Mephedrone pharmacokinetics after intravenous and oral administration in rats: relation to pharmacodynamics.

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Abstract

Rationale Mephedrone (4-methylmethcathinone) is a still poorly known drug of abuse, alternative to ecstasy or cocaine.

Objective The major aims were to investigate the pharmacokinetics and locomotor activity of mephedrone in rats and provide a pharmacokinetic/pharmacodynamic model.

Methods Mephedrone was administered to male Sprague-Dawley rats intravenously (10 mg/kg) and orally (30 and 60 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 Mephedrone plasma concentrations after i.v. administration fit to a two-compartment model (α=10.23 h^{-1} and β=1.86 h^{-1}). After oral administration, peak mephedrone concentrations were achieved between 0.5-1 h, and declined to undetectable levels at 9 h. Absolute bioavailability of mephedrone was of about 10% and the percentage of mephedrone protein binding was of 21.59 ± 3.67%. We have identified five Phase I metabolites in rat blood after oral administration. The relationship between brain levels and free plasma concentration was 1.85 ± 0.08. Mephedrone induced a dose-dependent increase in locomotor activity, which lasted up to 2 h. The pharmacokinetic-pharmacodynamic model successfully describes the relationship between mephedrone plasma concentrations and its psychostimulant effect.

Conclusions We suggest a very important first-pass effect for mephedrone after oral administration and an easy access to the central nervous system. The model described might be useful in the estimation and prediction of the onset, magnitude and time course of mephedrone pharmacodynamics as well as to design new animal models of mephedrone addiction and toxicity.

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

Introduction

Mephedrone is a synthetic ring-substituted cathinone closely related to the phenethylamine family, differing only by a keto functional group at the beta carbon. It can be purchased online or from street-dealers. On the Internet, mephedrone is often marketed as ‘plant food’, ‘bath salt" or ‘research chemical’. The rapid rise in popularity of mephedrone may reflect growing user dissatisfaction with the purity and availability of MDMA (ecstasy) or its chemical precursors and cocaine. Mephedrone appears to be used by several population groups, including people involved in the dance and music scene, as well as mainstream young adults, and adolescents (Schifano et al. 2011).

Mephedrone is known to have similar effects to other psychostimulant drugs (Brunt et al. 2012; Varner et al. 2012). There are two reported fatalities in the European Union in which mephedrone appears to be the sole cause of death and there are at least another 37 deaths in which mephedrone have been detected in post-mortem samples (Maskell et al. 2011). Mephedrone was the first cathinone derivative to be ‘risk-assessed’ by the extended Scientific Committee of the EMCDDA (European Monitoring Centre for Drugs and Drug Addiction).

Information on mephedrone human pharmacokinetics is limited to user reports. From these, it appears that both the desired and adverse effects of mephedrone are similar to those found for MDMA and cocaine (Wood et al. 2010b). However, its relatively short duration of action, which lead to repeated dosing, is more similar to cocaine (Wilkinson et al. 1980). In a recent study, 70 % of mephedrone users reported to be using it by nasal insufflation, while 30 % admitted to be taking it orally (Dickson et al. 2010; Winstock et al. 2011). There are numerous reports of individuals using mixed routes during a single session. Moreover, there are increasing reports of intravenous injection and there is also one case report from the UK of an individual who developed acute mephedrone toxicity after an intramuscular injection of dissolved powder (Wood et al. 2010a).

Users report on Internet forums that desired effects are typically seen within 15–45 minutes after oral ingestion and stated that the onset of action is slower when mephedrone is taken orally on a full stomach. Users also report onset of action to be within a few minutes following nasal insufflation, with peak desired effects after 30

minutes. Desired effects last approximately 2–3 hours; this leads users to redoes during a single session to extend the duration of the desired effects.

Studies on the pharmacokinetics of mephedrone in rats are very scarce. Meyer et al. (2010) suggested the most probable metabolites of mephedrone present in the urine. In this study mephedrone was administered orally and urine was collected over a 24-hour period. Hadlock et al. (2011) assessed mephedrone concentrations in rat brain and plasma 1 h after a binge treatment and more recently (Khreit et al. 2012) investigated the *in vitro* metabolism of mephedrone in rat isolated hepatocytes.

The current studies were designed to investigate the pharmacokinetic profile and pharmacodynamic response to mephedrone in rats after its acute administration by intravenous and oral route at different doses by using a highly sensitive and specific analytical methodology, based on liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS). Results from this study might provide useful tools in order to design new animal models of mephedrone addiction and toxicity. This objective was first pursued by constructing a pharmacokinetic/pharmacodynamics (PK/PD) model for mephedrone in rats in order to obtain basic guidelines for human risk assessment. In this study the following main pharmacokinetic parameters were estimated: total area under the curve ($AUC_{0-\infty}$), apparent volume of distribution at steady state (V_{ss}), total plasma clearance (Cl_p), metabolic clearance (Cl_{met}), and elimination half-life ($t_{1/2\beta}$). The pharmacodynamic studies described herein focused on the evaluation of the psychostimulant effect of this drug of abuse, and trying to establish a correlation between the hyperlocomotion induced by mephedrone and its plasma levels. Furthermore, another goal of this study was to analyze the metabolic pathway of mephedrone and its brain concentration after an oral administration as well as to establish a relationship between mephedrone brain concentration and its psychostimulant effect.

Materials and methods

Drugs

Pure racemic mephedrone and methylone HCl were synthetized and characterized by us as described previously (López-Arnau et al. 2012). Mephedrone solutions for injection were prepared in saline immediately before administration. Isoflurane was from

Laboratorios Dr. Esteve (Barcelona, Spain). Reagents required for LC/MS assays were obtained from Sigma-Aldrich (St. Louis, MO, USA).

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 guidelines of the European Community Council (86/609/EEC). 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.

Pharmacokinetic experiments

For intravenous pharmacokinetic analysis, mephedrone was administered at a dose of 10 mg/kg. For oral pharmacokinetic experiments, mephedrone was administered at doses of 30 and 60 mg/kg to rats previously fasted for 18 h.

Blood samples were collected from isoflurane anesthetized rats in a time schedule from 0.08 to 9 h and transferred to 1 ml glass tubes with 7.5% EDTA on ice. After each blood extraction, an equal volume of saline was infused to maintain homeostasis. In some animals, blood samples and the whole brain were obtained simultaneously 45 min after oral administration (dose of 30 mg/kg).

90 µl of plasma samples were mixed with 10 µl of internal standard (IS) solution (methylone, 200 ng/ml). The mixture was extracted with methanol (to a final concentration of 70%) and after centrifugation (10,000 x *g* for 5 min), supernatant was acidified (Sørensen et al. 2011), filtered (Microcon 30, Millipore, Bedford, MA, USA) and transferred to an auto sampler vial.

An HP Agilent Technologies 1100 LC system equipped with an auto sampler and a column oven set to 40 ^{o}C and coupled API 3000 triple-quadrupole MS (AB Sciex) 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 and controlled by Analyst software. 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 in the auto sampler and 5 µl 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). The turbo ion spray source was used in positive mode with a capillary voltage of +4500 V and N_2 as nebulizer gas. For mephedrone one transition was followed (*m/z* 178.1→160.0) (collision energy of 17 V). Two transitions were followed for methylone (*m/z* 208.1→190.1; 208.1→160.0) (collision energies of 17 and 22 V respectively), and both were used for the quantification.

Blood Metabolite determination

Blood samples were collected at 30, 60 and 120 min after oral administration at a dose of 30 mg/kg. The metabolite samples were treated as described above, without IS. For the metabolite identification, a Linear Trap Quadrupole Orbitrap Velos MS equipped with an ESI source was used, coupled to an Accela chromatograph, a refrigerated auto sampler and a photodiode array detector (Thermo Scientific, Hemel Hempstead, U.K.). 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 with an increasing linear gradient (v/v) of B (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. Data were acquired in Fourier transform mass spectrometry mode (FT MS). Operation parameters were as follow: source voltage, 3.5(kV) in positive or negative ion 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 using collision energy between 35 and 50%.

Protein binding and brain levels

In protein binding experiments samples were divided in two. One half was filtered through centrifugal filter units (Centrifree YM-30, Millipore) 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 mephedrone levels and protein binding assays were quantified as described in the pharmacokinetic experiments.

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 mephedrone levels, five standards were prepared, also daily, in 0.5 ml of brain homogenate (from 10 to 250 ng/ml). Methylone 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% and t0he intra- and inter-assay coefficients of variation (CV) were less than 15%.

Locomotor activity experiments

Prior to experiments, animals received two habituation sessions (48 and 24 h before testing). During these sessions, each rat was intravenously or orally administered saline and placed in a Plexiglas cage. This cage constituted the activity box that was later placed inside a frame system of two sets 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 were recorded and sent to a computerized system (SedaCom32, PANLAB, Barcelona, Spain). The interruption counts, over a 10 min-block, were used as a measure of horizontal locomotor activity. After intravenous or oral drug administration, locomotor activity was monitored for 180 min and 240 min, respectively. On the testing day, the animals received mephedrone intravenously (10 mg/kg), or orally (30 or 60 mg/kg), and were immediately placed in the activity box. Registration of horizontal locomotor activity then began.

Pharmacokinetic/pharmacodynamic modeling

PKPD analysis was carried out on mean and standard deviation data. Because experimental 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. Assessment of the relative goodness of fit of each proposed model to the observed data was based on the objective function value, the AIC (Akaike's information criterion), the residual and weighted residual plots and the errors in parameter estimation, expressed as the CV (in %). PK and PKPD analysis was achieved by use of the compartmental modeling SAAM II software system (SAAM Institute, Seattle, WA, USA).

Pharmacokinetic analysis.

The distribution and elimination characteristics of mephedrone were determined after the i.v. administration. Fixing the parameters obtained in the i.v. model, p.o. 30 and 60 mg/kg mephedrone profiles were analyzed simultaneously by using a bicompartmental 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, 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:

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

Where Cp is the total plasma drug concentration at time t, the terms 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, and absorption rate constant, k_a, was fitted. The area under the concentration-time curve ($AUC_{0-\infty}$) and area under the first moment of the plasma drug concentration-time curve ($AUCM_{0-\infty}$) were calculated by the following equations:

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

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

The values reported as the C_{max} and T_{max} are the actual observed values. The F (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 pharmacokinetics parameters were calculated with the following equations:

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

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

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

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

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

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

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

Where k_a and $t_{1/2,abs}$ 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. 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 mephedrone concentration at time t, the V_{max} the maximum metabolic capacity achieved by the metabolic system and K_m the Michaelis-Menten constant. Cl_{met} is the metabolic clearance calculated as follows (Barrett et al. 1998):

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

Pharmacokinetic/Pharmacodynamic analysis.

A link compartment representing stimulation of the locomotor behavior was used to describe the data (Sheiner et al. 1979). Integration of mephedrone pharmacokinetics and pharmacodynamics was based on the relationship between mean plasma mephedrone concentration-time profile for i.v. and oral dosages. PK/PD modeling was also performed by using SAAM II. 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 stimulation PK/PD model proposed by the sigmoid E_{max} equation is 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 mephedrone concentration is zero. EC_{50} is the concentrations that increase E_0 to 50% of the E_{max} or maximal response and "n" determines the sigmoid shape of the function (Hill coefficient) and contributes to the steepness of the slope. This produces a PD model that describes the effect as a function of time.

Results

Mephedrone pharmacokinetics

The observed plasma concentrations after i.v. administration of mephedrone at each time point are shown in Figure 1. Plasma mephedrone was almost undetectable at 4 h after administration of this dose. Estimated values for the main pharmacokinetic parameters of intravenous administration in rats are presented in Table 1.

The plasma concentrations versus time curve after intravenous administration of mephedrone were described by a two-compartment model with significantly different distribution and terminal elimination phases (α = 10.23 h^{-1} and β = 1.86 h^{-1}). Pharmacokinetic parameters showed that the $t_{1/2\beta}$ was 0.37 h. The Cl_p and the V_{ss} were of 1.69 l/h and 2.60 l/kg, respectively (Table 1).

For oral dosing conditions, pharmacokinetic parameters derived from the mephedrone plasma concentration curves are summarized in Table 2. C_{max} values were achieved rapidly showing a T_{max} within 0.43 to 0.93 h, plasma concentrations declined to undetectable levels at 9 h. Dose-normalized C_{max} at 60 mg/kg (16.00 ± 5.70) was greater than the observed at 30 mg/kg (11.00 ± 6.93) but did not reach significance. The absolute oral bioavailability was 7.30% and 11.20% for 30 and 60 mg/kg respectively.

Normalized $AUC_{0-\infty}$ values by the dose (9.82 ± 0.72 and 14.92 ± 2.87 for 30 and 60 mg/kg, respectively; $p<0.05$) show a non-lineal increase. V_{max} and K_m parameters and the calculated Cl_{met} were different for the two oral doses (Table 2). After oral administration, the value of Cl_{met} decreased from 77% of total plasma clearance (at the dose of 30 mg/Kg) to 20% (at the dose of 60 mg/Kg) showing an oral dose-dependent kinetic behavior.

Mephedrone protein binding and brain levels

Results from present assays evidenced a percentage of mephedrone protein binding of 21.59 ± 3.67%. Brain levels of 104.4 ± 17.7 ng mephedrone/g tissue (n = 3) were found. A relationship between brain levels and free plasma concentration yielded a ratio of 1.85 ± 0.08.

Identification of mephedrone and metabolites in rat blood

We have identified five metabolites of mephedrone. These metabolites were detected in all collected samples at 30, 60 and 120 min. A description of the identification of mephedrone and the observed metabolites is provided below.

Mephedrone

The calculated [M+H^+] m/z for mephedrone ($C_{11}H_{15}NO$) was 178.12319; the found [M+H^+] m/z was 178.12290 (0.29 mDa). The peak at m/z 160 is attributable to the typical H_2O loss (18 Da). The loss of methylamine group (31 Da) yielded a fragment with low intensity at m/z 147. The presence of the fragment at m/z 119 indicates the loss of C_3H_9N (59 Da), the intensity of which was found to be considerably low.

4-Methylcathinone (4-MC)

We identified the corresponding N-demethylation metabolite, 4-MC, with formula $C_{10}H_{13}NO$. The calculated [M+ H^+] m/z was 164.10699; the found [M+ H^+] m/z was 164.10728 (0.29 mDa). The peak at m/z 146 is corresponding to the H_2O loss (18 Da). The C_2H_6N loss (45 Da) gave a peak at m/z 119. Both losses suggest that this mass spectrum is in accordance with the metabolite structure proposed.

4-Hydroxymethylmethcathinone (*4-OH-MMC*)

We identified an allylic hydroxylation of mephedrone, the resulting chemical formula of which was $C_{11}H_{15}NO_2$. The calculated [M+ H^+] m/z was 194.11756; the found [M+ H^+] m/z was 194.11745 (0.11 mDa). The typical loss of water was also observed giving a

peak at m/z 176. Furthermore, we detected a double water-loss (36 Da), suggesting the possible presence of a hydroxyl group in this structure. The loss of C_3H_9N gave a peak at m/z 135, indicating that this fragment contains the hydroxyl group previously mentioned. Moreover, the double-loss of water leads us to conclude that this group can only be found in the allylic position (Fig. 2).

4-Carboxymethylmethcathinone (4-CMMC)

The structure corresponding to the oxidation of 4-OH-MMC was identified; the chemical formula for this compound was $C_{11}H_{13}NO_3$; the calculated [M+ H^+] m/z was 208.09737; the found [M+ H^+] m/z was 208.09702 (0.35 mDa). In positive ion mode, the spectrum showed a peak at m/z 149 (C_3H_9N loss) indicating the possible presence of a carboxybenzoylcation fragment. The detection of a typical carboxylic loss (44 Da) in negative ion mode confirmed the proposed structure.

3'-Hydroxy-4-methylmethcathinone (3'-OH-4-MMC)

A compound with chemical formula $C_{11}H_{15}NO_2$ was identified; the calculated [M+H^+] m/z was 194.11756; the found [M+H^+] m/z was 194.11774 (0.19 mDa). The typical loss of water was also observed giving a peak at m/z 176. Additionally, a water double-loss (36 Da) was detected indicating the possible presence of a hydroxyl group in this structure. The loss of the methylbenzoylcation fragment (119 Da) gave a peak at m/z 74, which corresponds to a hydroxylated immonium cation thus confirming the proposed structure.

?-Hydroxy-4-methylmethcathinone (?-OH-4-MMC)

The metabolite corresponding to an aromatic hydroxylation for mephedrone was detected. The chemical formula for this structure was also $C_{11}H_{15}NO_2$; the calculated [M+H^+] m/z was 194.11756; the found [M+H^+] m/z was 194.11773 (0.18 mDa). The peak at m/z 135 corresponds to the hydroxylated methylbenzoylcation and the single loss of water (peak at m/z 176) suggests that this group is in an undetermined aromatic position.

We ensured that the mass found did not correspond to endogenous compounds by comparing each metabolite mass from treated and untreated rat blood samples. Based on the found metabolites, we propose the following phase I metabolic pathway for mephedrone, displayed in Figure 3.

Locomotor activity

Intravenous administration of mephedrone induced a significant increase in rat locomotor activity (AUC Saline: 8683 ± 98; Mephedrone: 71248 ± 9518; n = 3, $p<0.01$; Student-t test, independent samples) that lasted for 120 min.

Similarly, an overall ANOVA demonstrated a significant effect of oral mephedrone on the locomotor activity in rats ($F_{2,8} = 11.261$, $p<0.01$). The post-hoc Tukey-Kramer tests, revealed that oral administration of mephedrone increased the locomotor activity in a dose-dependent manner (AUC saline: 20760 ± 2002; mephedrone 30 mg/kg: 39778 ± 10255, $p<0.05$; mephedrone 60 mg/kg: 79692 ± 22302, $p<0.01$; n = 3). As can be seen in Figure 4, this increase is due mainly to a different time-course profile. The higher dose (60 mg/kg) induced a maximum break response (2784 ± 901, n = 3) that did not differ significantly from that of 30 mg/kg (1802 ± 163, n = 3), but the disappearance of the effect becomes much slower; 60 min after the dose of 30 mg/kg breaks values were not significantly different from animals treated with saline. At the dose of 60 mg/kg, the psychostimulant effect of mephedrone persisted for 90 min.

Pharmacokinetic/Pharmacodynamic analysis.

A plot of locomotor activity versus mephedrone concentrations over time shows a direct concentration-effect relationship after i.v. administration (Fig 5a). After oral dosing (Fig 5b and 5c) a clockwise hysteresis loop was observed, mainly with the highest dose. The model shows a mean E_{max} value of 444.28 ± 200.10 and EC_{50} values ranging from 65.37 to 315.44 ng/ml (with a mean value of 0.86 μM) (Table 3). Good agreement between predicted and observed values was obtained in accordance to the quality model adequacy (mean objective function= 4.35, AIC = 9.54, determined CV <50% for all the estimated parameters).

Discussion and conclusions

The pharmacokinetics of mephedrone was investigated in adult male Sprague-Dawley rats. In this study the increase in locomotor activity induced by mephedrone after i.v. and oral administration was characterized and an integrated PK/PD model was provided.

A significant increase in locomotor activity (a measure for psychostimulant effects) occurred after mephedrone administration and is consistent with the onset of subjective effects in humans. Doses reported vary from 15 to 250 mg for oral ingestion and 5 to 125 mg for nasal insufflation with total doses typically ranging 0.5–2.0 g after redosing during a single session. The oral doses used in this study were chosen according to the FDA guidelines (Food and Drug Administration, Center for Drug Evaluation and Research, 2005) and are equivalent to 336 and 672 mg respectively.

The pharmacokinetics of mephedrone in plasma after i.v. and oral administration was well described by a two compartmental open model with and without Michaelis-Menten elimination, respectively. The goodness of fit and quality of estimated pharmacokinetic parameters were evaluated and confirmed by the objective function, AIC, plot of observed versus predicted concentrations and the variation coefficients with values lesser than 50%, except for the V_{max} value at the highest oral dose where the CV has not been estimated.

Our results show that the blood levels of mephedrone declined biphasically after intravenous administration. The large apparent V_{ss} indicates that, in rats, mephedrone is distributed extensively into tissues. This V_{ss} value was expected of a highly lipophilic molecule that must cross the blood brain barrier in order to exert its psychostimulant effect.

Compounds with a brain/plasma ratio value greater than 1 freely cross the blood-brain-barrier (Hitchcock and Pennington 2006). Hence, the obtained value of mephedrone brain/plasma ratio of 1.85 demonstrates a high and good central nervous system penetration. Moreover this result is in agreement with those obtained by Simmler et al. (2012) in an *in vitro* model of the human blood brain barrier.

Comparing the two oral doses, an evident non-linearity in the pharmacokinetics of mephedrone was detected using a Michaelis-Menten elimination equation. The non-linear pharmacokinetics described in the study allows to identify different V_{max} and K_m values, suggesting that the pathways involved in the metabolic clearance may be quantitatively and/or qualitatively different depending on the dose. Because the metabolic clearance decreased from 77% of total plasma clearance (at the dose of 30 mg/Kg) to 20% (at the dose of 60 mg/Kg), and taking into account the presence of phase I metabolites in plasma, a possible saturation of liver metabolic enzymes and pathways is suggested. These results are in agreement with the low oral bioavailability observed and suggest that mephedrone undergoes an extensive first pass effect after oral administration. Moreover, it is important to note that the low bioavailability of mephedrone explains its widespread use snorted rather than taken orally.

This study represents the first qualitative assessment of mephedrone metabolites in rats after oral intake. We identified the presence of five metabolites in rat blood at three time points after administration (30, 60 and 120 minutes). Based on the obtained data, we propose a first step of phase I metabolism for mephedrone, implying an N-demethylation reaction, yielding the corresponding methylcathinone metabolite. Mephedrone undergoes different oxidative reactions including aliphatic and aromatic hydroxylation leading to the corresponding 3'-hydroxy-methylmethcathinone or ?-hydroxy-4-methylmethcathinone metabolites. Khreit et al. (2012) proposed one of these metabolites as an unidentified compound named as "compound U". This is the first time that both mephedrone metabolites have been identified *in vivo*. We also identified 4-hydroxymethylmethcathinone, a metabolite resulting from an allylic hydroxylation of mephedrone, which would subsequently suffer an oxidation, leading to the final metabolite 4-carboxymethylmethcathinone

Intravenous and oral administration of mephedrone induced a psychostimulant effect, measured as an increase in the locomotor activity in male Sprague-Dawley rats. As we have described previously in mice (López-Arnau et al. 2012), after an oral administration to rats, a dose-dependent duration of the effect of mephedrone was found. It is important to note that the increase in the locomotor activity elicited by mephedrone is mainly due to a different time-course profile. The 60 mg/kg dose

induced a maximum break response which was not significantly different from that of 30 mg/kg, but the psychostimulant effect lasted longer. This can be explained by the saturation of mephedrone metabolism, as mentioned above.

Although more research on the pharmacology and toxicology of abused cathinones is needed (Baumann et al. 2012), this study shows new insights into the pharmacokinetic-pharmacodynamic relationship of mephedrone. By means of this relationship it is possible to perform an estimation of the EC_{50} in the effect compartment and E_{max} parameters, as well as an evaluation of the onset, magnitude and time course of the psychostimulant effect. The pharmacodynamics maximum effect was observed immediately after mephedrone administration and the decay of the stimulatory effect was evaluated from the plot of locomotor activity as a function of mephedrone plasma concentrations.

After the i.v. administration, the change in response was directly interpretable as function of mephedrone concentration with the use of a sigmoidal E_{max} model (Csajka and Verotta 2006). After oral dosage, plots of mephedrone effect versus observed plasma mephedrone concentrations revealed a clockwise hysteresis loop in both oral dosages. A clockwise hysteresis loop has been described after cathinone (Schechter 1990) or MDMA administration (Hysek et al. 2012) and could be attributable to the more rapid distribution of the drug to the brain than to venous blood (Porchet et al. 1987).

The high brain/plasma ratio obtained in this study suggests that mephedrone freely crosses the blood brain barrier, showing an extensive distribution within the brain. Previous *in vitro* results suggest that mephedrone inhibits dopamine, norepinephrine and serotonin uptake at concentrations around 1 μM (López-Arnau et al. 2012). These results are in close agreement with the EC_{50} values in the effect site calculated in the present study by means of the proposed PKPD model. The low bioavailability of mephedrone found after oral ingestion justifies why abusers preferably snort it. Mephedrone's half-life is shorter than that of MDMA (Fonsart et al. 2009), which causes users to often redose, thus contributing to the appearance of addiction. Finally, the non-linear kinetics after oral administration of mephedrone can cause a dramatic increase in plasma levels, leading to enhanced toxicity.

In conclusion, the present research provides, for a first time, useful information on the *in vivo* pharmacokinetics, pharmacodynamics and the pharmacokinetic-pharmacodynamic relationship of mephedrone in rats and will help design new experiments in rodents with kinetics-based data as well as offer a better understanding of the effects of this drug of abuse in humans.

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Statement of conflicts of interest The authors declare that they have no financial or commercial conflicts of interest.

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Legends for figures

Fig. 1 Semi-logarithmic plot of experimental and fitted mephedrone plasma levels after intravenous (10 mg/kg) and oral (30 and 60 mg/kg) administration. Rats received mephedrone at time 0, and blood specimens (0.2 ml) were collected through the external jugular vein from 0.08 to 8 h after administration. Mephedrone plasma levels were quantitated by LC-MS as described in Materials and Methods section. Data are mean for *n:* 4 to 5 rats/group.

Fig. 2 LC-MS Orbitrap data for fragmentation of mephedrone and their metabolites in rat plasma after a single oral dose of 30 mg/Kg at three different times after administration (30, 60 and 120 min). Scheme of proposed fragmentation patterns for product ions of mephedrone and its metabolites.

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

Fig. 4 Time-course of locomotor activity induced after oral (30 and 60 mg/kg) and intravenous (10 mg/kg) administration of mephedrone. For this behavior, the interruption counts were registered, displayed in a 30 min-block. Vertical axis shows breaks/animal in 30 minute intervals. Locomotor activity was monitored for 240 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 mephedrone versus observed locomotor activity measured each 10 min. Panel a: after intravenous administration (10 mg/kg). Panel b: after oral administration (30 mg/kg). Panel c: after oral administration (60 mg/kg). Data points show experimental time (in h) of pharmacokinetic and pharmacodynamic data.