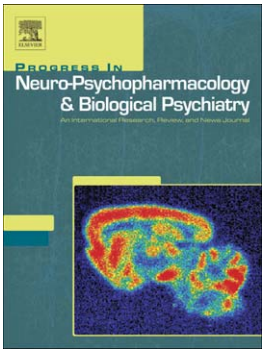

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An integrated pharmacokinetic and pharmacodynamic study of a new drug of abuse, methylone, a synthetic cathinone sold as "bath salts".

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ABSTRACT

Introduction: Methylone (3,4-methylenedioxymethcathinone) is a new psychoactive substance and an active ingredient of "legal highs" or "bath salts". We studied the pharmacokinetics and locomotor activity of methylone in rats at doses equivalent to those used in humans. *Material and methods:* Methylone was administered to male Sprague-Dawley rats intravenously (10 mg/kg) and orally (15 and 30 mg/kg). Plasma concentrations and metabolites were characterized by LC/MS and LC–MS/MS fragmentation patterns. Locomotor activity was monitored for 180-240 min. *Results:* Oral administration of methylone induced a dose-dependent increase in locomotor activity in rats. The plasma concentrations after i.v. administration were described by a two-compartment model with distribution and terminal elimination phases of α=1.95h^{-1} and β=0.72h^{-1}. For oral administration, peak methylone concentrations were achieved between 0.5- 1 h and fitted to a flip-flop model. Absolute bioavailability was about 80% and the percentage of methylone protein binding was of 30%. A relationship between methylone brain levels and free plasma concentration yielded a ratio of 1.42 ± 0.06, indicating access to the central nervous system. We have identified four Phase I metabolites after oral administration. The major metabolic routes are *N*-demethylation, aliphatic hydroxylation and *O*-methylation of a demethylenate intermediate. *Discussion:* Pharmacokinetic and pharmacodynamic analysis of methylone showed a correlation between plasma concentrations and enhancement of the locomotor activity. A contribution of metabolites in the activity of methylone after oral administration is suggested. Present results will be helpful to understand the time course of the effects of this drug of abuse in humans.

Key words Methylone; Pharmacokinetics; Locomotor activity; PK/PD modeling; Rat.

1. Introduction

A new generation of designer phenethylamine derivatives, known as "legal highs" or "research chemicals" has emerged and has been marketed as a legal alternative to "ecstasy" (3,4-methylenedioxymethamphetamine or MDMA) or cocaine. Their chemical structure is related to cathinone, the main psychoactive constituent of khat (Sørensen, 2011). These products are also advertised as "bath salts", the active ingredients of which include mephedrone, methylone and MDPV (3,4-methylenedioxypyrovalerone) alone or mixed (Spiller et al., 2011), available for purchase on line, at convenience stores or truck stops. In October 2011, the Drug Enforcement Administration (DEA) placed these three synthetic cathinones (mephedrone, methylone and MDPV) and their salts and isomers into the Schedule I of the Controlled Substances Act, to avoid an imminent hazard to the public safety. In October 2012, the DEA extended the temporary placement of methylone into this schedule.

The most commonly available cathinone derivatives sold on the recreational market appear to be mephedrone (4-methyl-methcathinone) and methylone (3,4-methylenedioxymethcathinone) (Brunt et al., 2010). Methylone was first synthesized as an antidepressant and taken orally or nasally, as drug of abuse, emerged under the trade name "explosion" around 2004 thus making it one of the first products to be marketed via on-line (Bossong et al., 2005).

Previous studies have described that cathinone users consider the effects of these new drugs of abuse to be superior to those of cocaine and MDMA (Winstock et al., 2010, Vardakou et al., 2011) and it may explain their rapid rise in popularity.

To our knowledge, very little is known about the pharmacology of methylone. Results from *in vitro* studies hypothesized that the mechanism of action of methylone, was similar to that of d-amphetamine (Cozzi et al., 1999, Baumann et al., 2012), by binding to the monoamine transporters (Nagai et al., 2007, Simmler et al., 2012). More recently, some studies on the pharmacological targets of cathinones have been published by our group (Martinez-Clemente et al., 2012) and others (Kehr et al., 2011, Hadlock et al., 2011, Motbey et al., 2011, Simmler et al., 2013). It has been demonstrated that methylone increased the spontaneous locomotor activity in mice in a dose-dependent

manner, this effect is prevented by ketanserin or haloperidol pre-treatment. Methylone compared to mephedrone and butylone, was the most potent cathinone to inhibit serotonin and dopamine uptake, an effect which partially persists after withdrawal (López-Arnau et al., 2012).

Human pharmacokinetics data on methylone are obtained from consumer reports (Shimizu et al., 2007, Boulanger-Gobeil et al., 2012), on-line reports also indicate that 150-300 mg is a common oral dose of methylone. Some reports describe that in humans the onset of effect of methylone appears at 15–30 min with a 2–3.5 h duration, but 6–24 h to return to "normal" status. Kamata et al. (2006) identified the characteristic human and rat urinary metabolites of methylone that were of great importance in forensic analysis (Zaitsu et al., 2009). Moreover, some human sudden deaths related to methylone intake have been reported (Cawrse et al., 2012, Pearson et al., 2012) and some reported cases met the Hunter criteria for serotonin syndrome (Boyer and Shannon, 2005, Warrick et al., 2012). Methylone concentration- and time-effect relationships in laboratory animals and/or humans after different doses and routes of administration have not been adequately characterized and further research will be necessary to develop adequate prevention and treatment policies.

The aim of the present study was to characterize the pharmacokinetic profile of methylone in rats after intravenous and oral administration and to correlate it with a pharmacodynamic evaluation of the psychostimulant effect of this drug of abuse, thus establishing a PK/PD model. Furthermore, another goal of this study was to analyze the *in vivo* Phase I metabolites in rat blood and the brain/plasma concentration of this cathinone after an oral administration to assess its penetration into the central nervous system. Because ethical considerations considerably limit the administration of this addictive substance to humans, animal models that mimic human use are essential. Accordingly, the oral doses used in this study were selected in the attempt to emulate those used by human drug abusers. To our knowledge, all published papers on pharmacokinetics of methylone are of a forensic nature (urinary collected specimens) (Crawse et al., 2012, Kamata et al., 2006) or from *in vitro* experiments (Mueller and Rentsch, 2012). This study constitutes the first approach to the kinetics of methylone based on *in vivo* blood sample data from laboratory animals and will be helpful to

design different treatment regimes in rodents in order to evaluate methylone's effects as well as to understand the time course of the effects of this drug of abuse in humans.

2. Material and methods

2.1. Drugs and Reagents

Pure racemic methylone and mephedrone HCl were synthetized and characterized by us, as described previously (López-Arnau et al., 2012). Methylone solutions for injection were prepared in saline immediately before administration. Isoflurane was from Laboratories Dr. Esteve (Barcelona, Spain). Reagents required for LC/MS assays were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Animals

The experimental protocols for the use of animals in this study were approved by the Animal Ethics Committee of the University of Barcelona, following the 86/609/EEC guidelines. Male Sprague-Dawley rats (Janvier, Le Génest, France) weighing 225-250 g were used. Animals were housed at 22 ± 1 ºC under a 12-h light/dark cycle with free access to food and drinking water.

2.3. Pharmacokinetic experiments

For oral pharmacokinetic experiments, methylone was administered at doses of 15 and 30 mg/kg to animals previously fasted for 18 h and for intravenous pharmacokinetic analysis, methylone was administered at a dose of 10 mg/kg. A total of 8-10 animals per dose were used.

Blood samples (150-200 µl) were collected from isoflurane anesthetized rats through a venipuncture of the external jugular vein in a time schedule from 5 min to 8 h (or 24 h in some cases) and transferred to 1 ml glass tubes (containing 10 µl EDTA 20 mg/ml) on ice. A total of 4-5 blood samples were obtained from one animal. After each blood extraction an equal volume of sterile saline was infused to maintain volume and osmotic homeostasis.

Blood samples were centrifuged at 1,000 x *g* for 10 min to obtain the plasma. 90 µl of plasma samples were mixed with 10 µl of internal standard (IS) solution (methylone, 200 ng/ml). The mixture was extracted by adding 250 µl of methanol up to a final concentration of 70%. The denatured proteins were precipitated by centrifugation at

10,000 x g for 5 min. 250 µl of clear supernatant was acidified with formic acid (50% v/v) to a pH of 2.5-3.0 to obtain stable extracts, because in non-acidified live-blood extracts, cathinones degraded relatively fast (Sørensen 2011). The mixture was transferred to an ultrafiltration filter cup and high-molecular-weight components were removed by means of filtration through a 30-kDa regenerated cellulose membrane (Microcon 30®, Millipore, Bedford, MA, USA). The ultrafiltration unit was centrifuged at 20,000 × g for 10 min and 100 µl of the filtrate were transferred to an auto sampler vial.

An HP 1100 Liquid Chromatography (LC) system equipped with an autosampler, a column oven set to 40 ^{o}C and coupled API 3000 triple-quadrupole mass spectrometer (MS), with a turbo ion spray source was used to quantify the corresponding cathinone. Chromatographic separation was achieved on a Luna HST C18 (100 *x* 2mm, i.d., 2.5 µm) column. The mobile phase was water (A) and methanol (B) with 0.1% of formic acid in both solvents. An increasing linear gradient (v/v) of B was used (t(min),%B), as follows, (0, 5), (20, 95), (22, 95), (22.5, 5) and (32.5, 5), at a constant flow rate (150 µl/min). The biological samples were refrigerated at 4 ^{o}C and 5µl were injected into the LC-MS/MS system. The LC-ESI (electrospray ionization)-MS/MS conditions were optimized by direct infusion of cathinone standards (1 µg/ml) dissolved in 50,50 (v/v) water (0.1% formic acid)/methanol (0.1% formic acid) into the MS at a constant flow rate (5 µl/min). Two transitions were followed for methylone (*m/z* 208.1 → 190.1 and 208.1→ 160.0) (collision energies of 17 and 22 V) and both were used for the quantification. For mephedrone one transition was followed (*m/z* 178.1→160.0, 17V).

2.4. Blood Metabolite determination

Blood samples were collected at 60, 120 and 180 min after oral administration at a dose of 30 mg/kg. Samples were treated as described above, without IS. For metabolite identification, a Linear Trap Quadrupole Orbitrap Velos MS equipped with an ESI source was used. This system was coupled to an Accela chromatograph, a refrigerated auto sampler and a photodiode array detector. Chromatographic separation was achieved on a Luna C18 (100 x 2.1mm, i.d., 3µm) column. The mobile phase was the same as the one used in the pharmacokinetic studies. In this case, an increasing linear gradient (v/v) of B was used (t(min),%B), as follows, (0, 2), (20, 95), (22, 95), (25, 2) and (30, 2) at a constant flow rate (150 µl/min). The injection volume was 10 µl. The data were acquired in Fourier transform mass spectrometry mode (FT MS) and ranged

from *m/z* 50 to 1,000 in both positive and negative ion modes. Operation parameters were as follow, source voltage, 3.5 (kV) in positive mode, S-Lens RF levels, 60%, capillary temperature was fixed at 275 ^{o}C, sheath gas at 40 (arbitrary units) and auxiliary gas at 10 (arbitrary units). MS^2 acquisition was carried out under collision-induced dissociation conditions using collision energy between 35 and 50%.

2.5. Protein binding and brain levels

Blood samples were obtained 45 min after oral administration (dose of 30 mg/kg) followed by decapitation and removal of the whole brains. In protein binding experiments blood samples were divided by half. One half was filtered through centrifugal filter units (Centrifree® YM-30, Millipore, Bedford, MA, USA) for comparison with the other unfiltered half. Plasma samples were extracted as described above. The extraction of brain samples was carried out as described by Hadlock et al. (2011) and brain methylone levels and protein binding assays were quantified as described in the pharmacokinetic experiments.

2.6. Calibration

Plasma and brains from untreated rats were used to obtain the calibration curves. In the plasma analysis, seven standards were prepared daily in 100 µl of blank plasma (from 10 to 6,000 ng/ml). To determine brain methylone levels, five standards were prepared, also daily, in 0.5 ml of brain homogenate (from 10 to 250 ng/ml). Mephedrone was used as IS at the final concentration of 200 ng/ml for plasma levels and 50 ng/ml for brain levels. The method showed linearity within the concentration range studied and the limit of quantification was considered lower than 10 ng/ml. Quality control samples were prepared at 50, 1,000, 5,000 ng/ml and 20, 50, 200 ng/ml for plasma and brain analysis, respectively. The accuracy of the assay was 90 - 110%. The intra- and inter-assay coefficients of variation (CV) were less than 15%.

2.7. Pharmacokinetic analysis.

Mean plasma concentration time profiles were analyzed by bi-compartmental modeling. The distribution and elimination characteristics of methylone were determined after the i.v. administration. Fixing the parameters obtained in the i.v. model, oral methylone profiles were analyzed simultaneously by using a bi-compartmental model with oral delay and Michaelis-Menten metabolism kinetics. The best fit line was selected after

visual inspection of the fitting, the analysis of the objective function and the AIC (Akaike's information criterion), the precision of the estimates (mean and CV) and the weighted residuals plot analysis.

The i.v. data were described by and open two-compartmental model and fit to the following equation,

$$Cp = Ae^{-\alpha t} + Be^{-\beta t}$$

Where Cp is the total plasma drug concentration at time t, A and B are the extrapolated zero intercepts, and α and β represent the apparent first-order elimination rate constants. The half-life ($t_{1/2\beta}$) for the elimination phase and the volume of distribution in the central compartment (*V*c) were calculated as follows, $t_{1/2\beta} = 0.693/k_{10}$ where k_{10} is an overall elimination rate constant, *V*c = Dose/(A+B). For the oral route, absorption rate constant, k_a, was fitted. The parameters Clp (total plasma clearance) and *V*ss (steady state apparent volume of distribution) were calculated using non-compartmental methodology. The area under the concentration-time curve ($AUC_{0-\infty}$) and area under the first moment of the plasma drug concentration-time curve ($AUMC_{0-\infty}$) were calculated by the following equations,

$$AUC_{0\to\infty} = \frac{A}{\alpha} + \frac{B}{\beta}$$

$$AUMC_{0\to\infty} = \frac{A}{\alpha^2} + \frac{B}{\beta^2}$$

The values reported as the Cmax and Tmax are the actual observed values. The *F* (absolute bioavailability) value for oral administration can be calculated by the following formula,

$$F = \frac{D_{iv} \cdot AUC_{oral\,(0\to\infty)}}{D_{oral} \cdot AUC_{iv\,(0\to\infty)}}$$

Where, for the oral and i.v. routes D_{oral} and D_{iv} are the respective doses, ($AUC_{0-\infty}$) oral and ($AUC_{0-\infty}$) i.v. are the respective AUCs from 0 to infinity.

Oral pharmacokinetic parameters were calculated with the following equations,

$$t_{1/2\,abs} = \frac{0.693}{k_a}$$

$$AUC_{0\to\infty} = \frac{F \cdot D}{V_c \cdot k_{10}}$$

$$AUMC_{0\to\infty} = \frac{F \cdot D}{V_c \cdot k_{10}^2}$$

$$MRT = \frac{AUMC_{0\to\infty}}{AUC_{0\to\infty}}$$

$$MAT = \frac{1}{k_a}$$

$$Cl_p = V_c \cdot k_{10}$$

$$V_{ss} = V_c \cdot \left(\frac{k_{12} + k_{21}}{k_{21}}\right)$$

Where k_a and $t_{1/2abs}$ are the absorption constant and the absorption constant half-life obtained after oral administration. MRT and MAT are the mean resident time and the mean absorption time, respectively. Initial estimates of Clp and Vss were those of i.v. route. The microconstants k_{12} and k_{21} used for the calculation of *V*ss are the terms that describe the distribution of the drug between the central and peripheral compartments. When Michaelis-Menten fitting was applied, the first order elimination constant from the central compartment was substituted by the following equation,

$$C_t = \frac{V_{max} \cdot C_t}{K_m + C_t}$$

Where C_t is the methylone concentration at time t, the *Vmax* the maximum metabolic capacity achieved and K_m the Michaelis-Menten constant. Clmet is the metabolic clearance calculated as follows (Barrett et al., 1998),

$$Cl_{met} = \frac{V_{max}}{K_m}$$

2.8. Locomotor activity experiments

Prior to experiments, all rats received two habituation sessions (48 and 24 h before testing). During these sessions, each rat received saline and was placed in a Plexiglas cage. This cage constituted the activity box that was later placed inside a frame system

of 16 infrared photocells (LE8811, PANLAB, Barcelona, Spain) mounted according to the *x, y* axis coordinates and 2.5 cm above the wire mesh floor. Occlusions of the photo beams (breaks) were recorded and sent to a computerized system (SedaCom32, PANLAB, Barcelona, Spain). The interruption counts, over a 10 min-block, were measured. After intravenous or oral drug administration, locomotor activity was monitored for 180 min and 360 min, respectively. On the testing day, the animals received methylone intravenously (10 mg/kg), or orally (15 or 30 mg/kg), and were immediately placed in the activity box. Registration of horizontal locomotor activity then began. Results are expressed as area under the curve (AUC), which was measured as the total changes from baseline at each recording interval over 360 min,

2.9. Pharmacokinetic/pharmacodynamics modeling

PK/PD analysis was carried out on mean and standard deviation data. Because experimental observed data were obtained in parallel assays, data fitting were performed with the aggregates of the different doses (data pooling) in order to estimate a unique set of parameters. PK and PK/PD analysis was achieved by use of the compartmental modeling SAAM II software system (SAAM Institute, Seattle, WA, USA).

2.10. Pharmacokinetic/Pharmacodynamics analysis.

A link compartment representing stimulation of the locomotor behavior was used to describe the data (Sheiner et al., 1979). Integration of methylone pharmacokinetics and pharmacodynamics was based on the relationship between mean plasma methylone concentration-time profile for i.v. and oral dosages. PK/PD modeling was also performed by using SAAM II. The pharmacokinetic model includes central and peripheral compartments, the inter-compartmental rate constants of absorption and elimination and the input rate for i.v. and oral administration. The effect site was connected by a fixed rate constant from the central plasma compartment. A dummy compartment provides the concentrations in the effect site (C_e). The simulation PK/PD model proposed is an additive sigmoid E_{max} equation expressed in terms of C_e such that,

$$E = E_0 + \frac{E_{max} \cdot C_e^n}{C_e^n + EC_{50}^n}$$

The baseline value E_0 is the effect when methylone concentration is zero. EC_{50} is the concentration that increases E_0 to 50% of the Emax or maximal response and "n" determines the sigmoid shape of the function (Hill coefficient).

3. Results

3.1. Methylone pharmacokinetics

The observed and model-fitted plasma concentrations of methylone at each time point are shown in Figure 1. The plasma concentrations versus time curve after intravenous administration of methylone were adequately described by a two-compartment model (α=1.95 h^{-1} and β=0.72 h^{-1}) (Fig. 1). Pharmacokinetic parameters (Table 1) showed that the $t_{1/2\beta}$ was about 1 h. The Vss and Clp resulted in values of 2.39 l/kg and 0.53 l/h respectively.

For oral dosing conditions, pharmacokinetic parameters derived from the methylone curves are summarized in Table 2. Cmax values were achieved rapidly, usually within 0.5 to 1 h, and declined to undetectable levels at 24 h. As might be expected, absolute AUC value increased proportionally with dose. Bioavailability was calculated of 78-89% after oral administration of the two doses. The standard errors of the majority of pharmacokinetic parameters were relatively small (coefficients of variation < 20%). Interindividual variability in the experimental Cmax value was evident from the high value of CV.

3.2. Methylone protein binding and brain levels

Results from blood samples obtained near Tmax yielded a percentage of methylone protein binding of 30.82 ± 2.23%. Whole brain levels of 349.2 ± 26.7 ng methylone/g tissue (n = 3) were found. The relationship between brain levels and free plasma concentration yielded a ratio of 1.42 ± 0.06, indicating access of methylone to the central nervous system.

3.3. Identification of methylone and metabolites in rat blood

In the present study, we have identified four metabolites that are detected in all collected samples at 60, 120 and 180 min. The identification of methylone and the observed metabolites by mass spectrometry is provided below.

3.3.1. Methylone

The calculated [M+H^+] m/z for methylone ($C_{11}H_{13}NO_3$) was 208.09737, the found [M+H^+] m/z was 208.09720 (0.17 mDa). The peak at m/z 190 is attributable to the typical H_2O loss (18 Da). The loss of methylamine group (31 Da) gave a fragment with low intensity at m/z 177. We also observed the loss of the methylenedioxy group (CH_4O_2, 48 Da). The presence of the fragment at m/z 149 indicates the loss of C_3H_9N (59 Da), the intensity of which was found to be considerably low.

3.3.2. 3,4- Methylenedioxycathinone (MDC)

We identified the corresponding N-demethylation metabolite, MDC, with formula $C_{10}H_{11}NO_3$. The calculated [M+ H^+] m/z was 194.08117, the found [M+ H^+] m/z was 194.08142 (0.25 mDa). The peak at m/z 176 is corresponding to the H_2O loss (18 Da). The C_2H_6N and CH_4O_2 loss (45 Da and 48 Da) gave two peaks at m/z 149 and 146, respectively, suggesting that this mass spectrum is in accordance with the metabolite structure proposed.

3.3.3. 4-Hydroxy-3-methoxymethcathinone (4-OH-3-MeO-MC) and 3-Hydroxy-3-methoxymethcathinone (3-OH-4-MeO-MC)

Two metabolites were detected with the same chemical formula $C_{11}H_{15}NO_3$ and mass spectrum, but with different retention times (Fig. 2). For 4-OH-3-MeO-MC and 3-OH-4-MeO-MC the calculated [M+ H^+] m/z was 210.11247, the found [M+ H^+] m/z was 210.11319 (0.72 mDa) and 210.11355 (1.08 mDa) respectively. Both compounds gave a peak at m/z 192 (H_2O loss). In this case, the loss of the methylenedioxy group was not found, however the peak at m/z 160 shows the CO_2H_6 loss (50 Da) reflecting the aperture of the methylenedioxy ring. Based on studies by Kamata et al. (2006), we assumed that 4-OH-3-MeO-MC was the first compound to be eluted out of the two, due to that the chromatography conditions were similar.

3.3.5. 3'-Hydroxy-methylenedioxymethcathinone (3'-OH-MDMC)

A compound with chemical formula $C_{11}H_{13}NO_4$ was detected, and the calculated [M+H^+] m/z was 224.09173, the found [M+H^+] m/z was 224.09154 (0.19 mDa). Typical loss of water was also observed giving a peak at m/z 206. Furthermore, a double water loss (36 Da) was detected, indicating the possible presence of a hydroxyl group in this structure. The peak at m/z 176, which shows the loss of 48 Da, can be attributed to the methylenedioxy group. In order to confirm the proposed structure, the

loss of the methylenedioxybenzoylcation fragment (149 Da) gave a peak at m/z 74, which corresponds to a hydroxylated immonium cation (Fig. 2).

We ensured that the mass found did not correspond to endogenous compounds by comparing each metabolite mass from treated and untreated rat blood samples. From the metabolites detected, the proposed *in vivo* phase I metabolic pathway for this cathinone is displayed in Figure 3.

3.4. Locomotor activity

Intravenous administration of methylone induced an increase in the rat locomotor activity (AUC Saline, 8683 ± 98, Methylone, 95078 ± 19953, n = 3, $p<0.01$, Student-t test, independent samples) that lasted for 150 min.

Similarly, an overall ANOVA demonstrated a significant effect of oral methylone in the locomotor activity in rats ($F_{2,8}$ = 6.015, $p<0.05$). The post-hoc Tukey-Kramer test revealed that methylone increased the locomotor activity in a dose-dependent manner (AUC saline, 20760 ± 2002, Methylone 15 mg/kg, 95767 ± 23537, $p<0.05$, Methylone 30 mg/kg, 133354 ± 32878, $p<0.05$, n = 3). As can be seen in Figure 4, this increase is due mainly to a different time-course profile. The higher dose (30 mg/kg) induced a maximum break response (2011 ± 750, n = 3) that did not differ significantly from that of 15 mg/kg (2005 ± 344, n = 3), but the disappearance of the effect becomes much slower. After the dose of 15 mg/kg, break values were not significantly different from animals treated with saline after 180 min. At the highest dose tested, the psychostimulant effect of methylone persisted for 270 min (Fig. 4).

3.5. Pharmacokinetic/Pharmacodynamic analysis.

A plot of locomotor activity versus methylone concentrations over time shows a direct relationship between concentrations and pharmacological effect after i.v. administration of methylone (Fig. 5A). A counter-clockwise hysteresis behavior was observed after the oral administration (Figs. 5B and 5C). With the developed PK/PD model the mean EC_{50} and E_{max} values obtained ranged from 3.1 to 6.7 ng/ml and from 508.42 to 1280.86 breaks, respectively (Table 3). Good agreement between the predicted and observed values was noted for the locomotor activity data (mean objective function of 7.60 ± 0.52 and mean AIC of 7.04 ± 0.57). Methylone plasma concentration profile and predicted %Emax versus time are shown in Fig 6. A delay between these plasma concentrations

and the effect can be observed at the dose of 15 mg/kg, but this delay is reduced at the highest dose. This does not occur when effect site-concentration is displayed. The values of methylone concentration in the effect-site were approximately twice when doubling the dose.

4. Discussion

To our knowledge, no research on the pharmacokinetics of methylone in rats is presently available. Therefore, in this study we have characterized the pharmacokinetics and pharmacodynamics of methylone in male Sprague–Dawley rats. High levels of locomotor activity, which measures the psychostimulant effect, occurred after methylone administration and are consistent with the onset of subjective and physiological effects in humans. The oral doses used, 15 and 30 mg/kg, are equivalent to 158 and 315 mg respectively, according to the FDA guidelines (Food and Drug Administration, Center for Drug Evaluation and Research, 2005), to those frequently declared as very usual in humans.

Our results show that the blood levels of methylone in rats declined in a biphasic fashion after intravenous administration at a dose of 10 mg/kg. The large V_{ss} indicates that methylone is distributed extensively into tissues and the Clp value explains the rapid elimination half-life (1 h). At administered oral doses, methylone displayed linear pharmacokinetics since the observed concentrations in blood were directly proportional to the administered dose. This observation suggests that, at present doses, the processes involved in the disposition of methylone were not saturated. At the highest dose tested, the reduction in the k_a value and increased tlag explained that Tmax moves from 30 to 60 min.

The terminal plasma half-life of methylone after oral administration was significantly higher than after i.v. administration, suggesting a pharmacokinetic flip-flop model. Flip-flop pharmacokinetics is a phenomenon often encountered with extravascular administered drugs (Yáñez et al., 2011). Flip-flop occurs when the rate of absorption is slower than the rate of elimination. When flip-flop is expected, a longer duration of sampling may be necessary. Accordingly, in some animals, we collected blood samples 24 h after drug administration. Our fitted model confirmed that a flip-flop phenomenon

was taken place, since absorption rate ($t_{1/2abs}$ between 2–3 h) of methylone is considerably slower than its elimination rate ($t_{1/2\beta}$ of 0.55 h).

It is well recognized that compounds with a brain/plasma concentration ratio greater than 1 freely cross the blood-brain-barrier (Hitchcock and Pennington, 2006). Hence, the obtained brain/plasma ratio for methylone at Tmax of 1.42 demonstrates the access to central nervous system.

The rate of clearance and protein binding are acknowledged could cause discrepancy between *in vitro* and *in vivo* values (Halifax and Houston, 2012). In this study, methylone, at a plasma concentration near T max, binds to serum proteins at a low rate of about 30% and the metabolic clearance averages a 29% of total clearance. The calculated Clp for methylone averaged 0.5 l/h after both oral doses. Because it appeared to exceed both hepatic and renal blood flow in the rat (Birnie and Grayson, 1952, Heller and Hollyová, 1977), these in vivo data suggest that the liver, kidney, and possibly other clearing organs are involved in methylone elimination at these two oral doses.

We have investigated the *in vivo* Phase I metabolism of methylone after oral administration of a 30 mg/kg dose. We identified the existence of four metabolites in rat blood at three times after administration (60, 120 and 180 minutes) and we propose a first step phase I metabolism for methylone consisting of demethylation reaction, yielding the corresponding methylenedioxycathinone metabolite. Moreover, we propose that, in rats, methylone is a substrate of an aliphatic hydroxylation process resulting in the corresponding 3'-hydroxy-methylenedioxymethcathinone. Mueller and Rentsch (2012) proposed these methylone metabolites by an automated online metabolism method using human liver microsomes, but this is the first time that 3'-hydroxy-methylenedioxymethcathinone metabolite has been identified *in vivo.* The same authors found a third metabolite corresponding to the reduction of the beta-keto group which we did not detect in rat blood probably due to its low half-life.

We have also identified two hydroxylated metabolites (4-hydroxy-3-methoxymethcathinone and 3-hydroxy-4-methoxymethcathinone) with the same chemical formula and mass spectrum, resulting from a demethylenated intermediate that had lost the methylenedioxy group to the respective diol and which has not been found, probably due to its short half-life. Moreover, the amounts of these metabolites were

very low. This is also in agreement with results from the same authors who found that over 80% of these two metabolites were conjugated when excreted through urine. Surprisingly, we have not found the corresponding demethyl compounds of the hydroxylated metabolites but these was also not found by Mueller and Rentsch (2012), which studied Phase I metabolites. The results indicate that the metabolism of methylone contributes significantly to its plasmatic clearance and this contribution is especially evident when the drug is administered orally, as a result of a hepatic first-pass effect, as is suggested by the differential pharmacokinetic profile observed in both routes of administration.

Pharmacodynamic experiments demonstrated that intravenous and oral administration of methylone induced a psychostimulant effect, measured as an increase in locomotor activity in rats. It is important to note that the increase in the locomotor activity elicited by oral administration of methylone is due mainly to a different time-course profile. The 30 mg/kg dose induced a maximum break response which was not significantly different to that of 15 mg/kg, but the psychostimulant effect lasted longer. This is in agreement with previous published results in mice (López-Arnau et al., 2012; Marusich et al., 2012).

The PK/PD relationship established in the study allows performing an estimation of the EC_{50} and Emax parameters, and provides information about the onset, magnitude and duration of the locomotor activity with relation to the time course of methylone plasma concentrations. An increase in locomotor activity was observed immediately after methylone administration in accordance with the immediate onset of its effects in humans (Shimizu et al., 2007). There is a delay between drug response and methylone plasma levels after oral administration. Thus, in presence of delay, the methylone plasma concentration profile may not be directly related to the pharmacological effect. When defining in the model an effect site-compartment, a close relationship between the effect and the obtained concentration curve becomes evident.

At the dose of 15 mg/kg, the plot of locomotor activity as a function of plasma methylone concentrations shows a counter-clockwise hysteresis loop. This hysteresis can be explained by the appearance of active metabolites (Mandema et al., 1992); by indirect mechanisms of drug action (Dayneka et al., 1993) or by an imbalance between the site of action (the brain) and the plasmatic compartment (Sheiner et al., 1979).

In this study the presence of some metabolites of methylone, similar to active metabolites of MDMA (De la Torre and Farre, 2004), strongly suggests their participation in the overall locomotor activity, but other possibilities cannot be discarded (Csajka and Verotta, 2006). Further studies with individual metabolites will determine which structural species have the highest likelihood of contributing to the locomotor activity caused by methylone.

Additionally, we have previously described that methylone inhibits monoamine uptake by competing with the substrate (López-Arnau et al., 2012). Consequently, it induces hyperlocomotion mainly by an indirect mechanism (increasing extra-cellular dopamine) and by also a direct mechanism through activation of 5-HT_{2A} receptors could be evidenced. The lower affinity of methylone for 5-HT_{2A} receptors than for dopamine transporter lead us to hypothesize that direct activation of this receptor type can contribute to the final effect when high concentrations in the effect-site compartment are achieved. Then, at the oral dose of 30mg/kg, the lack of hysteresis can be explained by a) this additional direct mechanism of action or b) the low imbalance between brain and plasma concentrations at this dose.

The variability obtained in the pharmacodynamic estimates can be explained by the use of an Emax model without a clear maximum effect, seeing as it was obtained experimentally and taking into account that in this model maximum effect must be reached (Schoemaker et al., 1998). The experimental design followed in this study does not allow the assessment of the within and between variability of the PK/PD relationship and this drawback may contribute to the overall variability of the pharmacodynamic parameter estimates.

5. Conclusion

PK/PD analysis of methylone showed a correlation between plasma concentrations and enhancement of the locomotor activity. The identification of some metabolites of methylone, similar to active metabolites of MDMA strongly suggests their participation in the overall locomotor activity. We have previously described that methylone induces hyperlocomotion mainly by an indirect mechanism (increasing extra-cellular

dopamine). At the highest oral dose assayed, direct activation of 5-HT_{2A} receptor seems also to contribute to the final psychostimulant effect. The present research provides, for a first time, useful information on the *in vivo* pharmacokinetics of methylone, and can help design new experiments in rodents with kinetics data as well as provide a better understanding of the effects of this cathinone in humans.

Contributors

EE and JC were responsible for the study concept and design. RLA, JMC. MC and DP assisted with data analysis. All authors critically reviewed content and approved final version for publication.

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Legends of Figures

Fig. 1. Time-course of experimental and fitted plasma methylone levels after intravenous (10 mg/kg) and oral (15 and 30 mg/kg) administration. Rats received methylone at time 0, and blood specimens (0.2 mL) were collected through the external jugular vein from 0.08 to 8 h after administration. Plasma levels of methylone were quantitated by LC-MS as described in Materials and Methods section. Data are expressed as mean for *n*, 4 to 5 rats/group. SEM values are not displayed for clarity.

Fig. 2. LC-MS Orbitrap data for fragmentation of methylone and their metabolites in rat plasma after a single oral dose of 30 mg/kg at three different times after administration (60, 120 and 180 min). Scheme of proposed fragmentation patterns for methylone and its metabolite 3'-OH-MDMC.

Fig. 3. *In vivo* metabolic pathways proposed for methylone in rat plasma after a single oral administration of 30 mg/kg.

Fig. 4. Time-course of locomotor activity induced after oral (15 and 30 mg/kg) and intravenous (10 mg/kg) administration of methylone. For this behavior, the interruption counts in the frame of the apparatus were registered and displayed in a 30 min-block. Vertical axis shows breaks/animal in 30 minutes intervals. Locomotor activity was monitored for 360 min and 180 min for oral and intravenous administration, respectively. Data are expressed as the mean ± SEM of values from 3 rats.

Fig. 5. Observed plasma concentrations of methylone *versus* observed %Emax in 10 min-block. Panel A, after intravenous administration (10 mg/kg). Panel B, after oral administration (15 mg/kg). Panel C, after oral administration (30 mg/kg). Data points show experimental time (in h) of pharmacokinetic and pharmacodynamic data.

Fig. 6. Methylone plasma concentration profile (⋯⋯), effect site-concentration curve (—) and predicted %Emax (----) *versus* time. Panel A, after intravenous administration (10 mg/kg). Panel B, after oral administration (15 mg/kg). Panel C, after oral administration (30 mg/kg).

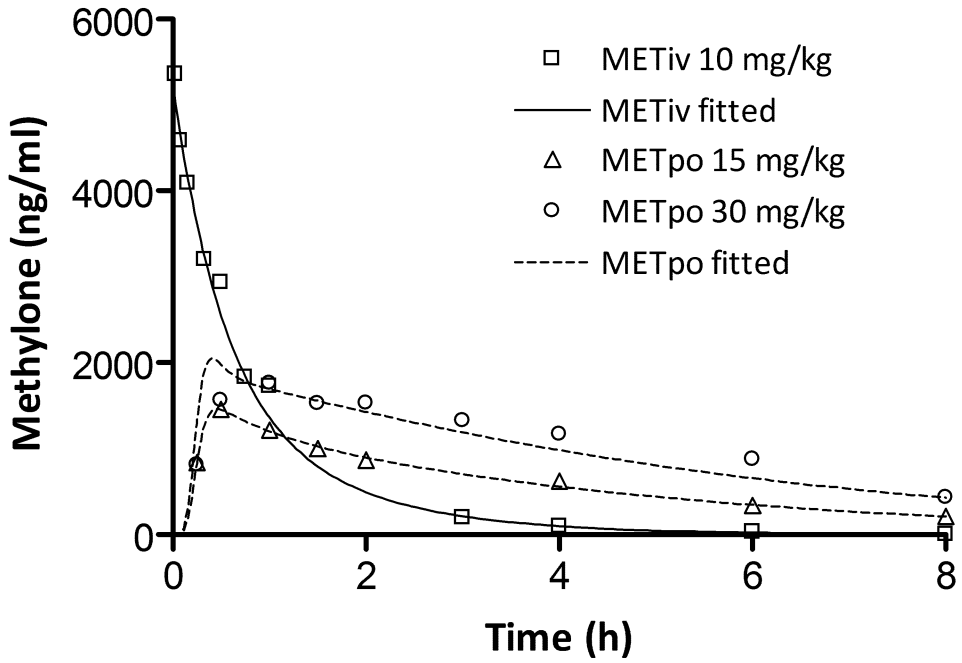


Figure 1

Drug and Metabolites	*R_t (min)*	*Monoisotopic mass*	*Precursor ion (m/z)*	*Product ions (m/z)*
MDMC	7,54	208,09720	208	190,177,160
MDC	7,14	194,08142	194	176,149,146
4-OH-3-MeO-MC	5,84	210,11319	210	192,160
3-OH-3-MeO-MC	6,16	210,11355	210	192,160
3'-OH-MDMC	10,34	224,09154	224	206,188,176,149,74

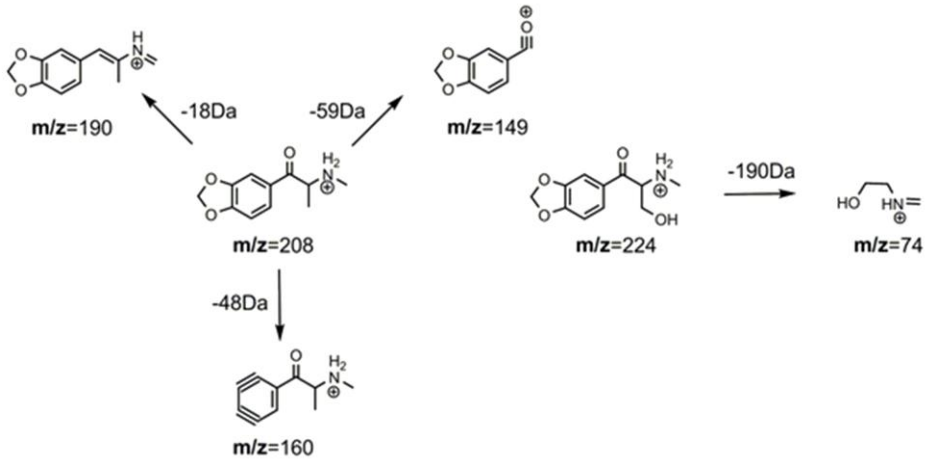


Figure 2

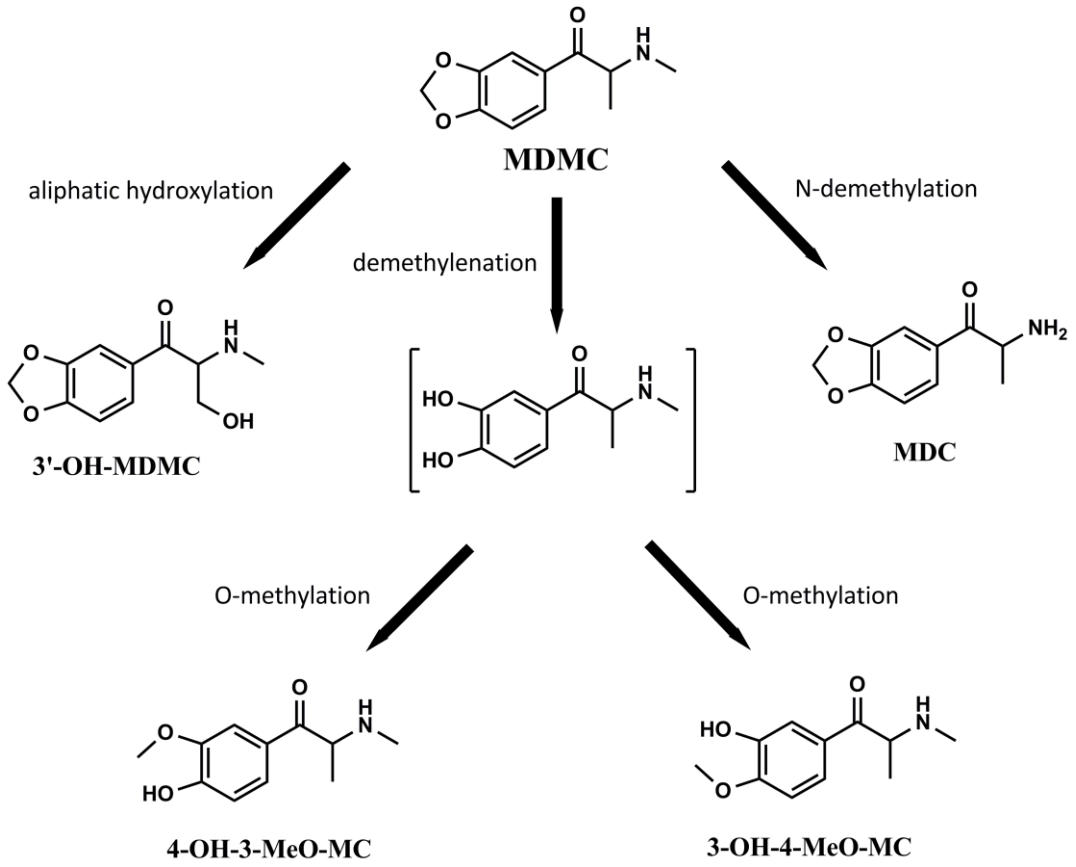


Figure 3

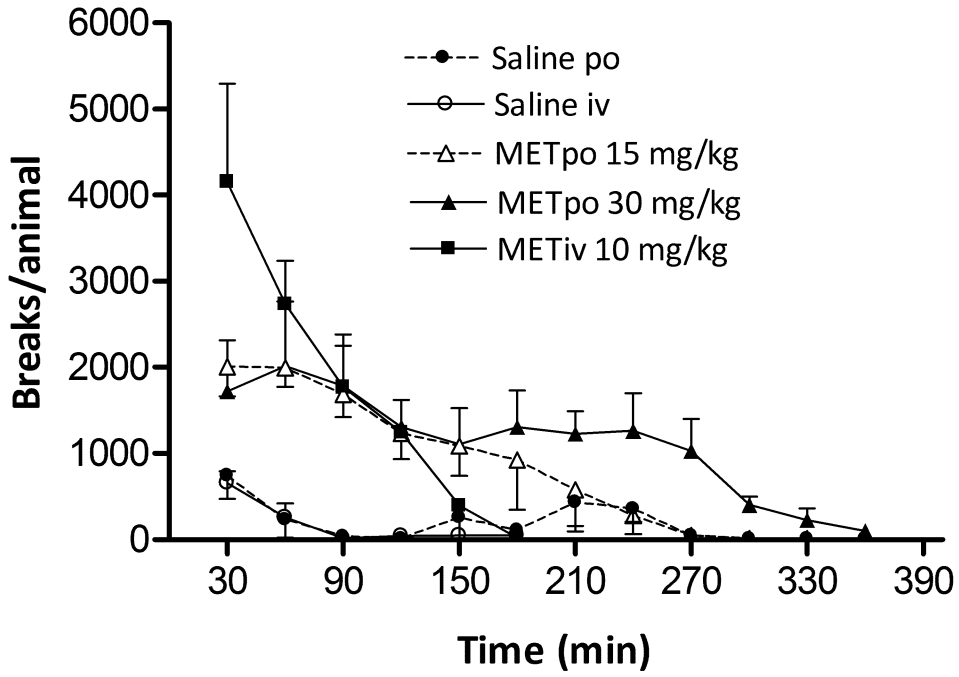


Figure 4

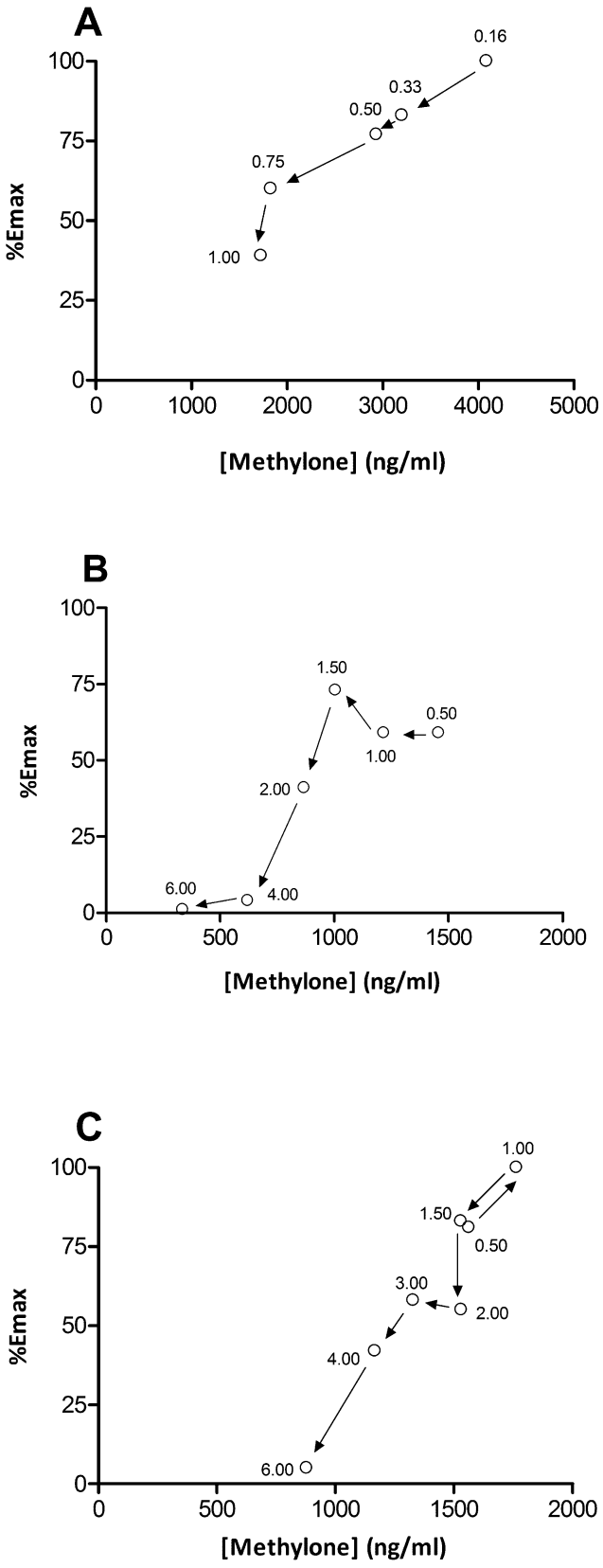


Figure 5

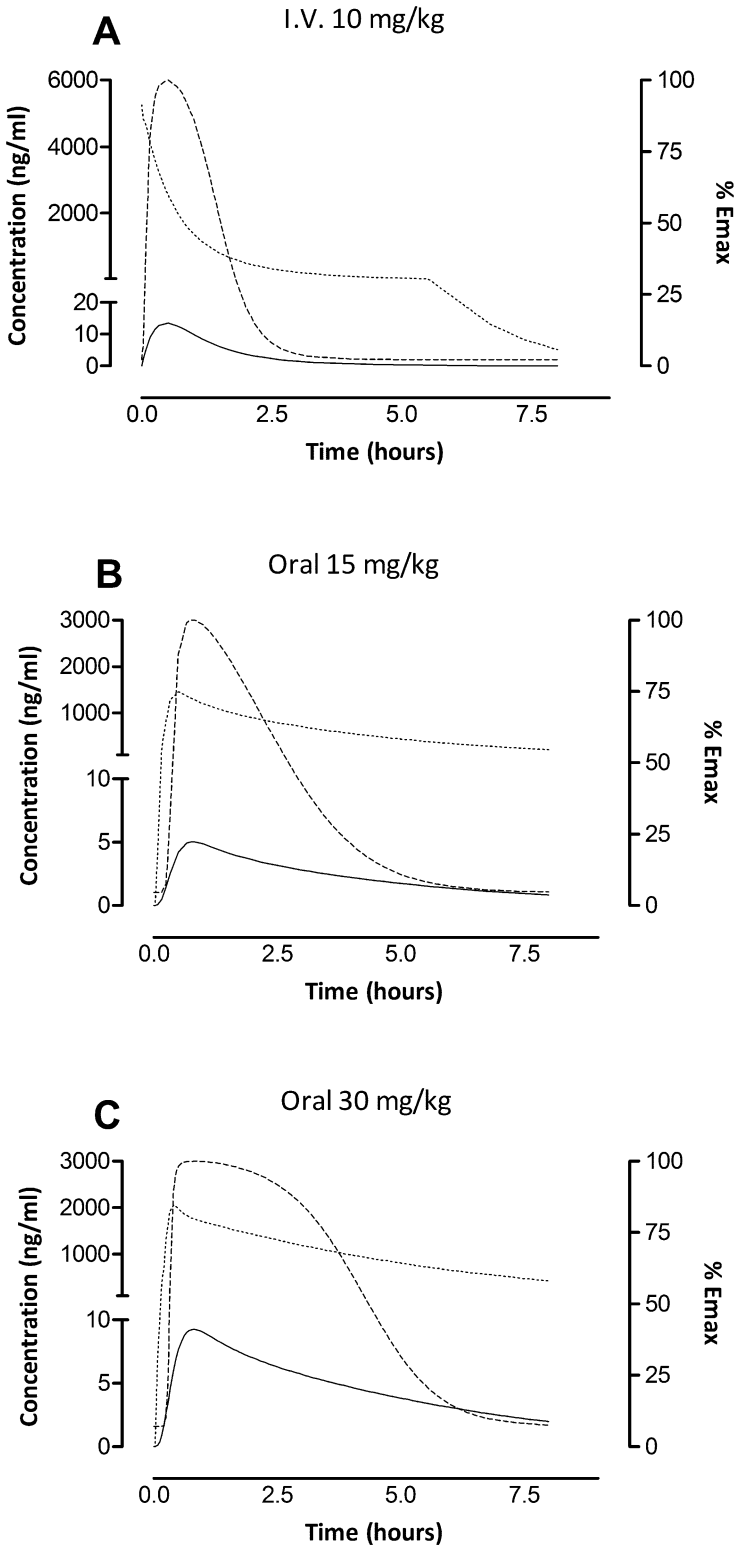


Figure 6

Table 1: Main pharmacokinetic parameter estimates of methylone after i.v. administration (10 mg/Kg) to male Sprague-Dawley rats.

Parameter	Units	Estimate	CV(%)
A	ng/ml	3483.84	12.3
B	ng/ml	1787.75	21.8
α	h^{-1}	1.95	11.7
β	h^{-1}	0.72	7.1
K_{10}	h^{-1}	1.22	4.2
K_{12}	h^{-1}	0.28	24.7
K_{21}	h^{-1}	1.15	16.4
AUC 0-∞	ng.h/ml	4251.89	1.9
AUC 0-t	ng.h/ml	4241.6	0.2
C^o_p	ng/ml	5271.60	4.7
V_c	ml	426.81	4.7
V_{ss}	ml	537.68	1.54
$t_{1/2\beta}$	h	0.95	7.1
CLp	ml/h	529.18	1.9

Table 2: Main pharmacokinetic parameter estimates of methylone after oral administration to male Sprague-Dawley rats at a dose of 15 mg/Kg (Value 15) and 30 mg/Kg (Value 30). Results are expressed as mean and the corresponding coefficient of variation (CV) in %.

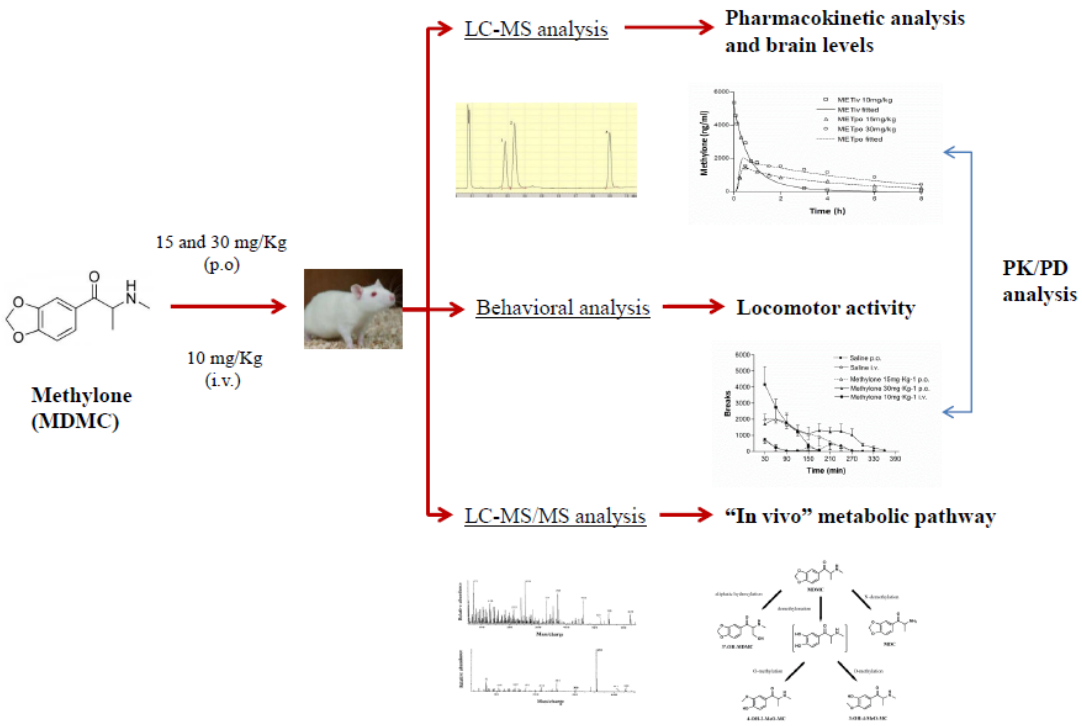
Parameter	Units	Value 15 (CV)	Value 30 (CV)
Cmax obs	ng/ml	1456.67 (15.8)	1896.00 (69.2)
Tmax obs	h	0.50 (0.0)	0.97 (55.3)
AUC 0-∞	ng.h/ml	5740.30 (24.4)	9988.80 (10.1)
AUC 0-t	ng.h/ml	4942.50 (2.4)	8092.60 (9.9)
$t_{1/2abs\ app}$	h	2.15 (7.0)	3.14 (10.7)
$t_{1/2\beta\ app}$	h	0.55 (4.5)	0.55 (4.5)
tlag	h	0.17 (7.7)	0.28 (9.8)
V_{ss}	ml	433.31 (4.9)	
F*	%	89.00 (-)	78.40 (-)
CLmet	ml/h	154.80 (18.7)	154.80 (18.7)
MRT	h	0.79 (4.5)	0.82 (4.2)
MAT	h	0.46 (7.0)	0.20 (10.7)
Km	μ/ml	2.68 (122)	2.68 (122)
Vmax	μg/h	414.30 (104)	414.30 (104)
CLp	ml/h	529.52 (2.05)	530.96 (1.9)

*Calculated as F = (AUC_0-oral *x* Dose i.v.)/(AUC_0-i.v. *x* Dose oral)

Table 3. Estimates of the pharmacodynamic parameters, according to the proposed additive sigmoid E_{max} equation PKPD model. In parentheses the corresponding coefficient of variation (CV) in %.

		dose		
Parameter	units	10 mg/kg i.v.	15 mg/kg p.o.	30 mg/kg p.o.
E_0	breaks	29.43 (112)	185.63 (28.20)	10.80 (*)
EC_{50}	ng/ml	6.68 (11.3)	1.32 (6.05)	0.90 (6.50)
E_{max}	breaks	1280.86 (8.97)	1985.30 (8.90)	1733.84 (6.60)
n	-	3.13 (**)	5.23 (**)	4.96 (**)
O.F.		6.91	5.68	5.25
AIC		7.77	6.60	7.85

O.F.: Objective Function; AIC: Akaike information criterion; (*): CV > 150%; (**) CV not determined



Graphical Abstract

Highlights

- We study the in vivo pharmacokinetics of methylone in rats
- Intravenous methylone kinetics were adjusted to a two-compartment model
- Bioavailability was about 80% and four Phase I metabolites were identified
- There exists a correlation between plasma concentrations and enhancement of the locomotor activity.