Original Paper

3-Methyl-methcathinone: Pharmacokinetic profile evaluation in pigs in relation to pharmacodynamics

Jakob A Shimshoni[1], Malka Britzi[2], Eyal Sobol[2], Udi Willenz[3], David Nutt[4] and Nir Edery[5]

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Abstract

3-Methyl-methcathinone (3-MMC) is a novel, synthetic cathinone analog, recently linked to poisoning events among recreational users. The lack of pharmacological data on 3-MMC, prompted us to explore its pharmacokinetic profile as well as its effect on feeding behavior, weight gain, and serum biochemistry. 3-MMC was administered to male pigs (n=3, three months old) as a single intravenous dose (0.3 mg/kg), followed by a multiple oral dose administration (3 mg/kg) for five days and plasma and tissue concentrations determined. Concomitantly a control group consisting of two healthy male pigs received saline solution instead of 3-MMC according to the same administration schedule. 3-MMC effects on complete blood count, biochemistry, feed intake, and body weight were examined. The pigs were sacrificed and submitted to a pathological and histopathological examination. 3-MMC displayed rapid absorption with a peak concentration achieved within 5–10 min after oral ingestion and a plasma half-life of 0.8 h. The bioavailability was about 7%. 3-MMC tissue levels were below detectable levels 24 h after the last oral dosage. No treatment-related clinical signs were observed and no histopathological findings were detected. 3-MMC caused significant change in daily feed intake and weight gain over time. The animals treated with 3-MMC displayed a lower rate of increase in mean body weight. Caution needs to be practiced in terms of extrapolating the present data to human safety, due to the low sample size, low dosage, and the relatively short study duration as well as the lack of data on abuse potential of 3-MMC.

Keywords

3-Methyl-methcathinone, mephedrone, pharmacokinetics, thermoregulation, feed intake, weight gain, pig

Introduction

Synthetic cathinones such as mephedrone are widely used recreational drugs due to their empathogenic and sympathomimetic stimulatory effects (Green et al., 2014; Kelly, 2011). The increased recreational misuse of the synthetic cathinone mephedrone, coupled with media concerns about possible harms prompted the UK government to ban a range of cathinone derivatives on 16 April 2010, under the 1971 Misuse of Drugs Act (European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), 2010, 2014; Nutt, 2011). Accordingly, in the USA, the Department of Justice and Drug Enforcement Administration (DEA) issued a final ruling, in October 2011, placing three synthetic stimulants, 4-methylmethcathinone (mephedrone), methylenedioxypyrrovalerone (MDPV) and 3,4-methylenedioxymethcathinone (methylone) under Schedule I of the Controlled Substances Act (DrugFacts: Synthetic Cathinones ("Bath Salts"), 2012); EMCDDA, 2010, 2014; Moris, 2010). In July 2012, the US President signed legislation permanently making mephedrone and MDPV illegal (DrugFacts: Synthetic Cathinones ("Bath Salts"), 2012).

Some synthetic cathinones increase the catecholamine extracellular levels in the central and peripheral nervous system by acting as substrates and /or inhibitors of monoamine transporters, thereby reducing the clearance of the monoamines from the synapse and stimulating non-exocytotic monoamine release by reversing the transporter efflux (Kelly, 2011; Shortall et al., 2013a, 2013b). Some of the synthetic cathinones such as mephedrone were shown to bind with relatively high affinity to 5-hydroxytryptamine receptor and α_1/α_{2a}-receptors, which contribute to their sympathomimetic effects (Shortall et al., 2013a, 2013b).

The most common routes of synthetic cathinones administration in recreational users are reported to be oral and insufflation, while repeated administration in a single session is very common (binge dosing), due to the short duration of psychoactive response (Green et al., 2014; Kelly, 2011). Common adverse effects of synthetic cathinones included cardiovascular (palpitations, chest pain), gastrointestinal (nausea, vomiting, abdominal pain), neurological/psychiatric (agitation, psychosis, memory loss, tremor, anxiety) and musculoskeletal (numbness and coldness of extremities) signs (Prosser and Nelson, 2012; Schifano et al., 2011). However, these

[1]Department of Toxicology, Kimron Veterinary Institute, Bet Dagan, Israel
[2]National Residue Control Laboratory, Kimron Veterinary Institute, Bet Dagan, Israel
[3]The Institute of Animal Research, Kibbutz Lahav, Israel
[4]Neuropsychopharmacology Unit, Imperial College London, London, UK
[5]Department of Pathology, Kimron Veterinary Institute, Bet Dagan, Israel

Corresponding author:
Jakob A Shimshoni, Department of Toxicology, Kimron Veterinary Institute, Bet Dagan, Israel.
Email: jakobshimshoni@gmail.com

symptoms occur mostly in users who simultaneously consume alcohol and/or other drugs (Green et al., 2014). Moreover, the purity as well as the dosage of the self-administered synthetic cathinones could not be established and, based on previous investigations of synthetic cathinone products sold online and on the street, all products consisted of multiple cathinone derivatives and some of the products contained local anesthetics at varying dosages (Leffler et al., 2014; Zamengo et al., 2014).

Mephedrone is one of the most common cathinone-derived designer drugs consumed by recreational users and consequently, the most studied synthetic cathinone in rodents in terms of pharmacological and toxicological effects (Dybdal-Hargreaves et al., 2013; Green et al., 2014; Kelly, 2011). Mephedrone's desired clinical effects sought by recreational users include euphoria and enhanced sex drive while, generally, most common unwanted side effects are exaggerations of its pharmacological effects, mostly excessive CNS and cardiovascular stimulation (Dybdal-Hargreaves et al., 2013; Green et al., 2014; Kelly, 2011; Prosser and Nelson, 2012; Schifano et al., 2011). There have been a number of deaths where mephedrone has been considered to be involved (Adamowicz et al., 2013; Gerace et al., 2014; Green et al., 2014; Leffler et al., 2014; Schifano et. al., 2012; Zamengo et al., 2014).

Mephedrone users reported taking between 150–250 mg in repeated administration, often resulting in ingestion of 0.5–1 g during a single session, with a clinical effect setting on between 15–45 min after oral administration and lasting up to 4–5 h (Green et al., 2014; Kelly, 2011).

After the initial ban on mephedrone, a new market for second generation synthetic cathinones quickly emerged. Mephedrone's structural isomer 3-methyl-methcathinone (3-MMC) is presently considered illegal in Israel, USA and the UK (Dangerous Drugs Ordinance; EMCDDA, 2014). A study conducted recently in Sweden reported numerous intoxication cases associated with 3-MMC exposure among recreational users (Adamovicz et al., 2013, 2014; Bäckberg et al., 2014). Due to the lack of toxicological and pharmacokinetic/pharmacodynamic data on 3-MMC and the emerging clinical case reports associated with 3-MMC intoxication, this prompted us to explore its pharmacokinetic profile as well as its effect on feeding behavior, weight gain and serum biochemistry.

Materials and methods

Drugs

Pure 3-MMC hydrochloride (98.8%) was obtained as a gift from Z-Chem (Amsterdam, the Netherlands) and its purity confirmed by standard analytical methods utilizing liquid chromatography tandem mass spectrometry (LC/MS/MS) and nuclear magnetic resonance. Physicochemical properties were determined according to the methods published by Zimmerman (1986).The melting point was determined using the Mettler Toledo FP62 instrument (Greifense, Switzerland), while cLog P was theoretically calculated by utilizing ACDlabs software (Toronto, Canada). The ultraviolent (UV) spectrum of 3-MMC hydrochloride dissolved in water was recorded with diode-array UV-detector, Agilent Technologies Deutschland GmbH, Waldbronn, Germany. 3-MMC hydrochloride solutions for injection and oral administration were prepared in pyrogen-free sterile 0.9% saline solution immediately before administration (Teva, Israel). Isoflurane and xylazine were purchased from Piramal Critical Care Inc. Bethlehem, Pennsylvania, USA. Reagents required for LC/MS/MS assay were obtained from Sigma-Aldrich (St. Louis, Missouri, USA).

Animals

Six healthy commercial male pigs (Landrace) weighting 28–34 kg, three months old were purchased from, and maintained throughout the experiments at, The Institute of Animal Research, Kibbutz Lahav, Israel. Management, treatment, sampling, and sacrifice of the pigs utilizing xylazine and isoflurane were performed according to Israeli laws and regulations. The animals were housed individually in pens in a humidity and temperature-controlled (22–24°C) vivarium with water freely available. The pigs had access to 2 kg dry feed in the morning and 2 kg dry feed in the afternoon (08:00 and 16:00) during the whole study. Due to an infection of the hind limbs of one of the pigs at the beginning of the study, the control group eventually consisted only of two healthy pigs, while the treatment group consisted of three pigs being randomly assigned to each group.

Pharmacokinetic study

The experiment was performed as a two-way design with a washout period of three days between intravenous (i.v.) bolus and oral administration (p.o.) within the same treatment group, so that each pig served as its own control for the calculation of the various pharmacokinetic parameters. A bolus dose of 0.3 mg/kg body weight (BW) 3-MMC hydrochloride dissolved in 10 mL saline for injection was injected into the jugular vein via a sterile central vein catheter (Novolab). Blood (4 mL) was collected from the jugular vein into heparin-coated tubes (Vacutest Kima) before administration (time 0 h) and at 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 10, 12, and 24 h post-administration. Blood samples were centrifuged at 3000 g for 10 min at 4°C and plasma was stored at −20°C until analysis within two weeks. Following a washout period of three days, the same pigs received a daily single oral dosage of 3 mg/kg via gavage (90 mg 3-MMC hydrochloride dissolved in 60 mL saline per animal) for five consecutive days and blood samples collected at 0, 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 10, 12, and 24 h after the first dosage administration. In the following days, blood samples were collected prior to the next oral administration. On the last oral dosage the same time schedule for blood collection was utilized as for the first oral dosage. For the control group (n=2), pure saline for injection instead of 3-MMC was given via i.v. bolus, followed by a multiple oral saline administration, according to the same drug regimen performed for the treatment group.

Pharmacokinetic parameter analysis

The pharmacokinetic parameters were calculated by noncompartmental analysis based on statistical moment theory using pharmacokinetic software WinNonlin version 4.1 (Pharsight Co., Mountain View, California, USA). Nonlinear curve fitting was performed by the method of least sum of squares utilizing WinNonline nonlinear estimation program. The distribution and elimination kinetics were determined after i.v. administration.

The half-life ($t_{1/2}$) and the volume of distribution (V) were calculated as follows:

$$t_{1/2}=\ln 2/\lambda,$$

where λ is the terminal slope of the concentration vs time curve following i.v. bolus administration

$$V=\text{Dose}\times\text{AUMC}/(\text{AUC})^2,$$

where AUMC is the area under the first moment of the plasma drug concentration time curve and AUC is the area under the concentration-time curve
Clearance (CL) was calculated according to the equation:

$$CL=\text{Dose}/\text{AUC}$$

The values reported for C_{max} and T_{max} are the predicted calculated values based on the Gauss-Newton algorithm utilizing WinNonline nonlinear estimation program. Bioavailability (F) was calculated using the following formula:

$$F=\text{Dose}_{oral}\times\text{AUC}_{oral}/\text{Dose}_{i.v.}\times\text{AUC}_{i.v.}$$

Absorption rate constant was determined by means of nonparametric numerical deconvolution as implemented in Win Nonlin (WinNonlin Enterprise Version 4.1 Pharsight Corporation, Mountain View, California, USA) using the automatic smoothing and initial rate not constrained to be zero options.

Analytical method validation

An overall validation of the method was performed using blank porcine plasma, brain and liver spiked with working standard solutions. The following set of parameters was included: recovery, linearity, accuracy, precision, limit of detection (LOD), and limit of quantification (LOQ). A calibration curve was established by spiking 200 µL plasma and 1 g homogenized tissue samples at six different concentrations of 3-MMC (0.5, 1, 10, 25, 50, 100 µg/kg) in six replicates. The calibration procedure was repeated for two additional consecutive days and mean linearity parameters calculated (Supplementary Material, Table S1). The extraction recoveries from plasma and tissue samples were calculated for low (1 µg/kg) and high (100 µg/kg) concentration levels in accordance with the guidelines defined by the European Union (European Commission, 2002). Analyte concentrations were determined by linear regression using the formula:

$$Y=mX+b;$$

where Y=normalized analyte area, X=analyte concentration in µg/kg

These data were used to determine the validation parameters. The extraction recoveries of the analytes were determined by comparing the results of the analysis of the spiked samples with those of the working solutions. The LOD and LOQ were calculated utilizing a specific calibration curve covering samples in the concentration range of 0.5–100 µg/kg according to the equations:

$$\text{LOD}=3\times(\text{SD}/m)\text{ and LOQ}=9\times(\text{SD}/m);$$

where SD= residual standard deviation of the regression line and m=slope of the calibration curve

A stability study of 3-MMC for the duration of 14 days at −20 ºC was established by spiking 3-MMC into blank pig plasma at low (1 µg/L) and high (100 µg/L) concentrations of six replicates each, according to the recommended guidelines reported by Hartmann et al. (1998). Concentration ratios between samples at day 0 and samples after 14 days storage at −20 ºC of 90–110% with 90% confidence interval within 85–115% have been regarded as acceptable criterion for stability.

Standard solutions

Stock solutions of 3-MMC was prepared in double-distilled water (DDW) at a concentration of 1.0 mg/mL and was found to be stable for three months when stored at −20 ºC. Working standard solutions were prepared by dilution in DDW to give the following concentrations: 10, 20, 200, 500, 1000, and 2000 µg/L.

LC/MS/MS analysis of 3-MMC in plasma, brain and liver tissues

3-MMC was analyzed by an in-house validated method according to the guidelines defined by the European Union (European Commission, 2002). Briefly, 0.2 mL plasma was acidified with 0.1 mL of 0.2% formic acid solution and plasma protein precipitated by addition of 0.4 mL acetonitrile. Following centrifugation at 14,000 rpm for 10 min, the upper phase was directly injected to the LC/MS/MS. For the analysis of 3-MMC levels in brain and liver tissues, 1 g tissue was homogenized in 1 mL 0.2% formic acid solution for 1 min followed by 10 min water-bath sonication. Sample workup was performed by vortex mixing 300 µL of supernatant with 400 µL of acetonitrile followed by centrifugation at 14,000 rpm for 10 min. Of the clear supernatant, 200 µL was transferred to an autosampler vial. The method utilized an Agilent 1100 (Agilent Technologies, Germany) liquid chromatography system (equipped with binary pump, degasser, column compartment and autosampler) combined with an Applied Biosystems ABI 3200 QTrap (Applied Biosystems, Toronto, Canada) mass spectrometer. LC separation was performed using a C18 Hypersil Gold column (3 µ, 100×2.1 mm, Thermo Electron Corporation, Bellefonte, Pennsylvania, USA). The mobile phase consisted of 0.2% formic acid (J.T. Baker, Deventer, the Netherlands) and acetonitrile (J.T. Baker, Deventer, the Netherlands). A gradient was applied by increasing acetonitrile concentration from 5% at 0 min to 80% at 2 min. This ratio was kept up to 4 min, then reversed to the initial conditions up to 4.5 min and allowed to stabilize for 3.5 min. The column compartment was kept at 30°C and the injection volume was 5 µL. Turbo ion spray for electrospray ionization with tandem mass spectrometry (ESI/MS/MS) in positive ion mode was operated at a temperature of 550°C. Multiple reaction monitoring (MRM) was applied. Precursor ion was 178.2 and precursor ions were 160.3 (quantifier) and 145.2 (qualifier).

Blood samples for complete blood count (CBC) and biochemistry analysis

Blood samples (3 mL) were collected from the jugular vein a day before the i.v. bolus administration, 48 h after the bolus injection and 24 h after the last oral dosage administered for five consecutive days. Blood samples were collected from the control group according to the same time schedule as the treatment group.

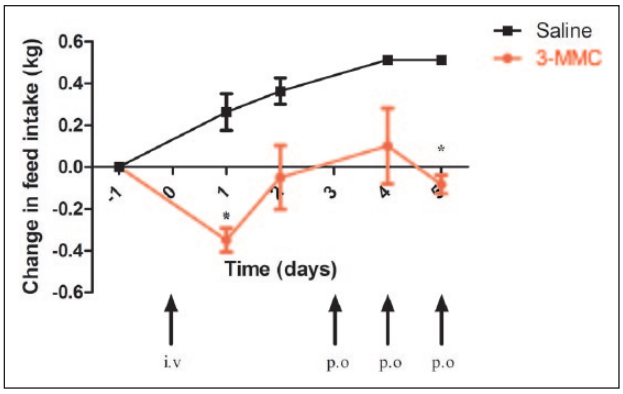

Figure 1. Effect of 3-methyl-methcathinone (3-MMC) on daily feed intake in individually housed pigs (n=3) in comparison to control group (n=2). Twenty-four hours prior to intravenous (i.v.) bolus administration (day −1) a mean baseline daily feed intake was determined for the 3-MMC group, while for the control group a baseline daily feed intake was obtained 48–72 h prior to the study beginning. Feed intake was assessed 24 h before i.v. bolus administration (day −1), 24 h after the single i.v. bolus dose (day 1) as well as on days 2, 4, and 5. Multiple oral 3-MMC administration commenced on day 3 for five consecutive days. Data are expressed as change in feed intake (mean in kg±standard error of the mean (SEM)) from the baseline reading taken before the i.v. bolus administration. Statistical analysis was performed using two-factor repeated analysis of variance (ANOVA) with Bonferroni's post-hoc test with a significance level of $^{*}p$<0.05.

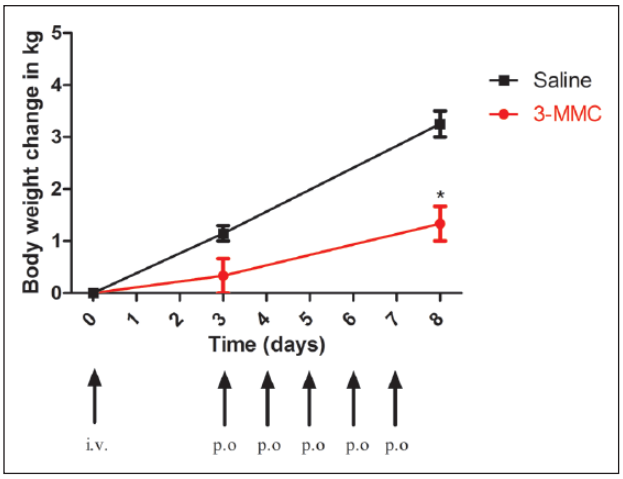

Figure 2. Effect of 3-methyl-methcathinone (3-MMC) on body weight in individually housed pigs (n=3, weight at day 0: 28–34 kg) in comparison to control group (n=2, weight at time 0: 30–31 kg). At day 0, a single dose of 3-MMC was administered intravenously (9 mg dissolved in 10 mL 0.9% saline) to three pigs, followed three days later by a multiple oral administration of 90 mg for five consecutive days to the same pigs. In the control group, the same procedure was applied, however with 0.9% saline. Body weight was assessed before i.v. bolus administration (day 0), before the first oral dose (day 3) and 24 h after the last oral dose (day 8). Data are expressed as change in mean body weight (mean weight in kg±standard error of the mean (SEM)) from the baseline reading taken before the i.v. bolus administration. Statistical analysis was performed using two-factor repeated analysis of variance (ANOVA) with Bonferroni's post-hoc test with a significance level of $^{*}p$<0.05.

Samples for CBC were collected in potassium-ethylenediaminetetraacetic acid tubes, and analyzed within 2 h from collection using a hematology analyzer (Advia 120, Siemens Medical Solutions Diagnostics GmbH, formerly Bayer HealthCare GmbH, Erfurt, Germany). Samples for serum chemistry were collected in plain tubes with gel separators. Samples were centrifuged within 30 min from collection, and harvested sera were immediately analyzed using a wet chemistry autoanalyzer (Cobas Integra 400 Plus, Roche, Mannheim, Germany, at 37°C) and reagents supplied by the manufacturer (Roche, Mannheim, Germany).The parameters determined for serum biochemistry are summarized below.

Effect of 3-MMC on daily feed consumption and body weight

In the treatment group a baseline for daily feed consumption was established 24 h prior to i.v. bolus administration (day −1), while the baseline for the control group was obtained as the mean value of the daily feed consumption 48–72 h prior to i.v. saline administration (Figure 1). Daily feed intake was recorded 24 h and 48 h after the single i.v. bolus dose (day 1 and 2 respectively). The effect of multiple oral dose administration (given on days 3,4,5,6, and 7) on feed consumption was recorded on days 3, 4, and 5. Daily feed intake was obtained by subtracting residual feed leftovers from the total daily feed supply (4 kg) 30 min after feeding the animals. Data are expressed as change in feed consumption (mean consumption kg±standard error of the mean (SEM)) from the baseline reading taken before the i.v. bolus administration and compared to values of the control group. BW was determined prior to i.v. bolus administration (day 0, baseline reading), as well as prior to the first oral dose administration (day 3) and 24 h after the last oral dose (day 8). Data are expressed as change in mean BW (mean BW in kg±SEM) from the baseline reading taken prior to the i.v. bolus administration and compared to control values (Figure 2).

Necropsy and gross pathology

A total of five pigs were submitted to the Kimron Veterinary Institute, Israel, for full necropsy 24 h after the last oral dose (day 8).

Histopathology

Tissues collected for histopathological examination from the pigs included sections of the heart, skeletal muscles (diaphragm, intercostal, triceps, vastus lateralis, longissimus dorsi, interspinalis, semispinalis, semimembranosus, semitendinosus, and biceps femoris), lung, pancreas, liver, kidney, spleen, brain, and small intestine and were preserved in 10% buffered formalin. Tissues were embedded in paraffin, sectioned at a 5 µm thickness and stained with hematoxylin and eosin.

Ethical animal use

Experiments were conducted according to the protocols approved by the local Helsinki Institutional Review Board for animal studies (Approval No. IL-14-03-94). Management, treatment,

Table 1. Physico-chemical and pharmacokinetic properties of 3-methyl-methcathinone (3-MMC) and mephedrone[a] hydrochloride.

Physico-chemical property[b]	3-MMC value±SE	Mephedrone value±SE
cLog P[c]	1.86±0.3	1.96
MW[d] (g/mol)	213.7	213.7
Melting point (°C)	193.2±0.2	241±3
pKa	7.84±0.1	8.69
Water solubility as hydrochloride (mg/mL)	2.0±0.1	–
UV-absorption max in water (nm)	206.2	263.5

MW: molecular weight; SE: standard error; UV: ultra-violet.
[a]Santali et al., (2011).
[b]Physico-chemical characterization of 3-MMC was performed according to the method described by Zimmerman (1986).
[c]Theoretically calculated by utilizing ACDlabs software (Toronto, Canada).
[d]Calculated MW.

sampling, and sacrifice of the pigs were performed according to Israeli laws and regulations by certified veterinarians in compliance with the Council Directive 2008/120/EC (European Commission, 2009).

Statistical analysis

The results of the biochemical and complete blood count analysis of the treatment group (n=3; the same animals serving as their on control) collected at different time points of the study, namely 24 h before i.v. bolus administration, 48 h after i.v. bolus and five days after multiple oral dose administration were directly compared to normal reference values of pigs raised at The Institute of Animal Research, Kibbutz Lahav. Feed consumption and BW were analyzed using mixed analyses of variance (ANOVAs) with a between-subjects factor of group (3-MMC treated pigs; saline treated pigs) and a within-subjects factor of time. Statistical power was calculated for the interaction of group factor×time by utilizing the software G*POWER 3.0.10. A posteriori power calculation was conducted to determine the power value at a given total sample size, significance value (0.05) and calculated effect size according to the partial eta equation:

$$\eta^2 = \text{Sum of square}_{(\text{interaction of group factor} * \text{within-subject-factor})} / \text{sum of square}_{(\text{interaction of group factor} * \text{within-subject-factor})} + \text{Sum of square}_{(\text{error})}.$$

Results

Physicochemical properties of 3-MMC

The physicochemical properties of 3-MMC and mephedrone hydrochloride are summarized in Table 1. The physicochemical properties of mephedrone were obtained from Santali et al., 2011.

3-MMC pharmacokinetic profile

Disposition and elimination pharmacokinetic parameters were derived from the mean plasma concentration vs time curve of three animals receiving i.v. bolus dose of 9 mg 3-MMC hydrochloride (Figure 3(a)). The plasma concentration time curve was analyzed by non-compartmental model and the corresponding parameters are summarized in Table 2. 3-MMC was well distributed into the extravascular tissues with a volume of distribution (V) of 240 L. The extensive total clearance of 199 L/h, resulted in a very short half live of 0.8 h, clearly reflecting the disappearance of the drug from plasma after four hours (Figure 3(a), Table 2). 3-MMC plasma concentrations were best fitted to the following one-compartment open body model equation:

$$C=157e^{-0.84\times t}$$

The mean observed 3-MMC plasma concentration vs time curve following oral dose administration (90 mg) enabled the extraction of C_{max} (27 µg/L) and T_{max} (0.08 h) values from the corresponding predicted plasma vs time curve, which was best fitted by the following one-compartment open body model equation:

$$C=44.8\times(e^{-1.2\times t}+e^{-40.9\times t})$$ (Table 2, Figure 3(a))

A mean calculated absolute oral bioavailability (F) value of 7% was obtained.

The absorption rate constant of 40.9 h^{-1} was determined by means of nonparametric numerical deconvolution of the observed oral plasma concentration time curve from the observed i.v. bolus plasma concentration time curve (Table 2, Figure 3(b)). Furthermore, more than 80% of the total amount absorbed, was absorbed within 12 min post oral drug administration (Figure 3(b)).

Analytical method validation

High recoveries were obtained from spiked blank plasma samples (97–89%, Supplementary Material, Table S1), while spiked blank brain and liver tissues yielded lower recoveries of 55–71% and 63–75 respectively (Supplementary Material, Table S1). The calibration curves for plasma, brain and liver samples presented excellent correlation between analyte concentration and peak area (R^2 for all curves were 0.999; Supplementary Material, Table S1). The LOD and LOQ values for 3-MMC in plasma were 0.1 µg/L and 0.3 µg/L respectively, whereas for the brain and liver samples five times higher LOD and LOQ values were determined (Supplementary Material, Table S1). The percent coefficient of variations (%CV) of within and between days for spiked plasma samples was 3.1–5.1% and 6.6–12.5% for liver and brain samples respectively (Supplementary Material, Table S2). The accuracy values for the analyte tested were in the range of 85–110%. Hence, in the examined range of concentrations analyzed (0.5–100 µg/L), the analytical method was considered accurate and precise. 3-MMC was found stable at −20 ºC for 14

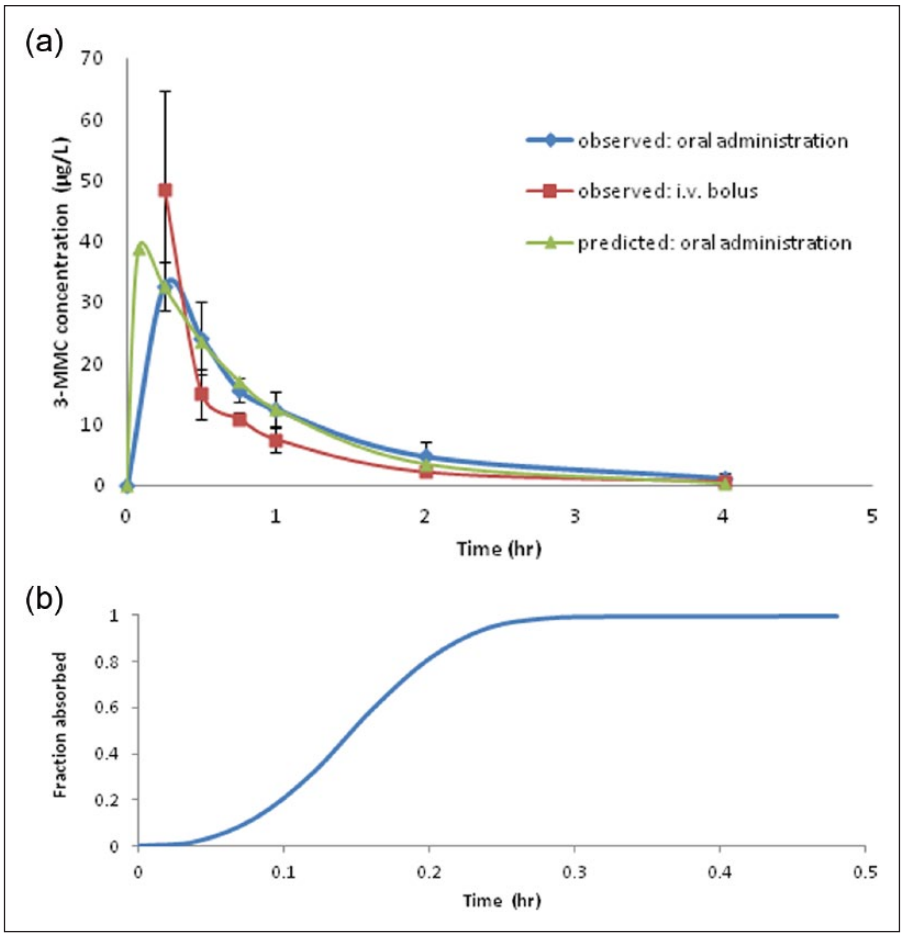

Figure 3. (a) Plasma concentration-time curves of 3-methyl-methcathinone (3-MMC) given as an i.v. bolus dose (9 mg) to three healthy male pigs (weight 28–34 kg, three months old); after a single oral dose (90 mg) with a washout period of three days between administrations; predicted concentration-time curve following single oral dose. (b) Mean fraction absorbed vs time (h) following oral dose administration of 3-MMC (90 mg) to three pigs. Data obtained by nonparametric numerical deconvolution as implemented in Win Nonlin (WinNonlin 4.1 Pharsight Corporation, Mountain View, California, USA) using the automatic smoothing and initial rate not constrained to be zero options.

Table 2. Pharmacokinetic parameters of 3-methyl-methcathinone (3-MMC) following consecutive single intravenous and oral administration to three male pigs (three months old, weighing 28–34 kg) with a washout period of three days between both administration modes.

Mode of administration	Pharmacokinetic parameter	3-MMC Mean±standard deviation	Mephedrone[a] Mean
Intravenous (i.v.) administration	Dose (D)	0.3 mg/kg	9 mg/kg
	Clearance (CL)	199±55 L/h	1.7 L/h
	Apparent volume of distribution (V)	240±84 L	0.5 L (at steady-state)
	Half-life $t_{1/2}$	0.83±0.1 h	0.4 h ($t_{1/2,\beta}$)
	k[b]	0.84±0.1 h^{-1}	–
	Area under the curve (AUC)	48±15 µg×h/L	1332 µg×h/L
Oral administration (p.o.)	Dose (D)	3 mg/kg	30 mg/kg
	AUC	31±11 µg×h/L	294 µg×h/L
	Bioavailability (F)	7.0±2	7.3
	T_{max}[c]	0.08 h	0.9 h
	C_{max}[c]	27±5 µg/L	331 µg/L
	k_a[c]	40.9 h^{-1}	0.3 h^{-1}

[a]The pharmacokinetic profile was determined in Sprague-Dawley rats and fits a two-compartment model (Martínez-Clemente et al., 2013).
[b]Elimination rate constant.
[c]Time at maximal concentration (T_{max}), the maximal concentration (C_{max}) and absorption rate constant (k_a) were obtained from the predicted concentration vs time curve calculated using the Gauss-Newton method utilizing the WinNonline nonlinear estimation program version 4.1.

Table 3. Serum biochemical markers of pigs 24 h before 3-methyl-methcathinone (3-MMC) administration; 48 h after single i.v. bolus dose administration (9 mg) and after five days of daily oral drug administration (90 mg).[a]

Biochemical marker	Before 3-MMC administration mean±STD (range)	48 h after single i.v. bolus (9 mg) mean±STD (range)	24 h post multiple oral dose administration (90 mg) mean±STD (range)	Reference range for healthy pigs
Alb (g/dL)	3.6±0.5 (3–4)	3.5±0.3 (3.2–3.7)	3.2±0.3 (2.9–3.5)	3.6–5.2
ALP (U/L)	223±121 (118–355)	151±19 (131–168)	127±36 (101.5–168)	60–280
ALT (U/L)	48±13 (34–60)	39±7 (33–47)	38±2 (36–40)	26–72
Amylase (U/L)	1488±448 (1096–1976)	2140±801 (1505–3040)	1886±411 (1616–2359)	896–3625
AST (U/L)	26±2.5 (24–29)	28±4 (24–32)	30±1 (29–31)	8–78
Bil (mg/dL)	0.01±0.001 (0.01–0.02)	0.06±0.04 (0.03–0.1)	0.04±0.02 (0.03–0.06)	0–0.2
Ca2+ (mg/dL)	11±0.4 (10.5–11.2)	10±0.3 (9.7–10.3)	9.7±0.2 (9.6–9.9)	9.8–12.4
Cholesterol (mg/dL)	88±9 (80–97)	86±9 (76–95)	81±21 (62–103)	63–112
CK (U/L)	659±47 (609–702)	270±49 (215–306)	313±124 (210–451)	263–4078
CL (mmol/L)	104±2 (102–105)	98±2 (97–100)	102±4 (98–105)	95–104
Creatinine (mg/dL)	1±0.1 (1.1–1.3)	1±0.3 (0.8–1.3)	0.9±0.2 (0.7–1.1)	0.7–2.1
GGT (U/L)	45±14 (32–60.2)	47±14 (34–62)	35±8 (29–43)	10–82
Glucose (mg/dL)	96±6 (91–103)	75±2 (73.5–77)	94±12 (86–108)	56–141
K (mmol/L)	4.4±0.2 (4.2–4.5)	4.4±0.2 (4.2–4.6)	4.5±0.3 (4.1–4.8)	3.4–6.8
Na (mmol/L)	143±2 (141–145)	139.6±0.7 (138.8–140)	138±2 (135–139)	135–151
Phosphate (mg/dL)	8±1 (7–9)	9.5±0.9 (8.5–10.3)	7.6±0.2 (7.4–7.7)	6.5–13.9
TP (g/dL)	6±0.6 (5.0–6.0)	6±0.9 (5.2–6.8)	6±0.6 (5.2–6.4)	5.7–8.5
TG (mg/dL)	46±23 (27–71)	23±12 (16–37)	24±10 (15–35)	6–49
Urea (mg/dL)	17±4 (13–21)	16±4 (12.5–21)	15±5 (10–19)	7–47

Alb: albumin; ALP: alkaline phosphatase; ALT: alanine transferase; AST: aspartate aminotransferase; Bil: total bilirubin; GGT: gamma glutamyl transferase; CK: creatinine kinase; CL: clearance; TP: total protein: TG: triglyceride; STD: standard deviation.

[a]Biochemical analysis was performed at the Biochemistry Laboratory, The Veterinarian Hospital, The Hebrew University, Bet-Dagan, Israel. Reference values were obtained from The Institute of Animal Research, Kibbutz Lahav, Israel.

days at the lower (1 μg/L) and upper (100 μg/L) concentration range of the calibration curve according to the acceptance criteria specified in the methods and materials section.

Analysis of plasma and tissue samples obtained from the with 3-MMC treated group

In all analyzed liver and brain tissues, 3-MMC levels were below the LOD 24 h after the last oral dosage. Plasma levels obtained following i.v. bolus administration (9 mg) as well as after the first oral dose administration (90 mg) displayed overlapping concentration range of 0.5–67 μg/L (Figure 3(a)).

Clinical chemistry and hematology

Serum biochemistry and complete blood count values were compared to the normal reference range provided by The Institute of Animal Research, Kibbutz Lahav (Table 3 and Supplementary Material, Table S3). No abnormal biochemical and complete blood count values were observed in comparison to the reference values.

Effect of 3-MMC on daily feed consumption and body weight

Figure 1 depicts the effect of 3-MMC on the mean change in daily feed intake following i.v.-bolus drug administration and a consecutive multiple oral dose administration over a time course of five days in comparison to the control group. The statistical analysis identified significant effect of 3-MMC treatment ($F=21.8$; $p=0.02$), the time effect ($F=4.8$; $p=0.01$) as well as of the interaction between 3-MMC treatment and the time post drug administration ($F=3.4$; $p=0.04$) on the change in feed consumption. However, only 24 h after i.v. bolus administration as well as 24 h after the second oral dose administration a significant effect of 3-MMC on the change in daily feed intake was observed (Figure 1; $p<0.01$). The post-hoc statistical power of the interaction between subject and within subject to detect changes in feed intake was 0.60 with an effect size of 0.53. BW change was recorded in the treatment and the control group prior to i.v. bolus drug administration (day 0) as well as prior to the first oral dose (day 3) and 24 h after the last oral dose (day 8) (Figure 2). The statistical analysis confirmed significant main effects of the time post drug administration ($F=77.5$; $p<0.0001$) and the interaction of drug treatment with time post-injection ($F=13.1$; $p=0.006$). Comparisons tests confirmed a significant effect of 3-MMC treatment on mean BW change on the eighth day as compared to control ($t=5.1$; $p<0.01$). The post-hoc statistical power of the interaction between subject (group factor) and within subject (time factor) to detect changes in body weight was 0.94 with an effect size of 0.81. The animals treated with 3-MMC displayed a lower rate of increase in mean body weight, gaining on average 1 kg weight by the end of the study, whereas the animals in the control group gained significantly more weight as measure on day 8, namely 3 kg body weight in average (Figure 2).

Pathological and histopathological examination

The i.v. bolus as well as the consecutive multiple oral dose administrations (90 mg for five days) were well tolerated. No treatment-related mortality and morbidity were observed during the study and no gross pathological findings were detected. Histopathological examination in two animals in the treatment and the control groups revealed mild diffuse hepatocellular vacuolation. In addition, mild multifocal collapse of alveolar walls as well as multifocal mild mononuclear infiltration involving the alveolar and interlobular septa (Interstitium) were observed in two animals in the treatment group, while in one animal in the control group mild hyperplasia of bronchiolar associated tissue was observed.No abnormal histopathological changes were observed in all other tissue samples analyzed.

Discussion

Due to the lack of sufficient toxicological, pharmacokinetic and pharmacodynamic data on cathinone isomers and analogues in large animal models, the aim of the present study was to investigate the 3-MMC pharmacokinetic profile as well as its effect on feeding behavior, weight gain and serum biochemistry in domestic pigs.

An animal model to study human diseases should accurately reproduce the various aspects of disease. Domestic pigs are closely related to humans in terms of anatomy, genetics, and physiology, and represent an excellent animal model to study various human diseases (Bassols et al., 2014; Kobayashi et al., 2012; Swindle et al., 2012). Indeed, experiments in pigs are much more likely to be predictive of therapeutic treatments in humans than experiments in rodents (Bassols et al., 2014; Kobayashi et al., 2012; Swindle et al., 2012). Consequently pigs are increasingly being used as an alternative to dogs or monkeys as the choice of non-rodent species in preclinical toxicological and pharmacokinetic testing of pharmaceuticals (Ferran et al., 2013; Swindle et al., 2012). Characterizing the pharmacokinetic profile of 3-MMC in pigs provides important information about potential hazardous for humans and a better understanding of 3-MMC's elimination kinetics from the body.

According to the literature, the oral dosage used in this study reflects a single oral dose of mephedrone and 3-MMC frequently consumed by recreational users (~3 mg/kg) (Erowid, 2014; Green et al., 2014; Kelly, 2011). Furthermore, the rationale for the chosen oral dosage regimen of once-daily drug administration for five consecutive days was to provide preliminary subchronic toxicological data that might inform future comprehensive toxicological studies. The i.v. bolus administration was essential in determining the absolute oral bioavailability. On the other hand, the dosage regimen applied within the study was not sufficient to reflect 3-MMC effects at higher dosages frequently consumed or after chronic exposure over a longer period of time or binge dosing frequently practiced among mephedrone users. Therefore caution should be practiced in extrapolating the present toxicological and pharmacokinetic/pharmacodynamic data to chronic recreational users exposed to larger 3-MMC doses and/or consuming simultaneously other drugs and alcohol.

A fully validated analytical method of 3-MMC was established in the range of 0.5–100 μg/kg in plasma, brain and liver samples (Supplementary Material, Tables S1, S2). The concentration range chosen covered the plasma levels obtained following the dosage regimens applied in this study. The new developed method met the requirements for specificity, linearity, accuracy, precision, and recovery in plasma and tissue samples in accordance with the method performance recommendations defined by the European Union (European Commission, 2002). The calculated LOD and LOQ values in this study were in the lower range of values reported for mephedrone in plasma, liver and brain matrices (Supplementary Material, Tables S1, S2; Martínez-Clemente et al., 2013; Wright et al., 2012).

The short half-life of 3-MMC determined in the pigs (0.8 h) following both modes of administration was consistent with the temporal profile of subjective effects reported by recreational users following consumption of its structural isomer mephedrone, and about twice the half-life of mephedrone observed in rats (Green et al., 2014; Martínez-Clemente et al., 2013; Wright et al., 2012). The large apparent volume of distribution of 3-MMC (8 L/kg) is usually expected of high lipophilic drugs; however 3-MMC at physiological pH is more than 50% ionized while the unionized form possess a relatively moderate lipophilic log P value of 1.8 (Table 1). The large apparent volume of distribution might be the result of low protein binding and possibly active transport into the tissue compartment (Martínez-Clemente et al., 2013; Rowland and Tozer, 1995). On the other hand, in all liver and brain samples analyzed 24 h after the last multiple oral dose administration, 3-MMC levels were below the LOD, clearly indicating the lack of accumulation and the rapid elimination from the central nervous system. As the liver and kidney drug levels usually reflect the plasma drug concentration, the lack of drug accumulation in the liver is not surprising. The extraordinarily high total clearance displayed by 3-MMC is in agreement with the reported total clearance of mephedrone observed in rats after body weight normalization (Martínez-Clemente et al., 2013). The total clearance determined in the present study (199 L/h) was more than twice the sum of the liver and kidney blood flow in pigs weighing 30–40 kg (88 L/h) and therefore additional elimination sites/mechanisms are expected to contribute to 3-MMC elimination for instance extrahepatic metabolism (Drougas et al., 1996; Lerman et al., 1999).

The low oral bioavailability observed in the three pigs following 3-MMC oral administration was identical to the bioavailability of mephedrone observed in rats (7%) and suggests that both drugs undergo an extensive first pass effect (Martínez-Clemente et al., 2013). The low bioavailability of 3-MMC and mephedrone may provide an additional explanation for their widespread use by insufflation. Other factors that contribute to the preferred insufflation route are the desire for rapid effect onset and cultural influences.

The pharmacokinetics of 3-MMC in plasma after i.v. bolus and oral administration was well described by a non-compartmental model. The goodness of fit and the quality of the estimated pharmacokinetic parameters following oral administration were evaluated and confirmed by the objective function Aikaike Information Criterion, plot of observed vs predicted concentrations and the variation coefficient with values below 25%.

The predicted fraction absorbed vs time curve clearly demonstrated a rapid absorption of 3-MMC, with more than 80% of the absorbed drug being absorbed within 12 min (Figure 3(b)). The predicted fraction absorbed vs time is in close agreement with the observed rapid onset of the psychostimulant (15–30 min) effect

for various mephedrone-like compounds reported by recreational users (Green et al., 2014).

No abnormal patho-and histopathological findings in the 3-MMC treated group were observed. Mild liver vacuolization was seen in all pigs of both study groups, representing a common feature in pigs raised in the aforementioned research facility. Clinically non-significant mild multifocal collapse of alveolar walls and multifocal mononuclear infiltration observed in two animals in the treatment group and a mild hyperplasia of bronchiolar associated tissue observed in one animal in the control group were considered secondary to mild lung infection unrelated to 3-MMC exposure. Furthermore, the complete blood count and plasma biochemistry results may indicate the lack of noxious effects on the liver, kidney, and white blood cells (Table 3 and Supplementary Material, Table S3).

3-MMC induced 50% decrease in plasma triglyceride levels following i.v. bolus injection, remaining at the same reduced levels by the end of the multiple oral drug regimen (Table 3). To the best of our knowledge, there are no reports of reduced triglyceride levels due to synthetic cathinone administration. In contrast, the lipid reducing activity of *Khat* leaves has been well documented in numerous studies, most probably due to the β-cathinone pharmacologic activity (Al-Habori et al., 2004). Whether the lipid-reducing activity of 3-MMC is of clinical importance could only be verified by additional studies utilizing a larger sample size and a longer drug administration period at multiple dosages.

A statistically significant reduced feed consumption was observed in the 3-MMC treated group 24 h after the i.v. bolus administration as well as 24 h after the second oral dose administration in comparison to the control group (Figure 1). The animals exposed to 3-MMC in the present study displayed significantly lower body weights on the eighth day of the experimental design as compared with the control group (Figure 2). This observation could be possibly explained by the reduced feed consumption of the treated group over the course of drug administration as compared to the control group. The outcome of reduced rate of BW increase might indicate potential use of certain mephedrone derivatives as appetite-suppressant agents. To the best of our knowledge, this is the first report demonstrating BW modulating activities of mephedrone-like compounds in animal models. The weight-and triglyceride reducing activity of 3-MMC might present potential therapeutic targets worthwhile exploring. On the other hand, it is important to emphasize the high abuse potential of numerous synthetic cathinones such as mephedrone and MDPV, limiting their utilization in clinical settings. Since the addictive potential of 3-MMC was not investigated in the present study, further research is warranted, to clarify this particular issue.

In conclusion, we have conducted the first large animal pharmacokinetic study in relation to feed intake and weight gain of 3-MMC finding little evidence of toxicity in pigs and a similar pharmacokinetic profile to that found in rats with a short half-life of 0.8 h. Caution needs to be practiced in terms of extrapolating the present data to human safety, due to the low sample size, low dosage and the relatively short study duration conferring a low statistical power for detecting changes in feed-intake, as well as rendering any solid conclusions regarding the undetected toxic effects of 3-MMC in the pathological/histopathological and serum biochemistry analysis. Comprehensive preclinical studies are required in-order to assess the 3-MMC safety profile before conducting trials in humans.

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