography-mass spectrometry, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci., 794 (2003) 99-108.

[61] T. LeGrand, S. Roy, C. Monchaud, C. Grondin, M. Duval, and E. Jacqz-Aigrain, Determination of ketamine and norketamine in plasma by micro-liquid chromatography-mass spectrometry, J. Pharm. Biomed. Anal., 48 (2008) 171-176.

[62] G. Geisslinger, S. Menzel-Soglowek, H.D. Kamp, and K. Brune, Stereoselective high-performance liquid chromatographic determination of the enantiomers of ketamine and norketamine in plasma, J. Chromatogr., 568 (1991) 165-176.

[63] M.E. Rodriguez Rosas, S. Patel, and I.W. Wainer, Determination of the enantiomers of ketamine and norketamine in human plasma by enantioselective liquid chromatography-mass spectrometry, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci., 794 (2003) 99-108.

[64] R. Moaddel, S.L. Venkata, M.J. Tanga, J.E. Bupp, C.E. Green, L. Iyer, A. Furimsky, M.E. Goldberg, M.C. Torjman, and I.W. Wainer, A parallel chiral-achiral liquid chromatographic method for the determination of the stereoisomers of ketamine and ketamine metabolites in the plasma and urine of patients with complex regional pain syndrome, Talanta, 82 (2010) 1892-1904.

[65] J.W. Jonker, E. Wagenaar, C.A. Mol, M. Buitelaar, H. Koepsell, J.W. Smit, and A.H. Schinkel, Reduced hepatic uptake and intestinal excretion of organic cations in mice with a targeted disruption of the organic cation transporter 1 (Oct1 [Slc22a1]) gene, Mol. Cell Biol., 21 (2001) 5471-5477.

[66] H. Koepsell, K. Lips, and C. Volk, Polyspecific organic cation transporters: structure, function, physiological roles, and biopharmaceutical implications, Pharm. Res., 24 (2007) 1227-1251.

[67] J. Muller, K.S. Lips, L. Metzner, R.H. Neubert, H. Koepsell, and M. Brandsch, Drug specificity and intestinal membrane localization of human organic cation transporters (OCT), Biochem. Pharmacol., 70 (2005) 1851-1860.

[68] T.K. Han, R.S. Everett, W.R. Proctor, C.M. Ng, C.L. Costales, K.L. Brouwer, and D.R. Thakker, Organic cation transporter 1 (OCT1/mOct1) is localized in the apical membrane of Caco-2 cell monolayers and enterocytes, Mol. Pharmacol., 84 (2013) 182-189.

[69] V. Massmann, B. Edemir, E. Schlatter, R. Al-Monajjed, S. Harrach, P. Klassen, S.K. Holle, A. Sindic, M. Dobrivojevic, H. Pavenstadt, and G. Ciarimboli, The organic cation transporter 3 (OCT3) as molecular target of psychotropic drugs: transport characteristics and acute regulation of cloned murine OCT3, Pflugers Arch., 466 (2014) 517-527.

[70] A. Amphoux, V. Vialou, E. Drescher, M. Bruss, C.C. Mannoury La, C. Rochat, M.J. Millan, B. Giros, H. Bonisch, and S. Gautron, Differential pharmacological in vitro properties of organic cation transporters and regional distribution in rat brain, Neuropharmacology, 50 (2006) 941-952.

[71] C. Schiller, C.P. Frohlich, T. Giessmann, W. Siegmund, H. Monnikes, N. Hosten, and W. Weitschies, Intestinal fluid volumes and transit of dosage forms as assessed by magnetic resonance imaging, Aliment. Pharmacol. Ther., 22 (2005) 971-979.

[72] T.K. Han, R.S. Everett, W.R. Proctor, C.M. Ng, C.L. Costales, K.L. Brouwer, and D.R. Thakker, Organic cation transporter 1 (OCT1/mOct1) is localized in the apical membrane of Caco-2 cell monolayers and enterocytes, Mol. Pharmacol., 84 (2013) 182-189.

[73] N. Tournier, L. Chevillard, B. Megarbane, S. Pirnay, J.M. Scherrmann, and X. Decleves, Interaction of drugs of abuse and maintenance treatments with human P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2), Int. J. Neuropsychopharmacol., 13 (2010) 905-915.

[74] C.Q. Xia, M.N. Milton, and L.S. Gan, Evaluation of drug-transporter interactions using in vitro and in vivo models, Curr. Drug Metab, 8 (2007) 341-363.

[75] J.W. Polli, S.A. Wring, J.E. Humphreys, L. Huang, J.B. Morgan, L.O. Webster, and C.S. Serabjit-Singh, Rational use of in vitro P-glycoprotein assays in drug discovery, J. Pharmacol. Exp. Ther., 299 (2001) 620-628.

[76] S.G. Aller, J. Yu, A. Ward, Y. Weng, S. Chittaboina, R. Zhuo, P.M. Harrell, Y.T. Trinh, Q. Zhang, I.L. Urbatsch, and G. Chang, Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding, Science, 323 (2009) 1718-1722.

[77] N.M. Hagelberg, M.A. Peltoniemi, T.I. Saari, K.J. Kurkinen, K. Laine, P.J. Neuvonen, and K.T. Olkkola, Clarithromycin, a potent inhibitor of CYP3A, greatly increases exposure to oral S-ketamine, Eur. J. Pain, 14 (2010) 625-629.

[78] C.A. Zarate, Jr., N. Brutsche, G. Laje, D.A. Luckenbaugh, S.L. Venkata, A. Ramamoorthy, R. Moaddel, and I.W. Wainer, Relationship of ketamine's plasma metabolites with response, diagnosis, and side effects in major depression, Biol. Psychiatry, 72 (2012) 331-338.

[79] S. Berggren, C. Gall, N. Wollnitz, M. Ekelund, U. Karlbom, J. Hoogstraate, D. Schrenk, and H. Lennernas, Gene and protein expression of P-glycoprotein, MRP1, MRP2, and CYP3A4 in the small and large human intestine, Mol. Pharm., 4 (2007) 252-257.

[80] Y. Yanagihara, M. Ohtani, S. Kariya, K. Uchino, T. Hiraishi, N. Ashizawa, T. Aoyama, Y. Yamamura, Y. Yamada, and T. Iga, Plasma concentration profiles of ketamine and norketamine after administration of various ketamine preparations to healthy Japanese volunteers, Biopharm. Drug Dispos., 24 (2003) 37-43.

Abbreviations

AE	Adverse event
BfArM	Federal Department for Drugs and Medical Devices
°C	Centigrade
e.g.	for example
MDCKII	Madin-Darby canine kidney
HEK	Human embryonic kidney
MPP^+	N-methyl-4-phenylpyridinium
BSP	Bromosulfophthalein
TEA	Tetraethylammonium
Rh123	Rhodamine-123
AMP	Adenosinmonophosphate
ATP	Adenosintriphosphate
BCA	Bicinchoninic Acid solution
K_m	Michaelis−Menten constant
V_{max}	Maximal uptake rate
GCP-V	Good Clinical Practice-Verordnung
GLP	Good Laboratory Practice
min	Minutes
h	Hour(s)
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
kg	Kilogram
mg	Milligram
µg	microgram
l	liter

ml	Milliliter
v/v	Volume/volume
LC-MS/MS	liquid chromatography tandem mass spectrometry
LC–MS	liquid chromatography mass spectrometry
HPLC-UV	High pressure liquid chromatography with ultraviolet detector
MRM	Multiple reaction monitoring
m^2	Quadrat meters
mmol	millimole
µmol/l	Micro mole/liter
ng/ml	Nano gram/milliliter
µm	Micrometers
l/kg	Liter/kilogram
SAE	Serious adverse events
SOP	Standard operating procedure
IR	Immediate release tablets
PR	Prolonged release tablets
IV	Intravenous
A_e	Amount excreted
AUC	Area under the curve
C_{max}	Maximum (serum)concentration
CL_R	Renal clearance
$CL_{intestinal}$	Intestinal clearance
CL_M	Metabolic clearance
F	Absolute bioavailability
λ_z	Terminal rate constant
$T_{½}$	Apparent terminal elimination half life
T_{max}	Time of maximal concentration in serum

V_{ss}	Volume of distribution at steady state
KET	Ketamine
n-KET	Norketamine
DHNK	Dehydronorketamine
HK	Hydroxyketamine
HNK	Hydroxynorketamine
TCA	Tricyclic antidepressant
OCT	Organic cationic transporter
OATP	Organic anion-transporting polypeptide
MATE	Multidrug and toxin extrusion protei
P-gp	P-Glycoprotein
NMDA	N-methyl-D-aspartate
CAD	Collision-activated dissociation
DP	Declustring potential
CE	Collision energy
EP	Entrance potential
CXP	Collision cell exit potential
CUR	Curtain gas
FDA	U.S. food and drug administration
DMSO	dimethylsulfoxide
EMA	European medicine agency

Acknowledgement

Firstly, I would like to thank Allah almighty who supported me during my whole life and helped me to finish this PhD.

I would like to express my sincere gratitude to my advisors: Prof. Werner Siegmund and Prof. Stefan Oswald for the continuous support during my PhD study and related research, for their patience, motivation, and immense knowledge. Their guidance supported me during my 4 years research as well as during the writing of my thesis.

My sincere thanks also goes to Dr. Markus Keiser who supported me in the in vitro study; my lab mates Dr. Ebahard Scheuch, Sabine Bade, Gita Schumacher and Danilo Wegner as well as the whole colleagues in the department of clinical pharmacology for all the work and fun we had in the last four years.

Beside my advisor and colleagues, I would like to thank the members of my faculty in Egypt (Future University in Egypt; FUE): on the top Dr. Hassan Azazy and Mr. Khaled Azazy who sponsored me with Azazy scholarship during my Ph.D; Prof. Ebada sarhan, Prof. Osama Salama, my dean Prof. Seham Elkheshen Prof. Manal Qandeel, Prof. Elmenshawy and my small family in pharmaceutical chemistry department for their insightful encouragement and support.

I cannot forget to thank Prof. Sakr whom I considered as a father and teacher.

Last but not the least, I would like to thank my parents, my sister, my small family especially my wife who left everything in Egypt and came to support me during my PhD, my son, and my daughter for supporting me spiritually throughout my PhD and my life in general.