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Investigating Gender Differences in the Metabolism of Ketamine

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ABSTRACT

Ketamine is a dissociative anesthetic that was developed for use as a short-acting anesthesia. It has a history of being a drug of abuse and is currently a Schedule III drug. Ketamine is being investigated as an antidepressant due to its fast-action and long-lasting effects in comparison to other antidepressants. The exact mechanism of action of ketamine on the brain that results in the antidepressant effects is currently unknown, as is the reason for differences in the way males and females respond to ketamine. Dr. Lori McMahon, Allie Widman, and Nateka Jackson in the Cell, Developmental, and Integrative Biology Department at University of Alabama at Birmingham are conducting a study to observe the differences in male and female responses to ketamine treatment in addition to investigating how ketamine interacts with the brain. In collaboration with the McMahon group, the aim of this study is to investigate the concentrations of ketamine and norketamine in different tissue samples at 3 different time periods after injecting ketamine. The objectives were to discern how the ketamine is being distributed after intravenous injection, how quickly it is metabolized, and how long it remains in the plasma, liver, hippocampus, prefrontal cortex, and cerebellum.

The results establish that the highest concentration of ketamine is in all tissues at time 0. There are no notable differences in the levels of norketamine and ketamine in male and ovariectomized female rats. Ketamine and norketamine were below the limit of quantitation within three hours of treatment in all tissues.

Future studies could include samples taken from female rates dosed with estrogen and progesterone and determine the concentrations of ketamine metabolites hydroxynorketamine and hydroxyketamine.

If ketamine becomes a widely used treatment for depression, it may become more widely abused. Therefore, documenting differences between genders in the pharmacokinetics and pharmacodynamics of the drug will aid the field of forensic toxicology.

Keywords: Ketamine, Norketamine, Depression, Metabolism.

ACKNOWLEDGEMENTS

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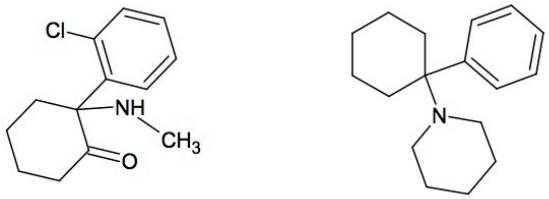
INTRODUCTION

Ketamine is a non-competitive N-methyl-D-aspartate (NMDA) glutamate receptor antagonist that acts as a dissociative anesthetic and antidepressant. The drug was first synthesized by Calvin Stevens in 1962[1] and approved for clinical use as an anesthetic in 1970. Soon after, it became a recreational drug of abuse due to its hallucinogenic and dissociative effects.[2] In the 1990s, ketamine became a popular 'club drug' and date rape drug,[2] under the street names Special K, Vitamin K, and K.[3] This lead to it being placed on the United States Controlled Substances list as a Schedule III substance in 1999.[4]

The structure of ketamine is similar to the drug phencyclidine (PCP), and produces similar effects in the body, with visual effects similar to those of LSD (Figure 1).[5] Ketamine was introduced in the early 1960s as an anesthetic alternative to PCP because it did not produce the same psychotic reactions.[6]



Ketamine PCP

Figure 1. The chemical structure of ketamine compared to PCP

Berman et al.[7] was the first to examine the acute effects of ketamine on depression in humans. This study involved seven patients with major depression. They received an intravenous treatment of ketamine hydrochloride (0.5 mg/kg) on two days that were separated by at least one week. They found that 50% of the subjects with major depressive disorder experienced greater than 50% improvement in their depression symptoms, as measured by the Hamilton Depression Rating Scale, within 72 hours of treatment. In 2006, Zarate et al.[8] showed similar results after giving subjects (n=18) with major depressive disorder (MDD) a single intravenous infusion of ketamine at a dose of 0.5 mg/kg over 40 minutes. The response rate to ketamine was 71% 24 hours after infusion. Diazgranados et al.[9] replicated the Zarate et al.[8] study in 2010, but the patients in the study had a diagnosis of bipolar depression instead of MDD and received infusions on two test days that were two weeks apart. The response rate to ketamine after 24 hours' post-infusion was 44%. Ketamine is now being used in some clinics as an antidepressant treatment, specifically MDD and bipolar depression (BD). It has also been shown that ketamine may be effective in treating cases of post-traumatic stress disorder, and acute suicidal ideation.[10]

Ketamine works differently than the majority of antidepressants in that it mainly targets the glutamatergic system in the body instead of the serotonergic and noradrenergic systems.[11] Ketamine also has a rapid onset of effects that ranges from 2-24 hours' post-infusion and these effects may last up to a week.[12] Other antidepressant treatments take much longer to achieve the desired effects. For example, it may be 2 weeks or more before monoamine oxidase inhibitor antidepressant treatments begin to take effect.[13] Studies have determined that the average time for full response to currently available

antidepressant treatments is 20 days.[13] Because ketamine's onset of effects is so rapid compared to what is currently available on the market, it is the focus of much research. There are many studies that focus on the, cellular, pharmacological, and brain circuit mechanisms of ketamine.[11]

One emerging area of research involving ketamine and other antidepressants is differences in the way females and males respond to treatments.[14] Women experience depression at about twice the rate of men.[14] Yet in many antidepressant studies, only male subjects are used, even though it is proven that women respond differently to several types of depression treatments.[14] Sex differences in antidepressant response are attributed to differences in the brain, both functional and anatomy, as well as different hormone environments.[15]

Beery and Zucker[16] brought sex bias in neuroscience and other biomedical research areas to light in 2011. They found that single-sex studies of males were predominate in many disciplines due the assumptions that the results from male studies apply to females or that female hormone cycles introduce unwanted variable concerns into the study.[16] In recent years however, the interest and number of studies that examine sex differences in response to anti-depressant treatment have increased.

Female rats have been found to be sensitive to lower doses of ketamine (2.5 mg/kg) than male rats.[15] The standard dose given for research on the antidepressant effects is 10 mg/kg.[6,15] Carrier and Kabbaj[15] found that ovariectomized female rats, like male rats, did not respond to ketamine at the dose of 2.5 mg/kg. When the female rats were given doses of estrogen and progesterone that mimic the 4-day estrous cycle, they showed an antidepressant response in a forced swim test after being dosed with 2.5

mg/kg of ketamine. This indicates that these hormones may play a role in the different antidepressant responses seen in males and females.[15]

A study conducted by Fransceschlli et al.[14] in 2015 also found that female mice were more sensitive to the rapid and sustained antidepressant effects of ketamine. The female mice responded faster and to lower doses of ketamine than male mice. At 30 minutes and 24 hours, females had a more efficient antidepressant response than male mice in a forced swim test, spending less time immobile than male mice. The data suggested that sex hormones play an important role in enhancing the antidepressant effects of ketamine.[14] Their conclusions were confirmed by Zanos et al. in 2016.[17] Sarkar & Kabbaj[18] showed that ketamine affects the spine density and synaptic proteins differently in males and female rats, which may also be a reason for increased sensitivity to ketamine's antidepressant effects in females.

Very little research has been conducted on the long-term effects of repeated ketamine treatments on the body and brain. The frequent use of ketamine, as seen in abusers of the drug, has produced symptoms of dependence, dissociation, amnesia, poor impulse control, and lower urinary tract dysfunction.[4] Ketamine's antidepressant effects take effect very quickly, but require repeated treatments in order to be sustained over a significant period of time.[19] After a single dose of ketamine, the average duration of effects in humans as shown in previous studies is five days.[20] A dose frequency study conducted in 2016 found that administering ketamine either two or three times per week for up to a 4 week period at a dose of 0.5 mg/kg was effective in maintaining antidepressant effects over a 15-day period in patients with treatment-resistant

depression. The results were similar in both the two and three times a week dosing groups.[20]

Thelen et al.[19] investigated the behavioral, neurochemical, and synaptic molecular effects of repeated ketamine treatments (10mg/kg per day over 21 days) in both male and female mice. There were opposite behavioral effects in male and female mice. The male mice demonstrated sustained antidepressant-like effects over the course of the treatment, while the repeated ketamine treatment in the female mice induced anxiety and depression effects as measured by an open field test and a forced swim test.

Ketamine is generally administered as a 1:1 racemic mixture of (R)- and (S)-ketamine hydrochloride.[10] There is an ongoing debate in the scientific community on which enantiomer of ketamine is more crucial in creating the long lasting antidepressant effects. It has been shown that the (S)-ketamine enantiomer has an affinity for the NMDA receptor that is approximately three times greater than (R)-ketamine, therefore an intranasal administration of (S)-ketamine is in development by a pharmaceutical company for treating depression[21]. However, (R)-ketamine was shown to have greater potency and longer lasting antidepressant effects than (S)-ketamine in animal models, which suggests that the NMDA receptor may not be the cause of the long-lasting antidepressant effects of ketamine.[21]

Ketamine is initially extensively metabolized by microsomal enzymes in the liver into a series of compounds.[3] In 1981, research was conducted using rat livers to help confirm what metabolites are formed in mammalian livers after ketamine is administered. This study was one of the first that found that ketamine breaks down into eight difference metabolites, with norketamine (NK) and dehydronorketamine (DHNK) being the primary

ones. The other metabolites that are formed in the liver include products of the alicyclic ring hydroxylation of ketamine and NK.[22] Another similar study confirmed that there were three hydroylated NK products formed in the liver in addition to NK and DHNK.[23] Early studies on ketamine indicated that DHNK was only an artifact created after being introduced to the high temperatures of a gas chromatograph/mass spectrometer (GC/MS).[3] However, subsequent studies have confirmed that DHNK is in fact produced in the body and not during the analysis process.[12]

Norketamine has been shown to be the major active metabolite in the body when ketamine is administered at anesthetic doses in humans and animals.[17] Norketamine is formed by a N-demethylation of the parent drug.[3] Another metabolite of ketamine is DHNK which is formed by the dehydrogenation of NK.[12]

It is estimated that ketamine has a half-life of about 2 hours and NK has a half-life of about 5 hours in blood.[11] Yet the antidepressant effects may persist up to a week after a single intravenous infusion[7, 8], even after all of the ketamine is supposedly cleared from the blood. One explanation may be that metabolites of ketamine are responsible for the sustained antidepressant effects.[11]

In the urine, approximately 2% is excreted as the parent drug, ketamine, 2% is excreted as NK, 16% is excreted as DHNK, and the rest is excreted as conjugates of hydroxylated metabolites.[2] Parkin, et al.[24] developed an analytical method to detect ketamine in human urine and found that ketamine could be detected in urine 3-5 days following administration, norketamine could be detected for 4-6 days, and DHNK could still be detected 6-10 days after administration.

The disposition of ketamine in the body is similar to some short-acting barbiturates.[25] Because ketamine and its metabolites are highly lipid soluble, they initially accumulate in highly permeable tissues. Therefore, the initial levels of ketamine and its metabolites are four to five times higher in these tissues, such as the brain, than in the plasma. The rush of ketamine to the brain, accounts for its dissociative and anesthetic effects.[25] It has also been proven that the brain does not metabolize ketamine and therefore any accumulation of metabolites in the brain is a result of metabolite formation outside of the brain.[26]

Like the majority of pharmaceuticals, ketamine's metabolites play an important role in the pharmacokinetics and pharmacodynamics of the drug. There is ongoing research being conducted to try and determine the exact molecular mechanisms causing the antidepressant effects, what metabolite is primarily responsible for ketamine's antidepressant properties, and if there are any differences in the concentrations of certain metabolites in males and females. Salat et al.[27] examined ketamine, NK, and DHNK and the role each of them play in the antidepressant effects. Ketamine and NK, when given at acute sub-anesthetic doses, reduced immobility time during a forced swim tests in rats. DHNK had no effect on immobility time during the forced swim test, which leads to the conclusion that DHNK does not play a role in creating antidepressant-like effects.

A study conducted by Zarate et al.[11] focused on ketamine, NK, DHNK, the six enantiomers of hydroxynorketamine (HNK), and hydroxyketamine (HK) concentrations in plasma taken from human participants as they related to strength of antidepressant effect and psychotic and dissociative symptoms. The structures of these metabolites as

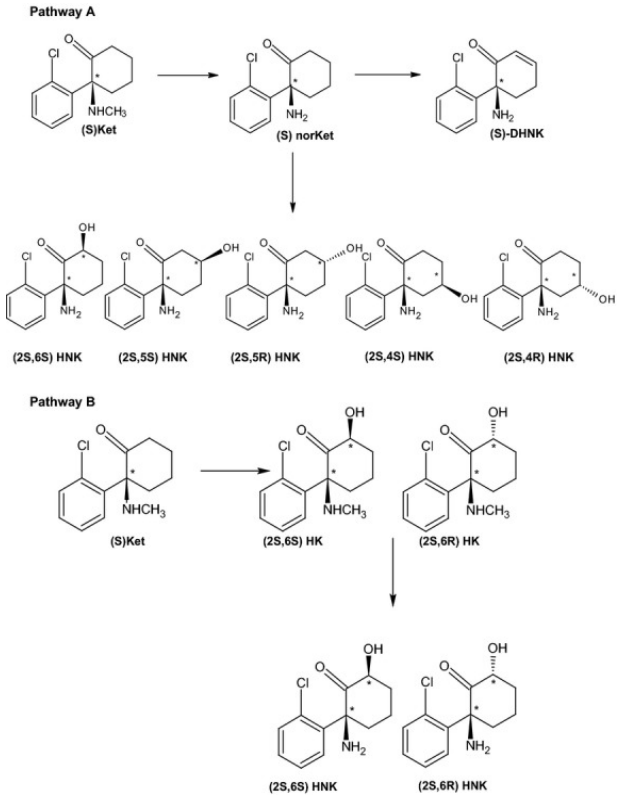


Figure 2. Metabolic pathway of ketamine.[28] From "The Distribution and clearance of (2S, 6S)-hydroxynorketamine, an active ketamine metabolite, in Wistar rats." By Moaddel, R., et al., 2015, *Pharmacology Research & perspectives*, 3(4). Reprinted with permission.

well as the proposed metabolite formation pathways are presented effects in Figure 2.[28] It was determined that the metabolite (2S,6S;2R,6R)-HNK was still present in the plasma 3 days after the ketamine infusion. The results showed that, in females, DHNK, (2S, 6S)-HNK, and (2S, 5S)-HNK levels were higher than they were in males and males had higher (2S, 6S)-HK levels than female participants. In addition, evidence was found that (2S, 5S)-HNK is a pharmacologically active metabolite that may also play a role in ketamine's antidepressant effects.

A study conducted by Zanos et al.[17] sought to investigate the roles of different metabolites in causing the antidepressant effects of ketamine. They explored differences in the pharmacokinetic profile of ketamine in males and females by examining the concentrations of ketamine, NK, and HNK in the brain and blood. They report that the

major HNK metabolite found in the plasma and brain of mice and plasma of humans after intraperitoneal administration is (2S,6S;2R,6R)-HNK. They found that in female mice, the concentration of (2S,6S;2R,6R)-HNK was 3 times higher in the brain compared to males. Therefore, based on the findings that ketamine is more potent in females than males, they hypothesized that this metabolite plays a crucial role in producing the antidepressant effects of ketamine. In contrast to Zarate et al., they concluded that the more potent enantiomer of HNK is (2R,6R)-HNK.

In some patients, ketamine can have adverse side effects including blurred vision, numbness, and psychotic features.[10] Therefore, discovering the metabolite of ketamine that causes the antidepressant effects would be important because it may not carry the same adverse side effects as the parent drug when administered. Newly reported research by Zanos et al.[17] demonstrates that when (2R,6R)-HNK is administered it produces the antidepressant effects associated with ketamine while lacking negative ketamine-related side effects.

In response to the finding that (2R, 6R)-HNK was responsible for the antidepressant effects of ketamine, Yang et al.[21], tested (R)-ketamine, (S)-ketamine, and (2R, 6R)-HNK in male mice. They found that (2R, 6R)-HNK showed no rapid or sustained antidepressant activity in the inflammation or social defeat stress models and (R)-ketamine showed greater potency as an antidepressant than (S)-ketamine. The researchers in this comparison study recommend further research comparing the antidepressant effects of (R)-ketamine and (2R,6R)-HNK.[21]

Many researches have expressed concern and criticism over ketamine being currently used as an off-label treatment for depression in some clinics based on their

opinion that there is insufficient medical evidence to support this application.[4] Some researchers have recommended repeated ketamine infusions to sustain the antidepressant effects, even though the long-term effects of this drug given at sub-anesthetic doses have not been extensively studied.[4] Because this is a drug of abuse that is being given to a vulnerable section of the population, the addition potential and tolerance effects should be considered before deciding on repeated ketamine infusions for treatment.[6] A report by the Canadian agency for Drugs and Technologies identified flaws in previous trials involving ketamine and pointed out that there is a lack of comparison of ketamine to other antidepressant treatments that are already validated. Therefore, they state it is unknown if ketamine treatment is better or worse than the antidepressants currently on the market.[4] Some critics also point out that there are other psychedelic drugs, such as amphetamines, that produce mood-elevating effects similar to ketamine after a single dose, but quickly fall back into a depressive state that may cause them to become addicted to the drug.[4] There is no other antidepressant that acts as an antidepressant at low doses and a drug of abuse at high doses, which also raises concerns about the safety of ketamine antidepressant treatment.

The purpose of this study was to investigate the levels of ketamine and NK in the plasma, liver, cerebellum, prefrontal cortex, and hippocampus of male and ovariectomized female rats after intravenous injection of ketamine. The concentrations were determined at three different points in time after dosing to determine where the ketamine was distributed, as well as how quickly the NK was being formed in each area of the body. The results from the male and female rats were also compared to identify any differences in how ketamine and NK are distributed.

MATERIALS AND METHODS

Sample Collection

The samples were collected from Sprague Dawley® (Charles River) male and ovariectomized female rats that were sacrificed at times 0 minutes, 30 minutes, and 3 hours after dosing. For each time period, three rats of each gender were dosed with an intravenous injection of ketamine at 10 mg/kg and a control rat was injected with a saline solution. Plasma, liver, cerebellum, prefrontal cortex, and hippocampus samples were collected for each rat. Samples were then placed in polypropylene tubes and stored at -80°C until analysis.

Sample Preparation

Samples were thawed, transferred into 2.0 mL polypropylene graduated tubes, and weighed before 10-15 metal beads (2.4mm) were added to each tube. Then, 500 mL of water and 50 μL of the internal standard, ketamine-d_4 (100 mg/L), were added to the sample. The tubes were vortexed until the tissue sample was fully homogenized. The tubes were then centrifuged at a speed of 12.5 x 1000 rpm for 15 minutes in an Eppendorf Minispin centrifuge. The supernatant was transferred to a new tube containing 1 mL of acetonitrile in order to precipitate the proteins. The tubes were then vortexed for a minute and centrifuged at the same speed for another 15 minutes. The aqueous layer was added to a test tube containing 2 mL of sodium acetate buffer (0.1 M) at a pH of 5 to begin the extraction procedure. The sodium acetate buffer was prepared by combining a 0.1 M

solution of sodium acetate and a 0.1 M solution of glacial acetic acid in a ratio of 1:2.39 in distilled water.

Analyte Extraction

The sample preparation and analyte extraction techniques were adapted from a method for extracting ketamine provided by DPX Technologies (Columbia, SC). Solid-phase extraction (SPE) dispersive pipette tips (5 ml, 5S-5TF25-02-030-050-5B DPX Technologies) were used to perform the extraction of ketamine and NK from the samples. The tips were conditioned by aspirating with 3 mL of methanol followed by 3 mL of water. Then the liquid sample was aspirated for approximately 15 seconds, and dispensed from the tips. This step was repeated four times. Next, 2 mL of the sodium acetate buffer were aspirated and dispensed one time, followed by 2 mL of methanol. Lastly, 3 mL of a 3% ammonium hydroxide solution in acetonitrile, at a pH of approximately 10, was aspirated and dispensed one time. The sample in the test tube was evaporated to dryness under nitrogen in an Organomation Associate's N-EVAP™112. The residue was dissolved in 100 μL of hexane and transferred to 200 μL microvials for GC/MS analysis with an Agilent 6890N Network GC system/5975 inert Mass Selective Detector with selected ion monitoring. The ions used for quantitation for ketamine, ketamine-d_4, and NK were 180, 184, and 166 respectively. Qualifier ions used for ketamine were 138 and 152 and for NK were 131 and 195. Exact details of the method used can be found in Appendix A.

Quantitation and Analysis

Quantitation of ketamine and NK in the samples was done by the use of a calibration curve created by preparing solutions containing a known concentration of ketamine and NK and taking them through the extraction process. This was done in order to account for any loss of the analytes occurring during the extraction process. Calibration curve standards were prepared at 1, 3, 5, 10, and 15 ng/μL each week from ketamine and NK standards (1mg/mL) purchased from Cerilliant and a calibration curve was prepared based on the results. The equation for the line of best fit was then generated in Microsoft Excel and used to calculate the concentrations in the rat samples. The calibration curves were linear for all of the analytes with r^2 values ranging from 0.98 to 0.99. The LOQ for both ketamine and NK was 1 ng/μL.

RESULTS

The average retention times for ketamine, d_4-ketamine, and NK are 8.16 ± 0.054, 8.10 ± 0.052, and 7.31 ± 0.070 minutes respectively. Representative chromatograms and mass spectra for each analyte can be found in Appendix B. Results are reported as μg/gram of sample because of the variations in the sample weights.

The results for the amount of ketamine in each tissue at each time point are presented in the graphs below (Figure 3). In all of the tissue samples, the ketamine amount is the greatest at time 0. After 30 minutes there is a very small amount of ketamine remaining and by 3 hours, ketamine is below the limit of quantitation for all samples. The amount of norketamine in each of the samples at each time point is presented in Figure 4. Norketamine is not detected in significant quantities in any samples at time 0 except for in the male liver samples where it is detected at an average of 1μg/gram of sample. Norketamine is not detected in the plasma at 30 minutes or 3 hours. In the liver, cerebellum, prefrontal cortex, and hippocampus, the concentration of NK at 30 minutes is right at or below 1 μg/gram. The highest concentrations of norketamine at 30 minutes are in the male hippocampus and male liver. By 3 hours, NK is below the level of quantitation (1μg/gram of sample) in all of the samples.

Each of the brain samples had significantly higher concentration of ketamine at time 0 than the plasma or liver samples. Out of the three sections of the brain, the prefrontal cortex had the most ketamine at time 0 (12-13μg/gram of sample) . By 30-minutes, no significant difference can be seen between the concentrations of ketamine and NK in the 3 sections of the brain.

There is no notable difference seen in the concentration of ketamine and NK between the male and ovariectomized female rat samples. The only slight difference is in the liver where at time 0 in females there was no NK, while in males norketamine was already forming. Out of all the tissues examined, NK appeared the fastest in the liver in males and stayed at relativity the same concentration in this tissue at time 0 and 30 minutes.

All of the control rat samples that had been injected with a saline solution only were negative for ketamine and norketamine. The results of the amounts of ketamine and NK compared on the same graph in each of the tissues and for each gender can be found in the bar graphs presented in Figure 5 below.

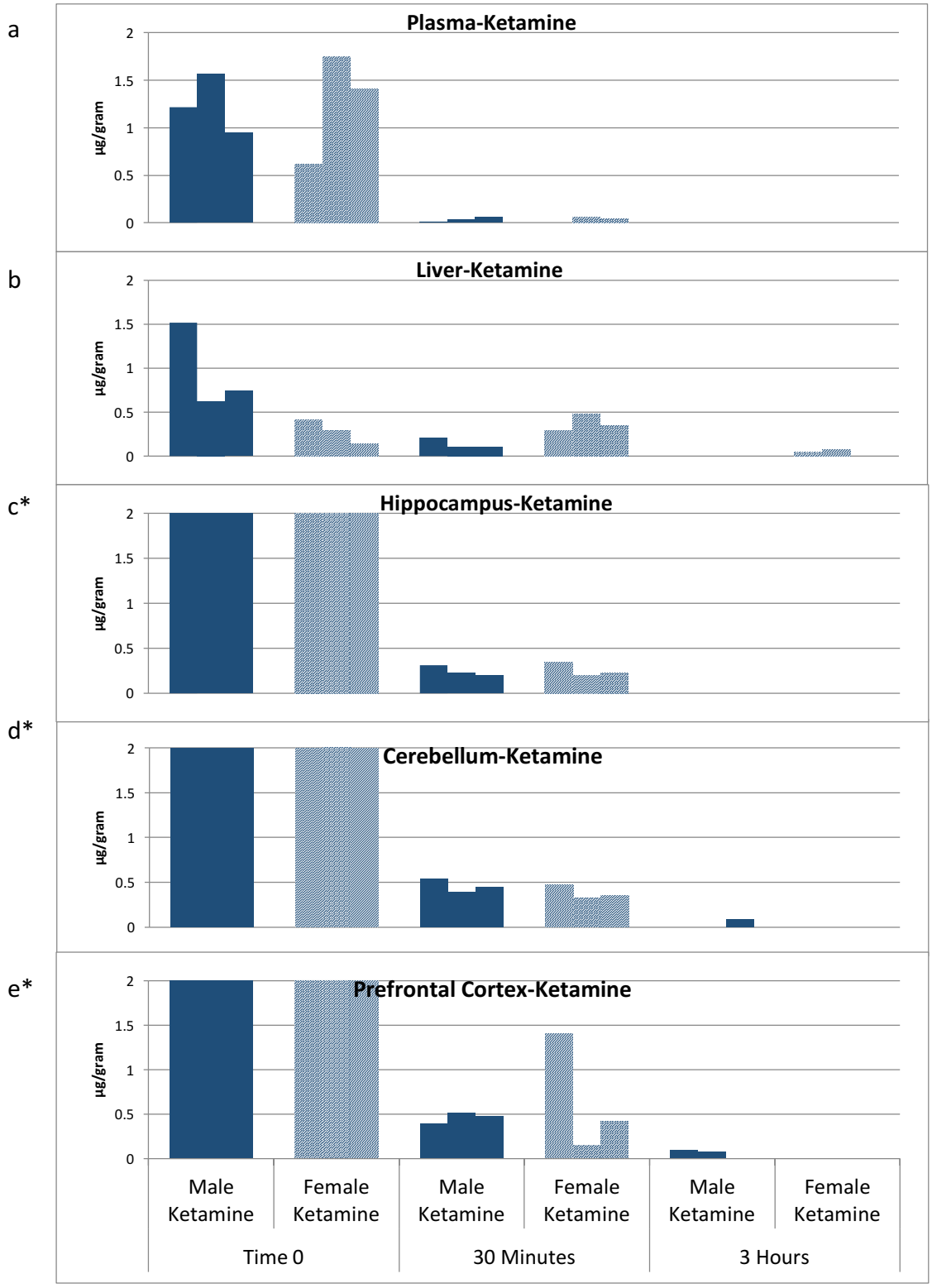


Figure 3. Graphs depicting the amount of ketamine in the five tissues examined in males and female rats at each time point. (a) Plasma amount, (b) Liver amount, (c*) Hippocampus amount, (d*) Cerebellum amount, and (e*) Prefrontal Cortex amount. *Amount of ketamine at time 0 in each of the brain sections for males and females are off scale. Please see Figure 5 for full scale.

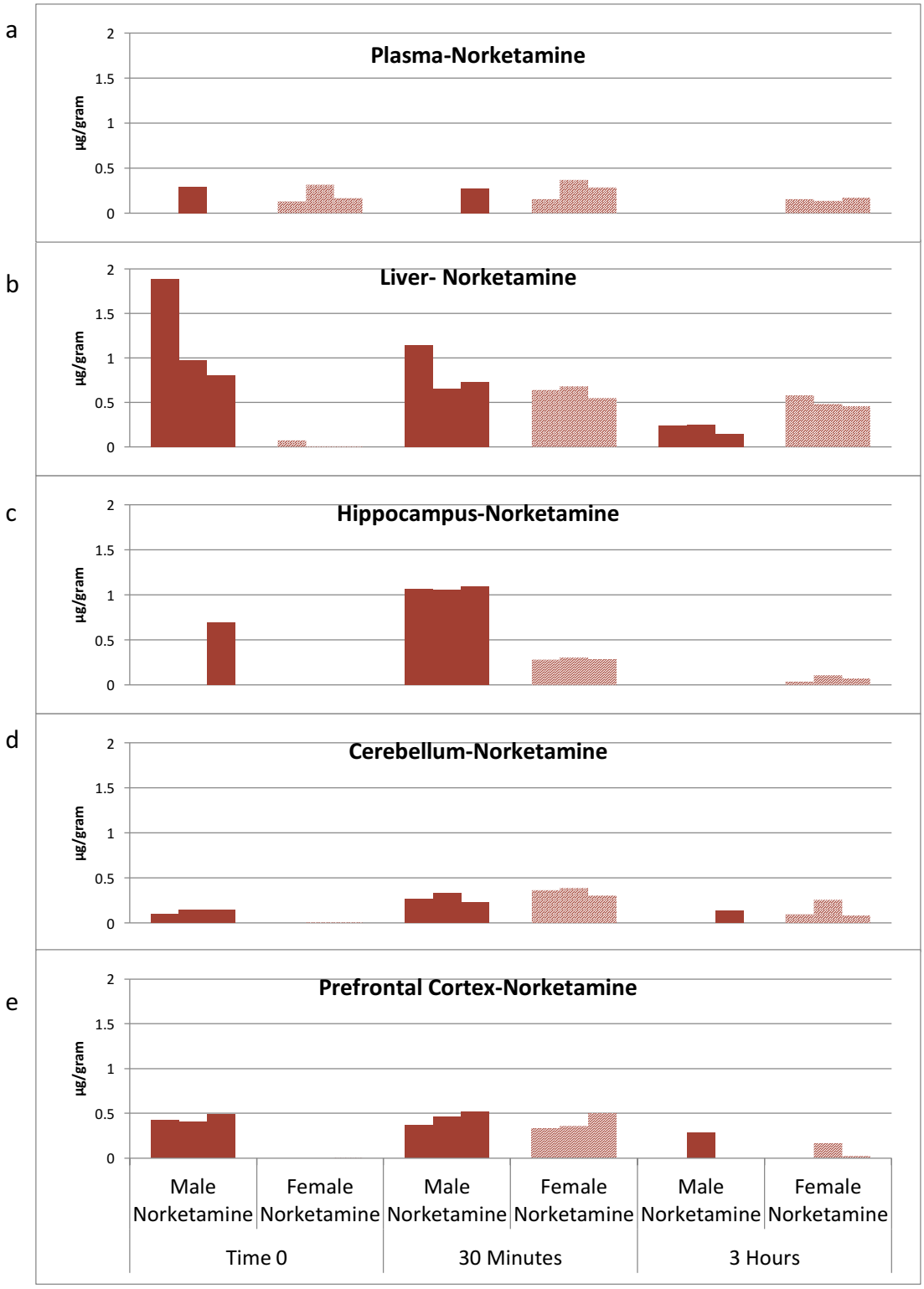


Figure 4. Graphs depicting the amount of norketamine in the five tissues examined in males and female rats at each time point. (a) Plasma amount, (b) Liver amount, (c) Hippocampus amount, (d) Cerebellum amount, and (e) Prefrontal Cortex amount.

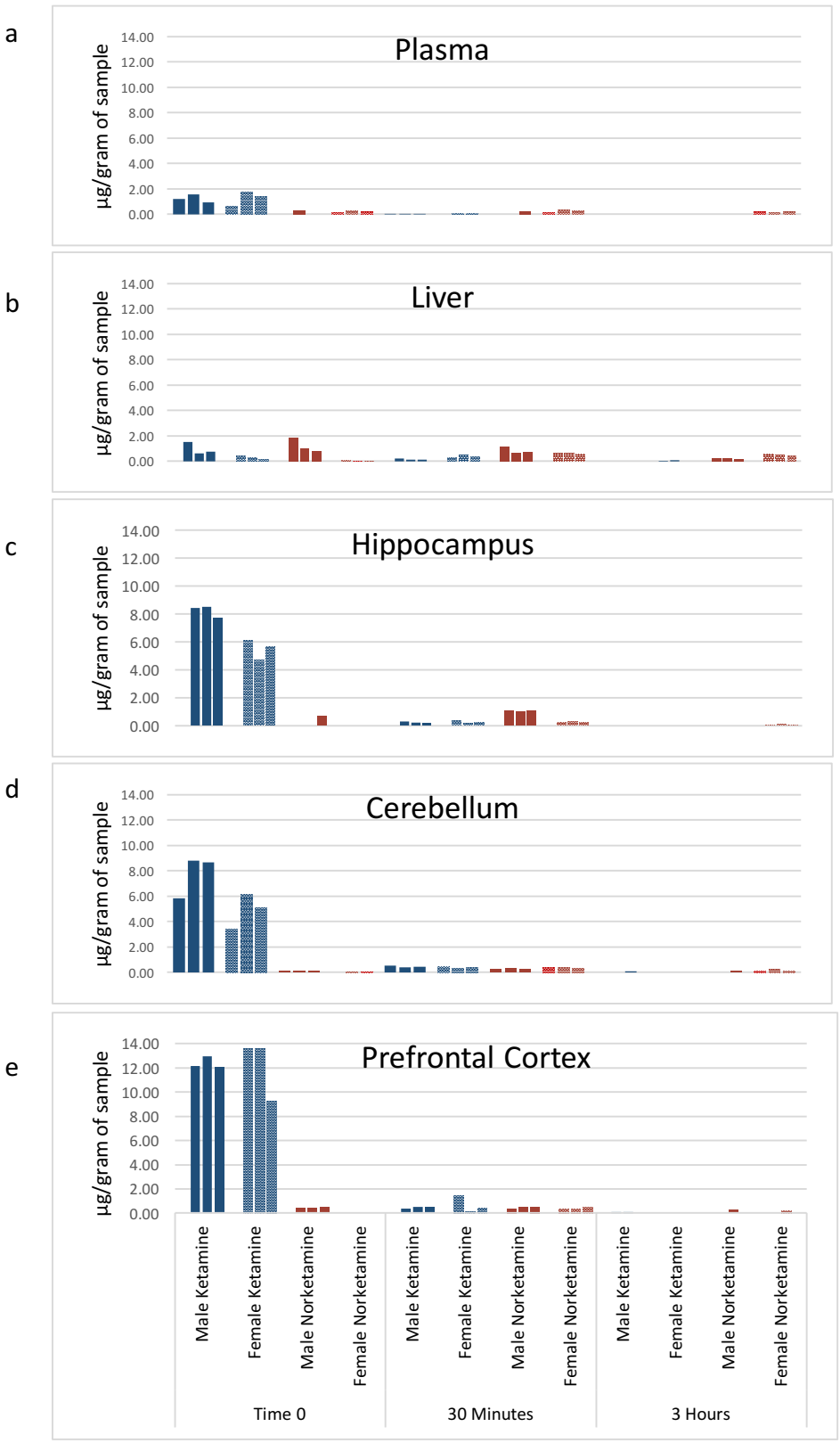


Figure 5. Graphs depicting the amounts of ketamine and NK in the five tissues examined in males and female rats at each time point. (a) Plasma concentrations, (b) Liver concentrations, (c) Hippocampus concentrations, (d) Cerebellum concentrations, and (e) Prefrontal Cortex concentrations.

DISCUSSION

These results show that ketamine reaches the brain tissue very quickly and that the initial concentration in the brain is much higher than in the plasma or liver. Ketamine and NK are also virtually cleared by 3 hours in all of the tissues. This is interesting to note as the antidepressant effects of ketamine have an onset time beginning around 2 hours after the dose is given.[12]

The high concentration in the brain samples at time 0 is not unexpected. Early studies of ketamine concentrations in the brain and plasma showed that ketamine enters the brain very rapidly after intravenous administration, achieves maximum concentration levels within a minute, and then declines.[26] Cohen et al.[26] also reported that the average brain: plasma ratio of ketamine was 6.5:1 consistently over a period of 10 minutes which indicated to them that the blood-brain barrier was almost non-existent for ketamine. It is reasonable that NK is detected the fastest in the liver because that is where the majority of the cytochrome P450 enzymes responsible for metabolism are located.[3]

With a sample size of 3, it is not clear if the variation seen between sample is due to individual rat variations between the rats or inconsistencies in the extraction procedure. Additional samples should be added to each set to determine if the pattern continues.

There was no notable difference in the concentrations of ketamine and NK in the male and ovariectomized female samples. This supports previous behavioral studies which have found that there is no difference in male and ovariectomized female anti-

depressant response to ketamine treatment. [14,15] This study will serve as a baseline for future studies of the effect of hormones on the metabolism of ketamine.

CONCLUSION

This study investigated the distribution of ketamine and its major metabolite NK in five different tissues of male and female rats. It was determined that ketamine appears in the brain tissues very quickly most likely due to its high lipophilicity. Ketamine is virtually cleared from all of the tissues examined by 3 hours. Norketamine is detected earliest in the liver of males and is also virtually undetectable in all of the samples by 3 hours. More data at each time point for each gender is needed in order to draw any additional conclusions concerning the variability of the samples in one time period. This study serves as a pilot project to establish a reliable testing method and baseline results for the amounts of ketamine and norketamine in male and ovariectomized female rats. Therefore, there are many ways that this study could be expanded upon. Dosing female rats with estrogen and progesterone and comparing them to male rat samples would help see if female hormones affect the metabolism or distribution of ketamine. In addition, quantifying additional metabolites of ketamine at each time point, especially enantiomers of HNK, would further help to see if there are any differences in male and female metabolite concentrations.

If ketamine becomes a widely used treatment for depression it will be important to document any differences in the pharmacokinetics and pharmacodynamics of the drug in males and females at this dose. This may become important information to know in the field of forensic toxicology in order to accurately interpret results involving impairment

from ketamine. As this drug is already a controlled substance, its more widespread use as an antidepressant may also increase its recreational use.

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APPENDIX A

GC/MS METHOD PARAMETERS

Table A1
SIM GC/MS parameters for ketamine and norketamine

Method Parameters	**Experiment Time:**	30 minutes
	Inlet Mode:	Split-Ratio 5:1
	Column Mode:	Constant Flow (1.0 mL/min)
	Injection Volume:	2 µL
	Oven Temperature Initial:	175°C for 10 Minutes
	Oven Program:	20°/min until temp. reaches 250°C-Hold for 16.25 min.
	Injector Temp.:	250°C
	Detector:	Mass Selective in Vacuum
	SIM Parameters:	**Norketamine**: Monitor ions 131, 166,170, and 195 from 5-8 minutes. **Ketamine and Ketamine-d_4**: Monitor Ions 138, 152, 180, and 184 from 8 min. to end **(dwell time of 50 msec for each ion)**
Column and Gas Information	**Column Information:**	Agilent HP-5MS: 30 m x 250 µm x 0.25 µm
	Carrier Gas:	Helium (UHP)
	Total Flow Rate:	1 mL/min.

APPENDIX B

EXAMPLE CHROMATOGRAMS AND MASS SPECTRA

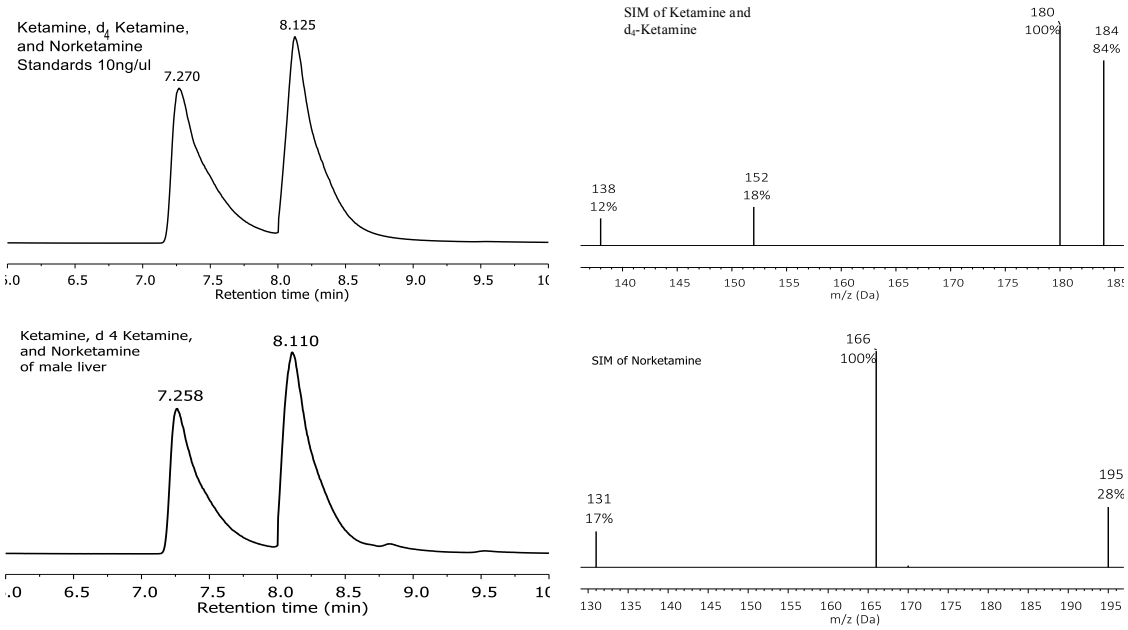

Figure B1. Representative chromatograms and mass spectrums for each analyte. Top chromatogram is from a standard sample and bottom chromatogram is from a 30-minute male liver sample. Norketamine has the retention time 7.270 min and Ketamine and d_4-Ketamine both elude at 8.125 min. in the top chromatogram and similar results are seen in the bottom chromatogram. Both mass spectrums were obtained from the standard sample. An extracted ion chromatogram was used to separate the two analytes that eluded at 8 minutes.