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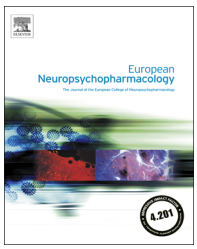

Human pharmacology of 3, 4-methylenedioxymethamphetamine (MDMA, ecstasy) after repeated doses taken 4 h apart Human pharmacology of MDMA after repeated doses taken 4 h apart

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Abstract
3,4-Methylenedioxymethamphetamine (MDMA, ecstasy) is a popular psychostimulant, frequently associated with multiple administrations over a short period of time. Repeated administration of MDMA in experimental settings induces tolerance and metabolic inhibition. The aim is to determine the acute pharmacological effects and pharmacokinetics resulting from two consecutive 100 mg doses of MDMA separated by 4 h. Ten male volunteers participated in a randomized, double-blind, crossover, placebo-controlled trial. The four conditions were placebo plus placebo, placebo plus MDMA, MDMA plus placebo, and MDMA plus MDMA. Outcome variables included pharmacological effects and pharmacokinetic parameters. After a second dose of MDMA, most effects were similar to those after a single dose, despite a doubling of MDMA concentrations (except for systolic blood pressure and reaction time). After repeated MDMA administration, a 2-fold increase was observed in MDMA plasma concentrations. For a

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simple dose accumulation MDMA and MDA concentrations were higher (+23.1% C_{max} and +17.1% AUC for MDMA and +14.2% C_{max} and +10.3% AUC for MDA) and HMMA and HMA concentrations lower (−43.3% C_{max} and -39.9% AUC for HMMA and -33.2% C_{max} and -35.1% AUC for HMA) than expected, probably related to MDMA metabolic autoinhibition. Although MDMA concentrations doubled after the second dose, most pharmacological effects were similar or slightly higher in comparison to the single administration, except for systolic blood pressure and reaction time which were greater than predicted. The pharmacokinetic-effects relationship suggests that when MDMA is administered at a 4 h interval there exists a phenomenon of acute tolerance to its effects.

1. Introduction

c(±)-3,4-Methylenedioxymethamphetamine (MDMA, ecstasy) is a synthetic amphetamine analogue widely used by young people. It exerts its effects by interacting with multiple neurotransmitter systems (the release of serotonin, dopamine and norepinephrine, and the re-uptake inhibition of these neurotransmitters) although it is typically referred to as a serotonergic drug. MDMA causes heightened feelings of well-being and euphoria (Camí et al., 2000; Camí and Farré, 2003). Ecstasy is associated with acute medical complications, long-term psychiatric disorders, and neuropsychological deficits (Greene et al., 2003; de Sola Llopis et al., 2008; Martín-Santos et al., 2010; Cuyàs et al., 2011). Its consumption is typically linked to intensive self-administration patterns: 'stacking' or taking several ecstasy tablets at once, and 'boosting' - repeatedly taking tablets during the evening. The most common pattern of use consists of one-half to five tablets taken 30 min to 2-4 h (h) apart (Parrott, 2005; Morefield et al., 2011; Licht et al., 2012; Ogeil et al., 2013). These kinds of dosing regimens could modify the MDMA pharmacological effects observed after a single dose, are associated with greater neuropsychological problems, and may place users at an increased risk of toxicity. Because the main desired effects of MDMA are reported to vanish 2 h after drug intake (Camí et al., 2000), while blood concentrations have not declined at that point (de la Torre et al., 2004), the patterns of use suggest that most subjects develop acute tolerance to MDMA effects.

Effects after repeated administrations have been investigated in several animal models where the dose and frequency of the applied administration protocols had profound effects on the severity of acute (hyperthermia) and long-term (neurotoxicity, cognition and behavior) responses (O'Shea et al., 1998; Green et al., 2004; Green et al., 2009; Plaza-Zabala et al., 2010; Viñals et al., 2013). In humans, several single dose placebo-controlled studies have been reported (de la Torre et al., 2000a; Pardo-Lozano et al., 2012). In addition, some repeated dose placebo-controlled studies have been published (Farré et al., 2004; Kuypers et al., 2007; Kuypers et al., 2008; Peiró et al., 2013), including the therapeutic use of MDMA in post-traumatic stress disorder (PTSD) (Mithoefer et al., 2011; Oehen et al., 2013).

The two previous studies carried out by the authors: the administration of two 100 mg doses of MDMA separated by 24 h (Farré et al., 2004); and one dose of 50 mg followed by 100 mg of MDMA within a 2 h interval (Peiró et al., 2013), showed some pharmacological tolerance to subjective effects, a possible sensitization phenomenon to some physiological effects, and an MDMA metabolic autoinhibition.

The present study was designed to determine the acute pharmacological effects and pharmacokinetics of two consecutive 100 mg MDMA doses separated by 4 h. The study design includes four experimental conditions (placebo plus placebo, placebo plus MDMA, MDMA plus placebo, and MDMA plus MDMA) which permit a more accurate evaluation of a potential tolerance phenomenon and metabolism inhibition.

2. Experimental procedures

2.1. Subjects

Ten healthy male volunteers were included in the study (mean age 23 years, range 20-25 years; mean weight 73.1 kg, range 62-86 kg; mean height 180 cm, range 170-189 cm). The subjects were recruited from the surrounding community by word of mouth. Eligibility criteria required the recreational use of MDMA on at least 6 occasions without any serious adverse reaction, and with no history of abuse or drug dependence according to DSM-IV for other substances with the exception of nicotine (in smokers). The participants had had previous experience with cannabis (75%), amphetamines other than MDMA (25%), and speed or cocaine (37%). All but two were smokers (less than 20 cigarettes per day). The subjects drank an average of 9.4 units of alcohol per week with a range from 1 to 25 (1 unit corresponds to 8 g of ethanol).

Prior to inclusion the volunteers were submitted to a general medical examination, including routine laboratory tests, urinalysis, and a 12-lead electrocardiogram (ECG), and a psychiatric interview (DSM-IV), to exclude any medical or psychopathological condition. Subjects were phenotyped for CYP2D6 activity using dextromethorphan as probe drug (de la Torre et al., 2005). Only CYP2D6 extensive metabolizers were included. The protocol was approved by the local Research Ethics Committee (CEIC-IMAS, Barcelona, Spain) and authorized by the Spanish Ministry of Health (AEMPS, Madrid, Spain). The study was conducted in accordance with the Declaration of Helsinki and Spanish laws concerning clinical trials. The volunteers were financially compensated for their participation in the study.

2.2. Drugs

(R,S)-MDMA was supplied by the Spanish Ministry of Health. Both placebo and MDMA capsules were prepared by the Pharmacy

Department of our institution to obtain identically appearing opaque, white, soft, gelatin capsules.

2.3. Study design

The study design was a double-blind, randomized, crossover, controlled trial with placebo. Treatments were randomly assigned using a balanced 4 × 4 Latin-square design. Sessions were conducted once per week, with at least 1-week washout period between them to minimize the influence of any carry-over effect. The four conditions in the study were (i) 100 mg MDMA followed by 100 mg MDMA 4 h later (M+M), (ii) placebo followed by 100 mg MDMA 4 h later (Pl+M), (iii) 100 mg MDMA followed by placebo 4 h later (M+Pl), and (iv) placebo followed by placebo 4 h later (Pl+Pl).

2.4. Experimental sessions

Subjects were admitted to the Clinical Research Unit facilities at 08:00 a.m. after an overnight fast. Upon arrival they were asked about any drug consumption or event that could affect their participation in the study. Volunteers were requested to refrain from taking any psychoactive drug for a minimum of three days prior to the study and throughout it, and from using caffeinated products and alcohol for 48 h prior to the experimental sessions. A urine sample was collected for drug testing (opiates, cocaine metabolite, amphetamines, and cannabinoids) (FPIA, Abbott Laboratories, Chicago, IL, USA). Participants were required to be drug free before inclusion in the experimental session. They remained seated in a calm and comfortable laboratory environment with an indwelling intravenous catheter inserted into a subcutaneous vein in the forearm of the non-dominant arm. A physician and a nurse were present during the entire session.

At the beginning of each experimental session baseline measure were taken. At 09:00 a.m. the participants at fasting state received the drug (placebo or MDMA 100 mg) with 100 milliliters (mL) of water. Four hours later, they received the second administration (placebo or MDMA 100 mg).

Two hours after the first and second administration a light breakfast was provided (a milk bun with 200 mL of water); a light meal was given 8 h after the first administration (salad, meat and fried potatoes with 400 mL of water). Adverse effects were assessed during each experimental session and the day after.

Prior to being included in the experimental sessions, volunteers completed a training session to familiarize themselves with testing procedures, questionnaires, and to obtain a regular performance in the psychomotor tasks.

2.5. Physiological measures

Non-invasive systolic blood pressure (SBP), diastolic blood pressure (DBP), heart rate (HR), oral temperature (T), and pupil diameter (PD) were recorded at -45 and -15 minutes (min), immediately prior to the first drug administration (time 0, baseline) and at 0.33, 0.67, 1, 1.5, 2, 3, 4, 4.33, 4.67, 5, 5.5, 6, 7, 8, 10, 12, and 24 h after the first drug administration. All measurements were carried out using a DinamapTM 8100-T vital signs monitor (Critikon, Tampa, Fla., USA). For safety reasons, ECG was continuously monitored during all the session using a DinamapTM Plus vital signs monitor (Critikon). Pupil diameter was recorded with a Haab pupil gauge (Pickworth et al., 1997).

2.6. Psychomotor performance measures

The psychomotor performance battery included the reaction time (RT), the digit symbol substitution test (DSST), and the Maddox-wing device (Camí et al., 2000; de la Torre et al., 2000a; de la Torre et al., 2000b; Hernández-López et al., 2002; Farré et al., 2004; Peiró et al., 2013). RT was assessed using the Vienna Reaction Unit (PC/Vienna System; Schufried, Austria). Results were expressed in milliseconds (ms) as the mean of the response time to 20 stimuli. The DSST is a subtest of the Wechsler Adult Intelligence Scale-Revised (Wechsler 1958). A computerized version was used (Farré et al., 2004; Peiró et al. 2013), and scores were based on the number of correct patterns keyed in 90 s (correct responses). The Maddox-wing device measures the balance of extraocular muscles and quantifies exophoria, as an indicator of extraocular musculature relaxation, and esophoria. Results were expressed in diopters along the horizontal scale of the device. (Hannington-Kiff 1970).

The psychomotor performance battery was performed at −45 min and at 1, 1.5, 2, 3, 4, 5, 5.5, 6, 7, 8, 10, 12, and 24 h after the first drug administration.

2.7. Subjective effects

Subjective effects were measured with visual analogue scales (VAS) and the Addiction Research Center Inventory (ARCI). A set of 21 different VAS [100 millimeters (mm)] labeled with different adjectives marked at opposite ends with “not at all” and “extremely” were employed (Camí et al., 2000; Farré et al., 1997). Subjects were asked to rate effects as “stimulated”, “high”, “any effect”, “good effects”, “bad effects”, “liking”, “drowsiness”, “changes in distances”, “changes in colors”, “changes in shapes”, “changes in lights”, “hallucinations - seeing lights or spots”, “changes in hearing”, “hallucinations - hearing sounds or voices”, “dizziness”, “hallucinations - seeing animals, things, insects or people”, “confusion”, “fear”, “depression or sadness”, “different, changed or unreal body feeling”, and “different or unreal surroundings”.

The validated Spanish 49-item short version of the ARCI (Lamas et al., 1994), a true-false questionnaire with empirically derived scales that are sensitive to the effects of a variety of drugs of abuse, was used (Haertzen 1974; Martin et al., 1971). The questionnaire included five scales: PCAG (pentobarbital-chlorpromazine-alcohol group, a measure of sedation); MBG (Morphine-Benzedrine group, a measure of euphoria); LSD (lysergic acid diethylamide group, a measure of dysphoria and somatic symptoms); BG (Benzedrine group, a stimulant scale consisting mainly of items relating to intellectual efficiency and energy); and A (Amphetamine, an empirically derived scale sensitive to the effects of d-amphetamine).

The VAS and ARCI were administered at -45 min and at 0.33, 0.67, 1, 1.5, 2, 3, 4, 4.33, 4.67, 5, 5.5, 6, 7, 8, 10, 12, and 24 h after the first drug administration.

2.8. Pharmacokinetics

Blood samples for the determination of the plasma concentrations of MDMA and its metabolites, 4-hydroxy-3-methoxymethamphetamine (HMMA), 4-hydroxy-3-methoxyamphetamine (HMA), and 3,4-methylenedioxyamphetamine (MDA) were collected during each experimental session at -5 min (0 h), 0.33, 0.67, 1, 1.5, 2, 3, 4, 3, 4, 4.33, 4.67, 5, 5.5, 6, 7, 8, 10, 12, and 24 h after the first drug administration. Plasma concentrations were measured by gas chromatography coupled to mass spectrometry (Pizarro et al., 2002).

2.9. Cortisol and prolactin concentrations

Blood samples for the determination of cortisol and prolactin were collected at -5 min (0 h), 1, 2, 3, 4, 5, 6, 7, 8, 10 h after the first drug administration. Plasma cortisol concentrations were measured by fluorescence polarization immunoassay (FPIA) (Abbott Laboratories) according to the manufacturer's instructions. Prolactin plasma concentrations were determined by a microparticle enzyme immunoassay (MEIA) (Abbott Laboratories) using an Axsym

Table 1 Physiological parameters, psychomotor performance, subjective effects and hormone results (n=10, mean, standard deviation) after a single dose (Pl+M) versus a repeated dose (M+M) administration.

Variable	Parameter	ANOVA	Tukey	Pl+M	Pl+M	M+M	M+M
		P	Multiple comparisons	Mean	SD	Mean	SD
Physiological							
SBP	AUC	<0.001	A, B, C	44.86	28.88	77.32	22.54
	Peak	<0.001	A, B	26.20	7.90	32	9.39
	T-C	<0.001	4.33 h*, 4.67 h**, 5 h**, 7 h*, 8 h*				
DBP	AUC	<0.001	A, B	19.58	27.34	36.92	18.69
	Peak	<0.001	A, B	12.40	13.85	20.90	6.81
	T-C	<0.001					
HR	AUC	<0.001	A, B	56.03	61.43	50.55	43.41
	Peak	0.001	A, B	25.45	25.26	25.30	19.51
	T-C	<0.001	4.33 h*, 4.67 h*, 5 h*				
T	AUC	0.129		0.91	1.11	1.38	1.17
	Peak	0.141		0.47	0.53	0.62	0.50
	T-C	0.005	4.33 h*, 5 h**, 5.5 h**				
PD	AUC	<0.001	a, B	8.03	2.17	10.85	2.67
	Peak	<0.001	A, B	3.25	1.06	3.33	0.76
	T-C	<0.001	4.33 h**, 4.67 h**, 5 h**				
Psychomotor							
RT decision	AUC	<0.001	B, C	39.83	79.74	149.75	119.25
	Peak	0.001	B, C	17.75	35.69	58.10	46.71
	T-C	0.003	5 h**, 5.5 h** ,6 h**, 7 h**, 8 h*				
RT total	AUC	<0.001	B, C	57.23	129.71	167.3	127.83
	Peak	0.001	B, C	22.60	51.80	68.3	49.36
	T-C	<0.001	5 h**, 5.5 h** ,6 h**, 7 h**, 8 h**				
Maddox	AUC	0.014	b	-3.84	4.42	-9.33	10.04
	Peak	0.028	b	-1.60	1.78	-3.20	3.77
	T-C	<0.001	5 h**, 5.5 h**, 6 h**, 7 h**				
Subjective							
Stimulated	AUC	<0.001	A, B	58.33	38.85	80.00	42.10
	Peak	<0.001	A, B	36.60	21.30	53.50	24.80
	T-C	<0.001	5 h**				
High	AUC	0.001	A, B	63.31	49.92	82.06	43.04
	Peak	<0.001	A, B	40.00	25.60	53.80	27.1

	T-C	<0.001	5 h**				
Any effect	AUC	<0.001	A, B	72.96	48.79	86.29	40.17
	Peak	<0.001	A, B	45.60	27.04	56.70	25.04
	T-C	<0.001	5 h*				
Good effects	AUC	<0.001	A, B	69.04	43.48	83.14	41.92
	Peak	0.001		45.8	29.04	55.40	27.34
	T-C	<0.001	5 h*				
Liking	AUC	0.003	a, B	60.10	44.96	75.32	52.86
	Peak	0.002	A, B	42.40	27.75	48.80	32.10
	T-C	<0.001					
Changes in colors	AUC	0.023	a, b	16.43	21.02	7.30	12.50
	Peak	0.045		17.60	25.02	6.60	11.92
	T-C	0.002	5h**				
Changes in lights	AUC	0.007	A, B	35.55	33.82	21.83	29.84
	Peak	0.001		28.40	23.84	16.20	18.52
	T-C	<0.001	5 h**, 5.5 h*				
Changes in hearing	AUC	0.024	a	15.68	22.04	7.89	12.27
	Peak	0.022	a	14.20	15.68	10.80	12.80
	T-C	0.015					
Different body sensation	AUC	0.009	a, b	42.01	40.82	53.47	45.91
	Peak	0.005	a, B	33.50	28.64	42.00	30.37
	T-C	0.001					
Different surroundings	AUC	0.045	a	29.54	42.75	14.23	26.37
	Peak	0.024	a	21.90	29.21	8.10	13.54
	T-C	0.001	5.5 h**, 6 h*				
ARCI-MBG	AUC	<0.001	A, B	13.68	5.43	16.17	9.39
	Peak	<0.001	A, B	8.00	3.83	7.80	3.88
	T-C	<0.001					
ARCI-LSD	AUC	<0.001	A, B	7.68	4.57	9.03	5.72
	Peak	<0.001	A, B	21.9	29.21	8.10	13.54
	T-C	<0.001	4.67**				
ARCI-BG	AUC	<0.001	A, B	3.98	4.73	4.34	4.57
	Peak	0.033	b	2.40	3.44	2.60	2.41
	T-C	0.006					
ARCI-A	AUC	<0.001	A, B	12.15	3.84	12.36	4.55
	Peak	<0.001	A, B	5.80	2.15	5.30	1.89
	T-C	<0.001					

Table 1 (*continued*)

Variable	Parameter	ANOVA	Tukey	Pl+M	Pl+M	M+M	M+M
		P	Multiple comparisons	Mean	SD	Mean	SD
Hormones							
Cortisol ($n=9$)	AUC	<0.001	A, B	20.45	19.00	39.04	24.42
	Peak	<0.001	A, B	12.34	6.30	17.44	7.57
	T-C	<0.001	5 h**				
Prolactin	AUC	<0.001	A, B	-2.79	1.12	41.38	29.10
	Peak	<0.001	A, B	20.12	13.88	15.59	7.73
	T-C	<0.001	6 h**, 7 h**				

A: Pl+Pl versus Pl+M, $p<0.01$; a: Pl+Pl versus Pl+M, $p<0.05$; B: Pl+Pl versus M+M, $p<0.01$; b: Pl+Pl versus M+M, $p<0.05$; C: Pl+M versus M+M, $p<0.01$; c: Pl+M versus M+M, $p<0.05$.

p Value of *T-C* corresponds to *p* value in time × condition interaction (exception: RT total *p* corresponds to *p* of condition).

Abbreviations: AUC=area under the curve; 4-8 h, Peak=peak effects from 4 to 8 h; and *T-C*=time-course from 4 to 8 h indicating time points where differences between conditions Pl+M and M+M were statistically significant; SD=standard deviation; *p*=statistical significance level.

*$p<0.05$.

**$p<0.01$.

instrument and following the manufacturer's instructions. Details of both assays have been previously published (Farré et al., 1997; Mas et al., 1999; de la Torre et al., 2000a, 2000b).

2.10. Statistical analysis

2.10.1. Effects

Values from physiological and psychomotor performance measures, subjective variables, and hormones were transformed to differences from baseline. Due to the fact that the primary outcome was the effects reported after the repeated dose, we only analyzed those observed after the second administration. A preliminary statistical analysis (data not shown) demonstrated no differences in MDMA effects between the M+Pl and Pl+M conditions. The peak effect in the 4 h following the second administration (maximum absolute change from baseline values from 4 to 8 h) and the area under the curve (AUC) of effects versus time (from 4 to 8 h), calculated by the trapezoidal rule, were determined for each variable. These transformations were analyzed by one-way repeated measure analysis of variance (ANOVA) with drug conditions as factor (Pl+Pl, Pl+M or M+M).

When ANOVA results showed significant differences among treatment conditions, post hoc multiple comparisons were performed using the Tukey test. Furthermore, a detailed comparison of time course of effects from 4 to 8 h was conducted using repeated measures two-way ANOVA, with treatment condition and time as factors. When treatment condition or the treatment condition × time interaction was statistically significant, multiple Tukey post hoc comparisons were performed at each time point using the mean square error term of the treatment condition × time interaction. All statistical tests were performed using PASW Statistics 18.0 (SPSS Inc., Chicago, IL, USA). A value of $p<0.05$ was considered statistically significant.

2.10.2. Pharmacokinetics

Non-compartmental analysis was performed in order to obtain the following pharmacokinetic parameters: maximum concentration in the concentration-time profile (C_{max}), time after dosing required for the maximum concentration (T_{max}), half-life ($t_{1/2}$), area under the curve from time point 4 to 12 h (AUC_{4-12}) for Pl+M and M+M conditions, apparent volume of distribution (Vd), and plasmatic clearance (CL). The pharmacokinetic parameters were determined using pharmacokinetic functions for Microsoft Excel (Microsoft Corporation, Redmond, CA, USA). A preliminary statistical analysis (data not shown) demonstrated no differences in MDMA and metabolite concentrations between Pl+M and M+Pl.

The metabolic ratios HMMA+HMA+MDA/MDMA, HMMA+HMA/MDA+MDMA, and MDA/MDMA were compared in order to evaluate the rate of the O- and N-demethylenation of MDMA.

Paired Student's *t* test for the pharmacokinetic parameters and metabolic ratios results, and Wilcoxon test for T_{max}, were used for statistical analysis to compare single versus multiple dose of MDMA (Pl+M versus M+M). Differences associated with $p<0.05$ were considered to be statistically significant.

3. Results

3.1. Global results

Table 1 shows a summary of the physiological, psychomotor, and subjective effects where at least one statistical difference (peak or AUC) was found in the ANOVA analysis among the three treatment conditions (Pl+Pl, Pl+M, M+M). Table 1 also includes the time course points that showed significant differences in ANOVA and the post-hoc Tukey test for the comparison of Pl+M versus M+M. Time course of

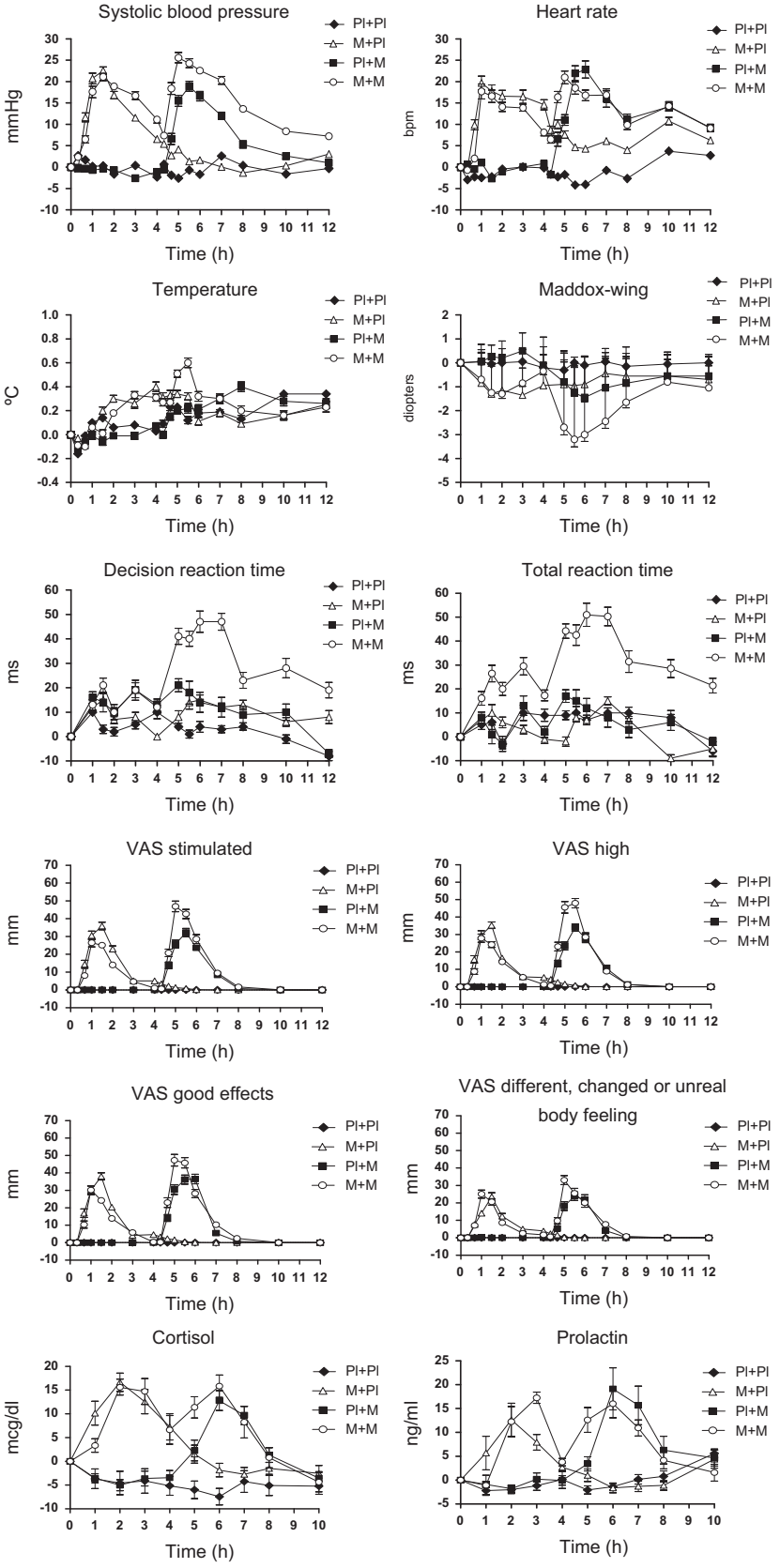

Figure 1 Time course of physiological effects, psychomotor performance, visual analog scale measurement for subjective effects and hormone concentrations over a maximum period of 24 h following two repeated doses of 100 mg of MDMA (n=10, mean, standard error; n=9 for cortisol).

Table 2 Exposition and metabolic rates of MDMA and metabolites (HMMA, HMA and MDA) results (n=10, mean, standard deviation) after a single dose (Pl+M) versus a repeated dose (M+M) administration.

Variable	Pl+M		M+M		M+M versus Pl+M
	Mean	*SD*	*Mean*	*SD*	*Increment × times*
MDMA					
C_{max} (ng/ml)	220.29	55.13	458.00	96.30	2.08***
AUC (ng/ml h^{-1})	1200.63	290.58	2595.98	627.85	2.16***
T_{max} (h)	6	(5-7)	5.25	(4.7-7)	NA
Ke (h^{-1})	0.09	0.02	0.08	0.03	0.89
$t_{1/2}$ (h)	7.81	1.76	10.06	4.83	1.29
CL (L/h)	41.43	14.06	31.37	10.46	0.76*
Vd (L)	450.40	129.78	424.68	152.91	0.94
MDA					
C_{max} (ng/ml)	9.42	2.85	20.55	5.80	2.18***
AUC (ng/ml h^{-1})	58.56	17.62	137.54	40.14	2.35***
T_{max} (h)	9.6	(5.5-12)	8	(4.7-12)	NA
Ke (h^{-1})	0.05	0.02	0.14	0.19	2.80
$t_{1/2}$ (h)	15.21	6.88	12.23	8.94	0.80
CL (L/h)	344.21	139.56	389.98	218.55	1.13
Vd (L)	6705.42	2042.60	4862.82	2414.68	0.73
HMMA					
C_{max} (ng/ml)	325.36	145.52	306.40	134.67	0.94
AUC (ng/ml h^{-1})	1712.28	672.57	1905.54	867.51	1.11
T_{max} (h)	6	(5.5-7)	5	(4-7)	NA*
Ke (h^{-1})	0.13	0.03	0.10	0.03	0.77*
$t_{1/2}$ (h)	5.53	1.11	7.46	2.01	1.35*
CL (L/h)	37.68	11.63	47.55	18.89	1.26*
Vd (L)	304.51	127.96	491.85	162.27	1.62**
HMA					
C_{max} (ng/ml)	6.39	2.26	7.58	2.78	1.19
AUC (ng/ml h^{-1})	36.49	11.82	46.93	15.17	1.29*
T_{max} (h)	8	(6-12)	10	(4-12)	NA
Ke (h^{-1})	0.08	0.08	0.05	0.03	0.63
$t_{1/2}$ (h)	17.82	15.33	17.67	7.63	0.99
CL (L/h)	634.15	386.83	918.79	503.22	1.45
Vd (L)	10170.71	4049.88	19305.27	7230.54	1.90**
Metabolic ratios					
MDA/MDMA	0.05	0.01	0.05	0.01	1.00
(MDMA+HMA+HMMA)/MDMA	1.67	0.89	0.86	0.43	0.51**
(HMMA+HMA)/(MDA+MDMA)	1.54	0.85	0.77	0.41	0.5**
(HMA+HMMA)/MDMA	1.62	0.89	0.81	0.43	0.5**

T_{max} is shown as median (range) values. Parameters were calculated from 4 to 12 h (corresponding to 8 h after second administration). Abbreviations: AUC=area under the curve; 0-8 h, Peak=peak effects from 0 to 8 h; SD=standard deviation; p=statistical significance level.
NA: not applicable. Statistical significance level obtained with T student test (a Wilcoxon test for t_{max}).
*$p<0.05$
**$p<0.01$.
***$p<0.001$.

effects are shown in Figure 1 (all conditions included). Pharmacokinetic parameters, and MDMA and metabolite plasma concentrations, over time are presented in Table 2 and Figure 2, respectively (only Pl+M and M+M). No serious adverse events were observed and none of the participants had hallucinations or psychotic episodes. All subjects completed the study.

3.2. Physiological effects

The prototypical effects of MDMA (increase in DBP, HR, T, and PD) were observed in the three conditions where the active drug was administered. Differences between single dose (Pl+M) and multiple dose (M+M) appeared mainly in SBP (AUC was 44.86 mmHg × h with 100 mg of MDMA and

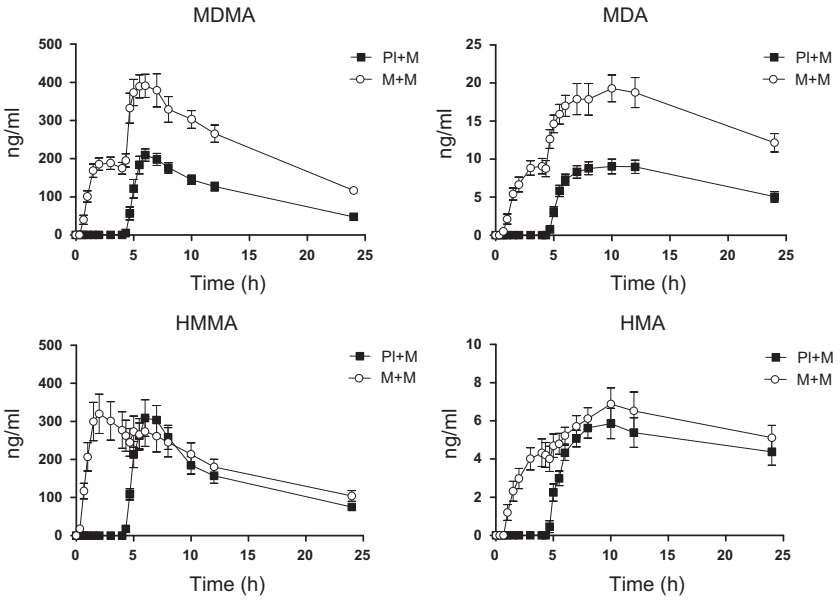

Figure 2 Plasma concentration over time curves of MDMA and its metabolites after a single dose (Pl+M) versus a repeated dose (M+M) administration (n=10, mean, standard error).

77.32 mmHg × h after M+M), with a marked increase in the M+M condition (at 4.33, 4.67, 5, 7, and 8 h after administration). For HR and PD differences between the single and multiple doses were observed during the first hour after administration. Maximum values appeared earlier with the multiple dose probably due to the effects of the second MDMA dose being added to the residual effects of the first one. The increase in T was higher after repeated MDMA administration in comparison with single dose (differences at 4.33, 4.67, and 5.5 h after administration).

3.3. Psychomotor performance

Psychomotor performance deteriorated with repeated MDMA administration in comparison to placebo, but no significant differences were found between placebo and the single MDMA dose. Significant differences between single and repeated dose (Pl+M and M+M) were observed in the AUC and peak effect for RT decision and RT total (RT, AUC and peak effect values were triplicated after repetition in comparison with single dose) and also at different time points after administration (5–8 h). MDMA repeated doses induced more esophoria, measured by the Maddox-wing device, than a single MDMA dose at several time points (5–7 h after administration). No significant impairment of DSST was found comparing both active conditions (Pl+M versus M+M).

3.4. Subjective effects

Subjective effects increased in the conditions where MDMA was administered, peaked between 1 and 2 h after administration, and returned to basal values 4 h after drug administration. Statistical differences were found in most of the comparisons with placebo. Although the intensity of effects after the second dose was higher than the single dose (M+M versus Pl+M), no statistical differences were found in terms of AUC or peak effects. For several outcomes (stimulated, high, any effect, and good effects) higher scores in the repeated doses condition were found to be statistically significant 1 h after the second administration (5 h). Unexpectedly, changes in the scores of colors, lights, and different surroundings at 5–6 h after administration were higher with the single dose condition and significantly different from the multiple dosage one. ARCI scales between both conditions did not vary (Pl+M and M+M) with the exception of the LSD scale (disphoria) at 4.67 h.

3.5. Hormones

Data concerning cortisol are only available for nine subjects due to technical problems during analysis and/or not enough sample volume for complete analysis. AUC and peak hormones concentrations were similar in both conditions (Pl+M versus M+M). Only a few time points showed statistically significant differences. The increase in cortisol was higher 1 h after repeated administration in comparison with the single dose (5 h). On the other hand, a lower concentration of prolactin was found 2 and 3 h after the repeated dose in comparison with the single one (6 and 7 h).

3.6. Pharmacokinetics

Results are reported comparing Pl+M to M+M conditions. All subjects presented quantifiable concentrations of MDMA 4 h after the administration of the first 100 mg dose (Pl+M) (Figure 2). MDMA and MDA concentrations (C_{max} and AUC) after the second dose (M+M) were higher than that observed after the 100 mg single dose (Pl+M) (see Table 2). Both values for MDMA and MDA were higher than those expected from a simple dose accumulation (+23.1% C_{max} and +17.1% AUC; +14.2% C_{max} and +10.3% AUC, respectively).

No changes were observed in $t_{1/2}$, T_{max}, Vd of MDMA between both experimental conditions (see Table 2).

Following the second MDMA dose, HMMA plasma concentrations increased by only 11% (AUC) in comparison with the first dose, while HMA concentrations increased by only 29% (AUC, $p<0.05$). HMMA and HMA values did not increase as expected due to simple dose accumulation (−43.3% C_{max} and -39.9% AUC for HMMA; -33.2% C_{max} and -35.1% AUC for HMA). A significant decrease in all metabolic ratios was found with the exception of the metabolic ratio MDA/MDMA (see Table 2).

4. Discussion

Our results suggest there exists a phenomenon of acute tolerance to MDMA effects after repeated doses taken 4 h apart because most subjective and physiological effects with single and multiple dose were similar, despite higher MDMA concentrations with multiple dose, but effects on blood pressure and reaction time were potentiated. The administration of a second dose resulted in higher MDMA concentrations than expected due to metabolic autoinhibition.

A few experimental studies have been conducted administering multiple doses of MDMA (Farré et al., 2004; Kuypers et al., 2007; Kuypers et al., 2008; Peiró et al., 2013), nevertheless, our study design is unique (4 treatment arms) and probably the optimum form to assess the presence of acute tolerance. In addition, the selected dosing interval (4 h) coincides with the time point where the subjective effects have disappeared, thus increasing the probability of MDMA users taking a second dose (Camí et al., 2000).

A similar study with a lower dosing (75 mg and 50 mg of MDMA) and two experimental conditions (double dosing versus double placebo) has been carried out. As it investigated the cognitive impact of repeated doses and their interaction with sleep deprivation it was performed in the evening. The researchers concluded that evening doses of MDMA selectively impaired impulsivity, psychomotor and memory performance, and that this impairment was additional to the effect of sleep deprivation on memory performance (Kuypers et al., 2007, 2008).

Effects of the drugs can be influenced by the setting of administration. MDMA multiple doses administration had also been studied in real recreational settings instead of research units. Two studies determined MDMA plasma concentrations (Irvine et al., 2006; Morefield et al., 2011) and physiological effects (Irvine et al., 2006) of ecstasy users in this context. Although in a typical session, MDMA consumption differs due to variation in dosage and the number of pills consumed, multiple ecstasy intake (between 3 and 5 pills) produces an MDMA plasma mean concentration of 500 mcg/L. In both articles, higher MDMA plasma concentrations, considered to be within the toxic range, were detected. They did not, however, induce acute MDMA complications which suggest that repeated MDMA use results in a tolerance to its prototypical effects.

4.1. Pharmacological effects

The administration of a single dose of 100 mg of MDMA produced the previously described typical physiological (increase of SBP, DBP, HR, T, and PD) and subjective effects (euphoria, stimulation, feelings of well-being, disphoria, and mild changes in perceptions) (Mas et al., 1999; Camí et al., 2000; Hernández-López et al., 2002; Farré et al., 2004, 2007; Pardo-Lozano et al., 2012).

On the other hand, placebo did not produce any noticeable effects compared with baseline values (the M+Pl condition showed some carry-over effect only in a few physiological outcomes that can be observed in Figure 1).

In contrast to typical stimulants, such as amphetamine, and concurring with previously published data (Camí et al., 2000; Kuypers et al., 2007), psychomotor performance was impaired after MDMA consumption. RT was notably increased after the multiple dose in comparison with the single one. A previous study showed the same trend with lower doses (50 mg+100 mg) (Peiró et al., 2013). Regarding cortisol, slight changes were observed after repeated MDMA administration at some time points. In contrast, when MDMA was given 24 h apart higher concentrations of cortisol were found after the second dose in comparison with the single one (1–2 h, 4–6 h, $AUC_{0-6\,h}$) (Farré et al., 2004). One possible explanation is the dosing time, morning–afternoon as opposed to morning–morning. Concurring with the previously mentioned study with a repeated dose 24 h apart, we did not observe a significant change in prolactin values.

In summary, the pharmacological effects reported after a repeated administration were only slightly higher than those observed with the single dose; our results replicate previously published data employing different time intervals and dosing (Farré et al., 2004; Peiró et al., 2013).

4.2. Pharmacokinetics

The effects observed following the second MDMA dose should be interpreted taking into account the plasma concentrations of the single one (Pl+M). MDMA and MDA plasma concentrations observed after the second dose can be explained considering dose proportionality. The maximal concentrations of MDMA reported after the single dose were in the range of those described in previous studies following the administration of 100 mg (de la Torre et al., 2000a, 2000b; Hernández-López et al., 2002; Peiró et al., 2013). The C_{max} (458 ng/ml) values after repeated MDMA administration (M+M) also concur with previously reported data following dose proportionality; after two 100 mg doses (232 ng/ml) taken 24 h apart (Farré et al., 2004) and 50 and 100 mg doses (311.16 ng/ml) taken 2 h apart (Peiró et al., 2013). These concentrations are similar to those observed in recreational settings (dance parties) where subjects ingested repeated doses of ecstasy (between 1.5 and 5 pills) (Irvine et al., 2006; Morefield et al., 2011). The increase of MDMA availability can be explained by its non-linear pharmacokinetics due to MDMA induced inhibition of CYP2D6 (de la Torre et al., 2000a; Farré et al., 2004; Yang et al., 2006; Yubero-Lahoz et al., 2011; O'Mathuna et al., 2008). With reference to HMMA concentrations, a considerably different pattern was observed. After the second dose, HMMA plasma concentration increased by only 11% (AUC), while C_{max} did not change. The significantly marked reduction in HMMA and metabolic ratio can be explained by the MDMA autoinhibition of

CYP2D6 (de la Torre et al., 2000a; Farré et al., 2004; Yang et al., 2006; Yubero-Lahoz et al., 2011; Peiró et al., 2013). This point is pharmacologically relevant since the metabolic dispositions of several relevant drugs (opiates, antidepressants, antiarrhythmic, and antivirals) are regulated by CYP2D6. Similar results were observed for HMA concentrations. In the case of MDA, similar concentrations were obtained in previous studies when the same dose of MDMA was administered (de la Torre et al., 2000a, 2000b). The increase observed in MDA plasma concentrations is most probably related to a higher availability of substrate (MDMA) for N-demethylation rather than to any metabolic interaction.

4.3. Pharmacological effects in relation to pharmacokinetics

The pharmacological effects after the second administration should have been higher taking into account the double MDMA concentrations achieved following the second dose (increase of 208% in C_{max} and 216% in AUC). Nevertheless, for most subjective and physiological variables, the pharmacological effects observed were similar. Only for SBP and RT were effects with a repeated dose significantly higher than those obtained with the single one. This phenomenon, particularly with respect to pleasant effects (euphoria (MBG), getting high, and stimulation), could indicate some degree of adaptation or possible tolerance.

In other laboratory studies (Hysek et al., 2011, 2012; Peiró et al., 2013), lower than expected pharmacological effects, based on plasma exposure, have been described. A similar decrease in effects has been reported by many recreational users (Parrott, 2005). Rapid or acute tolerance has been observed in animals for both MDMA and other amphetamines after a second dose (Frederick et al.,1995), and in humans after the administration of two or more repeated doses of amphetamines (Pérez-Reyes et al., 1991; Comer et al., 2001). Acute tolerance in humans could be related to different mechanisms. Serotonin exhaustion due to increased release, the inhibition of re-uptake, and the decrease in formation by the inhibition of tryptophan hydroxylase following the first dose of MDMA would diminish the amount of neurotransmitter available for release following the second dose (Hysek et al., 2014). Furthermore trafficking of serotonin transporters (internalization from the plasma membrane to the cell interior) leading to less drug-induced serotonin release or desensitization of postsynaptic receptor sites could also be responsible of the acute tolerance phenomenon (Baumann et al., 2008). On the other hand, chronic tolerance to some MDMA-related subjective effects has also been described in clinical research participants receiving MDMA (Kirkpatrick et al., 2014). Our participants' consumption of MDMA before the study ranged from 6 to 20 occasions lifetime. The small sample size and the narrow range of previous consumption did not allow us to study the influence of prior MDMA use in the results obtained.

It should be emphasized that while most subjective effects of MDMA were similar, or even blunted, after a second dose the drug, effects on blood pressure and reaction time were potentiated. These findings suggest that users who "stack" or "bump" doses of MDMA to overcome tolerance to subjective effects are at risk for hypertensive effects or impaired performance (impaired driving) that could be dangerous. Taking into account that ecstasy (MDMA) use is concentrated in young adults (in Europe it is estimated that 1.3 million adults aged 15–34 years used ecstasy last year [EMCDDA 2014]), who usually take several pills in one session, directed advise to avoid this pattern of consumption should be given to reduce potential life-threatening episodes. Users also combine ecstasy pills with others psychostimulants (amphetamine derivatives and/or cocaine), alcohol or marijuana increasing the risk of serious adverse drug reactions.

4.4. Limitations of the study

The main limitation of the study is the number of subjects included. A sample size of 10 participants may not have had enough statistical power to show differences in some variables, although statistical differences were found in others. The experimental design chosen is, however, complex and our sample size makes the study feasible. Other limitations appear in relation to the instruments used to measure the outcomes. An increase in the concentration of MDMA, which may normally cause an increase in effects, might not have been detected by some of the questionnaires (e.g. ARCI) because any additional increases in effects are not possible to describe. On the other hand, the limitations to detect changes could be associated with the achievement of a ceiling effect in the organic response. As an example, the increase in PD cannot probably be further enhanced by a multiple dose of MDMA.

In conclusion, MDMA inhibits its metabolism when consumed at 4 h repeated doses. It does not produce the expected physiological and subjective effects according to the MDMA concentrations attained. The most plausible explanation for this observation is the occurrence of an acute pharmacological tolerance phenomenon. Consumers of various doses in one night who are looking for constant levels of well-being should keep in mind that they may be suffering from cardiovascular toxicity and altered psychomotor performance.

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Contributors

MF, PNR, JC, and RT were responsible for the study design. MF, AT, PNR, MP, and MT collected the data. MF, AT, PNR, CPM, and EP undertook the statistical analysis of the data. MF, RT, SY, CPM, and EP wrote the first draft of the manuscript. JC provided critical revision of the manuscript for important intellectual content. All

authors critically reviewed content and approved the final version submitted.

Conflict of interest

The authors state that there were no conflicts of interest directly relevant to the content of the study.

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