

Reinforcing Effects of Binary Mixtures of Common Bath Salt Constituents: Studies with 3,4-Methylenedioxypropylamphetamine (MDPV), 3,4-Methylenedioxymethcathinone (Methylone), and Caffeine in Rats

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'Bath salts' use is associated with high rates of abuse, toxicity, and death. Bath salt preparations often contain mixtures of drugs including multiple synthetic cathinones (eg, 3,4-methylenedioxypropylamphetamine (MDPV) or 3,4-methylenedioxymethcathinone (methylone)) or synthetic cathinones and caffeine; however, little is known about whether interactions among bath salt constituents contribute to the abuse-related effects of bath salts preparations. This study used male Sprague–Dawley rats responding under a progressive ratio schedule to quantify the reinforcing effectiveness of MDPV, methylone, and caffeine, administered alone and as binary mixtures ($n = 12$ per mixture). Each mixture was evaluated at four ratios (10 : 1, 3 : 1, 1 : 1, and 1 : 3) relative to the mean ED_{50} for each drug alone. Dose-addition analyses were used to determine the predicted, additive effect for each dose pair within each drug mixture. MDPV, methylone, and caffeine maintained responding in a dose-dependent manner, with MDPV being the most potent and effective, and caffeine being the least potent and effective of the three bath salts constituents. High levels of responding were also maintained by each of the bath salts mixtures. Although the nature of the interactions tended toward additivity for most bath salts mixtures, supra-additive (3 : 1 MDPV : caffeine, and 3 : 1 and 1 : 1 methylone : caffeine) and sub-additive (3 : 1, 1 : 1, and 1 : 3 MDPV : methylone) interactions were also observed. Together, these findings demonstrate that the composition of bath salts preparations can have an impact on both their reinforcing potency and effectiveness, and suggest that such interactions among constituent drugs could contribute to the patterns of use and effects reported by human bath salts users. *Neuropsychopharmacology* (2018) **43**, 761–769; doi:10.1038/npp.2017.141; published online 2 August 2017

INTRODUCTION

Abuse of 'designer drugs' such as synthetic derivatives of cathinone has become a serious public health problem worldwide. These synthetic cathinones were marketed as safe and legal alternatives to illicit stimulants and are often sold as 'bath salts' preparations; however, increases in poison control center calls and emergency room visits (Substance Abuse and Mental Health Services Administration, 2013) has led to the Drug Enforcement Administration placing 13 of the most commonly detected synthetic cathinones under Schedule I regulations. Frequently sold as powders or capsules, bath salts are often administered via intravenous (IV), oral, or nasal routes with users often administering multiple doses within a session (Forrester, 2012; Baumann *et al*, 2013; Johnson and Johnson, 2014). Analysis of bath salts preparations obtained in the US suggests that 3,4-methylenedioxypropylamphetamine

(MDPV) and 3,4-methylenedioxymethcathinone (methylone) were two of the most widely available and abused synthetic cathinones when these drugs first emerged (Spiller *et al*, 2011; Shanks *et al*, 2012; Seely *et al*, 2013). Analogous to other abused stimulants, MDPV and methylone interact with dopamine, norepinephrine, and serotonin transporters (DAT, NET, and SERT, respectively) where MDPV functions as a DAT-selective cocaine-like transporter *inhibitor* and methylone functions as a non-selective amphetamine-like transporter *substrate* (Baumann *et al*, 2013; Eshleman *et al*, 2013; Simmler *et al*, 2013).

Bath salts preparations frequently contain multiple psychoactive compounds, including mixtures of multiple synthetic cathinones or a synthetic cathinone and caffeine, and the composition of these preparations varies not only with regard to the identity and purity of the psychoactive ingredient(s), but also varies across time within a single 'brand' (Brandt *et al*, 2010; Davies *et al*, 2010; Spiller *et al*, 2011; Shanks *et al*, 2012; Caudevilla-Gálligo *et al*, 2013; Seely *et al*, 2013; Zuba and Byrska, 2013). Caffeine is also commonly identified in combination with other stimulant drugs (eg, cocaine, 3,4-methylenedioxyamphetamine (MDMA) and methamphetamine; Goh *et al*, 2008;

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Lapachinske *et al*, 2015; Vidal Giné *et al*, 2016). One study that analyzed the contents of seized drug preparations found that MDPV, methylone, and caffeine were three of the most frequently detected constituents, regardless of formulation (eg, pills, powders, and so on), and that 65–80% of pills contained both MDPV and caffeine (Seely *et al*, 2013). Perhaps relatedly, human bath salts users report effects that range from highly pleasurable (eg, euphoria and alertness) to aversive (eg, agitation, paranoia, tachycardia, and death) (Ross *et al*, 2011; Spiller *et al*, 2011; Forrester, 2012; Johnson and Johnson, 2014). Although bath salts preparations typically contain more than one psychoactive ingredient, most preclinical research has focused on characterizing the effects of individual cathinones, rather than modeling bath salts as drug mixtures.

Thus, the primary goal of the current study was to determine whether the reinforcing effects of MDPV and methylone are altered when they are administered as binary mixtures with other common bath salts constituents (ie, MDPV+caffeine, methylone+caffeine, and MDPV+methylone). Although the mechanism of action of caffeine (adenosine A1 and A2A receptor antagonist) differs from stimulants like MDPV and methylone, caffeine has cocaine-like and methamphetamine-like discriminative stimulus effects (Garrett and Griffiths, 2001; Justinova *et al*, 2003; 2009; Collins *et al*, 2016). In addition, pretreatment with caffeine has been shown to increase/enhance cocaine self-administration in both rats and monkeys (Horger *et al*, 1991; Schenk *et al*, 1994; Comer and Carroll, 1996). Although these findings suggest that caffeine might do more than mimic the effects of drugs such as cocaine or methamphetamine, relatively little is known about the reinforcing effects of drug mixtures containing cocaine and caffeine. Similarly, despite the fact that bath salts preparations often contain caffeine, it is currently unknown whether caffeine alters the reinforcing effects of synthetic cathinones, such as MDPV and methylone.

Dose-addition analyses are a powerful approach to determine the nature of drug–drug interactions (eg, additive, supra-additive, or sub-additive) when both drugs produce the same pharmacologic effect. Because the composition of bath salts preparations varies greatly and because the nature of drug interactions is known to vary depending upon the proportion at which the constituents are mixed, each binary bath salts mixture was evaluated at four fixed-dose ratios (10 : 1, 3 : 1, 1 : 1, and 1 : 3) relative to the ED₅₀ of each drug. Dose-addition analyses were used to test the general hypothesis that the reinforcing effects (potency and effectiveness) of bath salts mixtures containing multiple synthetic cathinones or a synthetic cathinone and caffeine are greater than would be predicted for an additive interaction.

MATERIALS AND METHODS

Subjects

Male Sprague–Dawley rats (275–300 g) were obtained from Harlan (Indianapolis, IN) and maintained in a temperature- and humidity-controlled environment, on a 10/14-h dark/light cycle. All rats were singly housed and had free access to tap water and Purina rat chow throughout the study. All experimental procedures were conducted in accordance with

the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio, and the Eighth Edition of the Guide for Care and Use of Laboratory Animals (National Research Council, 2011).

Surgery

Rats were prepared with chronic indwelling catheters in the left femoral vein under 2% isoflurane anesthesia as previously described (Gannon *et al*, 2017a). Penicillin G (60 000 U/rat) was administered subcutaneously immediately following surgery to prevent infection, and all rats were allowed 5–7 days to recover before commencement of experiments. Catheters were flushed daily with 0.2 ml saline (before operant sessions) and 0.5 ml heparinized saline (100 U/ml, after operant sessions).

Apparatus

All operant sessions were conducted in standard operant conditioning chambers (Med Associates, St Albans, VT) located inside sound-attenuating cubicles. A set of green, yellow, and red LED lights was located above each of two levers, and a white house light was located at the top center of the opposite wall. A variable speed syringe driver delivered drug solutions through Tygon tubing connected to a stainless steel fluid swivel and spring tether held in place by a counterbalanced arm.

Self-Administration

Training. Rats were trained to respond under a fixed ratio (FR) 1 schedule of reinforcement for MDPV (0.032 mg/kg/inf) or methylone (0.32 mg/kg/inf) during daily 90-min sessions. A yellow LED above the active lever (left or right; counterbalanced) signaled drug availability, and completion of the response requirement resulted in a drug infusion (0.1 ml/kg over ~1 s) and initiated a 5-s timeout (TO) signaled by the illumination of the red, yellow, and green LEDs above the active lever and the houselight. Responding on the inactive lever and responding during TOs (active and inactive levers) were recorded but had no scheduled consequence. This schedule (FR1 : TO 5 s) was in place for 10 sessions, and all rats met acquisition criteria (>20 infusions and >80% of the responses emitted were on the active lever for 2 consecutive days). Subsequently, the response requirement increased to an FR5 for at least an additional 10 sessions, and upon meeting stability criteria ($\pm 20\%$ of the mean number of infusions for 3 consecutive days, with no increasing or decreasing trend) all rats were transitioned to a progressive ratio (PR) schedule of reinforcement.

Reinforcing effectiveness of individual bath salts constituents. For the remainder of the experiment, responding was maintained under a PR schedule of reinforcement under which response requirements for each successive infusion incremented according to the following equation: ratio = $[5e^{(\text{inf} \times 0.2)}] - 5$. The maximum session duration was 12 h, but sessions were terminated if a ratio was not completed within 45 min (ie, 45-min limited hold). Two groups of rats were trained to respond for 0.032 mg/kg/inf MDPV, with one group ($n = 12$) used to evaluate MDPV (0.0032–0.32 mg/

kg/inf), caffeine (0.1–1.78 mg/kg/inf), and their binary mixtures, and the other group ($n=19$) used to evaluate MDPV (0.0032–0.32 mg/kg/inf), methylone (0.1–1.78 mg/kg/inf), and their binary mixtures. A third group of rats ($n=15$) was trained to respond for 0.32 mg/kg/inf methylone and was used to evaluate methylone (0.032–1.78 mg/kg/inf), caffeine (0.1–1.78 mg/kg/inf), and their binary mixtures. The first dose evaluated was 0.032 mg/kg/inf MDPV (MDPV-trained rats) or 0.32 mg/kg/inf methylone (methylone-trained rats), with all remaining doses evaluated in a random order. Each dose (or dose pair) was available for at least two consecutive sessions and until responding met stability criteria (± 2 infusions from the previous session). All doses (dose pairs) for a particular drug (drug mixture) were evaluated prior to evaluating the next drug (drug mixture). Dose–response curves of individual constituents were established twice (once before and once after evaluation of drug mixtures) to determine whether responding changed over time. Because the study design required that 12 rats complete all portions of the study, and because toxicity was observed when rats were responding for methylone, seven rats were added to the MDPV+methylone group, and three rats were added to the methylone+caffeine group.

Drug Mixtures

Composition of binary bath salts mixtures. bath salts mixtures were constructed using the concept of dose-equivalence (see Tallarida and Raffa, 2010). Accordingly, dose–response curves for individual subjects were first normalized to the dose condition that maintained the greatest number of infusions (ie, E_{max}) and saline, with the number of infusions maintained by saline serving as the 0% effect level, and the difference in the number of infusions maintained by the E_{max} and saline serving as the 100% effect level. Normalized dose–response curves were then fit using a linear regression of the data spanning the 20%–80% effect levels (no more than one data point $> 80\%$ and one data point $< 20\%$) to obtain ED_{50} s, slopes, and y-intercepts for each pair of constituent drugs (ie, MDPV and caffeine, methylone and caffeine, or MDPV and methylone). Mixtures were designed at four ratios (10:1, 3:1, 1:1, and 1:3) relative to the mean ED_{50} for the group (ie, dose of each drug that maintained 50% of the maximal effect level). Since caffeine failed to maintain 50% of the maximum effect of either MDPV or methylone, the dose that maintained 50% of the maximal effect of caffeine (0.56 mg/kg/inf) was used to determine the composition of MDPV+caffeine and methylone+caffeine mixtures. In order to fully evaluate the reinforcing effects of the bath salts mixtures, each fixed dose ratio mixture (eg, 10:1, 3:1, 1:1, or 1:3) included a series of fixed dose pairs that spanned the 0–100% predicted effect levels (see Supplementary Table 1 for more details).

Dose-addition analyses. Predicted additive effect levels for each dose pair were calculated by first converting the unit dose of each constituent into dose equivalents of the training drug (eg, caffeine into MDPV equivalents for the MDPV+caffeine mixtures) using the following function (equation 1):

$$\text{Dose}_{\text{eqA}} = [(\text{Slope}^{\text{B}} \times \text{Dose}^{\text{B}}) + (\text{int}^{\text{B}} - \text{int}^{\text{A}})] / \text{Slope}^{\text{A}} \quad (1)$$

where Slope^{A} and Slope^{B} are the slope parameters and int^{A} and int^{B} are the y intercepts derived from the linear portion of the dose–response curves of drugs A (the training drug) and B, respectively, and Dose^{B} is the unit dose of drug B that is present in each dose pair. Summing the unit dose of drug A and the B_{eqA} allows each dose pair to be expressed in terms of the total dose equivalents of drug A. The total equivalent dose for each dose pair was then used to calculate the predicted effect for an additive interaction using the following function (equation 2):

Predicted additive effect level

$$= (\text{Slope}^{\text{A}} \times \text{Total dose}_{\text{eqA}}) + \text{int}^{\text{A}} \quad (2)$$

where $\text{total dose}_{\text{eqA}}$ is the total dose equivalents of drug A (ie, $\text{Dose}^{\text{A}} + \text{Dose}_{\text{eqA}}$ from equation 1). Predicted additive dose–response curves were determined for individual subjects for each drug mixture, and are graphically represented as the mean (\pm SEM) of the total drug equivalents (mg/kg/inf) and the mean (\pm SEM) predicted effect level (normalized to the E_{max} for Drug A) for each fixed-dose pair of each drug mixture.

Statistical analyses. Dose–response curves for individual constituents were analyzed by one-way repeated measures ANOVA with *post hoc* Dunnett's tests. All dose–response curves were analyzed by linear regression to obtain measures of potency (ED_{50}) and effectiveness (E_{max}) in individual subjects. For each constituent drug, the E_{max} (\pm SEM) values were compared between groups (ie, MDPV in MDPV+caffeine and MDPV+methylone groups, caffeine in MDPV+caffeine and methylone+caffeine groups, and methylone in MDPV+methylone and methylone+caffeine groups) of rats using a Student's *t*-test, while significant differences in ED_{50} s between groups were indicated by nonoverlapping 95% confidence intervals. For each bath salts mixture, potency (observed ED_{50} /predicted ED_{50}) and effectiveness (predicted E_{max} /observed E_{max}) ratios were calculated for individual subjects at each of the four dose ratios (10:1, 3:1, 1:1, and 1:3). Dose ratios for which the 95% confidence interval did not include 1 were considered to be significantly different than additive, with ratios less than 1 indicative of a supra-additive interaction, greater than 1 indicative of a sub-additive interaction, and inclusive of 1 indicative of an additive interaction.

RESULTS

Reinforcing Effects of Individual Constituents

Figure 1 (top row) shows the number of infusions maintained by the bath salts constituents alone for rats that responded for MDPV ($F(5,55) = 231.0$, $p < 0.001$; *post hoc* 0.01–0.178 mg/kg/inf $p < 0.05$) and caffeine ($F(4,44) = 15.1$, $p < 0.001$; *post hoc* 1–1.78 mg/kg/inf $p < 0.05$; left panels), methylone ($F(5,55) = 60.0$, $p < 0.001$; *post hoc* 0.1–1.78 mg/kg/inf $p < 0.05$), and caffeine ($F(4,44) = 23.5$, $p < 0.001$; *post hoc* 1–1.78 mg/kg/inf $p < 0.05$; middle panels) and MDPV ($F(5,70) = 259.3$, $p < 0.001$; *post hoc* 0.01–0.178 mg/kg/inf $p < 0.05$) and methylone ($F(4,56) = 96.6$, $p < 0.001$; *post hoc* 0.32–1.78 mg/kg/inf $p < 0.05$; right panels). Normalized dose–response curves for the self-administration of bath

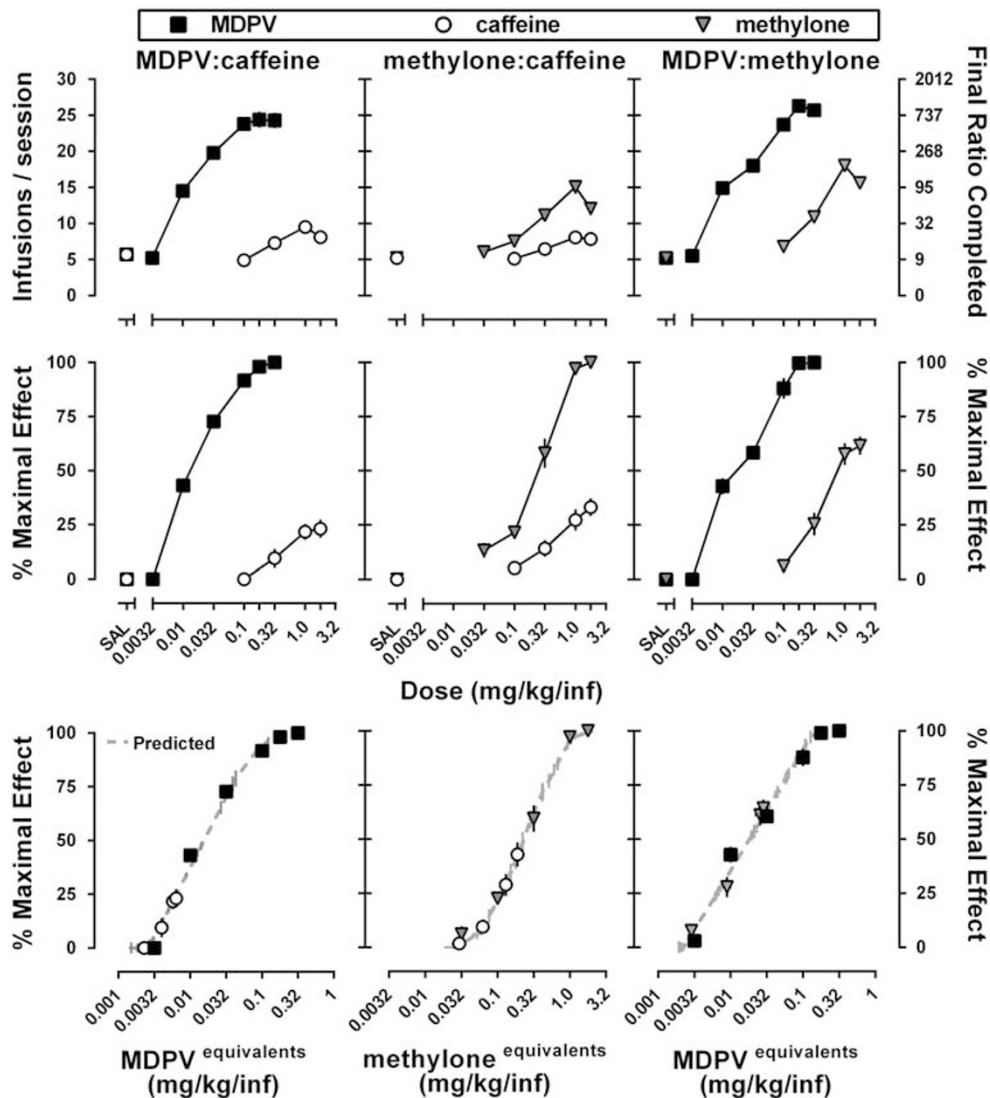


Figure 1 Dose–response curves for the self-administration of MDPV (squares), caffeine (circles), and methylone (triangles) under a progressive ratio schedule of reinforcement ($n = 12$ per group). Abscissa: first and second rows—‘SAL’ represents data obtained when saline was available for infusion, whereas doses refer to the unit dose of each drug available for infusion expressed as mg/kg/inf on a log scale. Third row—doses refer to the MDPV (first and third columns) or methylone (middle column) equivalents available for infusion expressed as mg/kg/inf on a log scale. Ordinate: first row—total infusions \pm SEM obtained during the session. Second and third rows—percent of the maximal effect \pm SEM, normalized to the drug in each pair that maintained the most responding (100%) and saline (0%).

salts constituents are shown in the lower two panels of Figure 1. All three bath salts constituents maintained responding in a dose-dependent manner; however, differences in potency (rank order: MDPV > methylone = caffeine) and effectiveness (rank order: MDPV > methylone > caffeine; maximum number of infusions) were observed. Because each drug was evaluated in two groups of rats, between-group comparisons of maximum number of infusions earned (E_{max}) and potency (ED_{50}) were also made. Although the E_{max} for MDPV (26.4 ± 1.0 for MDPV+caffeine and 26.3 ± 1.0 for MDPV+methylone) and caffeine (8.7 ± 0.4 for MDPV+caffeine and 8.3 ± 0.5 for methylone+caffeine) were comparable between groups (Table 1), methylone maintained significantly less responding ($t = 2.77$, $p < 0.05$) in rats from the methylone+caffeine group (14.8 ± 0.8) than in rats from the MDPV+methylone group (18.1 ± 0.9). Differences

in potency were not observed for any constituent. For the majority of the drugs, slope, ED_{50} , and E_{max} values did not differ for dose–response curves generated before and after evaluation of mixtures; however, for rats from the MDPV+caffeine group, caffeine’s effects (E_{max} and slope) were significantly decreased upon redetermination (data not shown).

Self-administration of methylone alone (1.0 and 1.78 mg/kg/inf) was lethal in 2 of 19 MDPV-trained rats and 1 of 15 methylone-trained rats.

Reinforcing Effects of Binary Mixtures of MDPV and Caffeine

Predicted and observed dose–response curves for mixtures of MDPV+caffeine are shown in Figure 2 (top row). When

Table 1 Reinforcing Effectiveness of bath salts Constituents MDPV, Methylo, and Caffeine Alone and in Binary Mixtures

	MDPV : caffeine		Methylo : caffeine		MDPV : methylo	
	E_{max} (\pm SEM)	Final ratio (\pm SEM)	E_{max} (\pm SEM)	Final ratio (\pm SEM)	E_{max} (\pm SEM)	Final ratio (\pm SEM)
Constituent 1	26.4 (1.0)	1168 (168)	14.8 (0.8)	118 (18)	26.3 (1.0)	1207 (177)
Constituent 2	8.7 (0.4)	27 (3)	8.3 (0.5)	24 (3)	18.1 (0.9)	271 (70)
10 : 1 Mixture	25.5 (0.8)	947 (156)	16.2 (0.7)	138 (20)	24.1 (1.5)	1007 (350)
3 : 1 Mixture	24.0 (0.7)	672 (76)	17.7 (1.0)	208 (38)	19.1 (0.6)	246 (34)
1 : 1 Mixture	21.6 (0.9)	445 (75)	16.6 (0.8)	157 (26)	18.6 (1.1)	266 (64)
1 : 3 Mixture	19.3 (0.6)	256 (30)	12.8 (0.4)	62 (6)	16.7 (0.9)	169 (36)

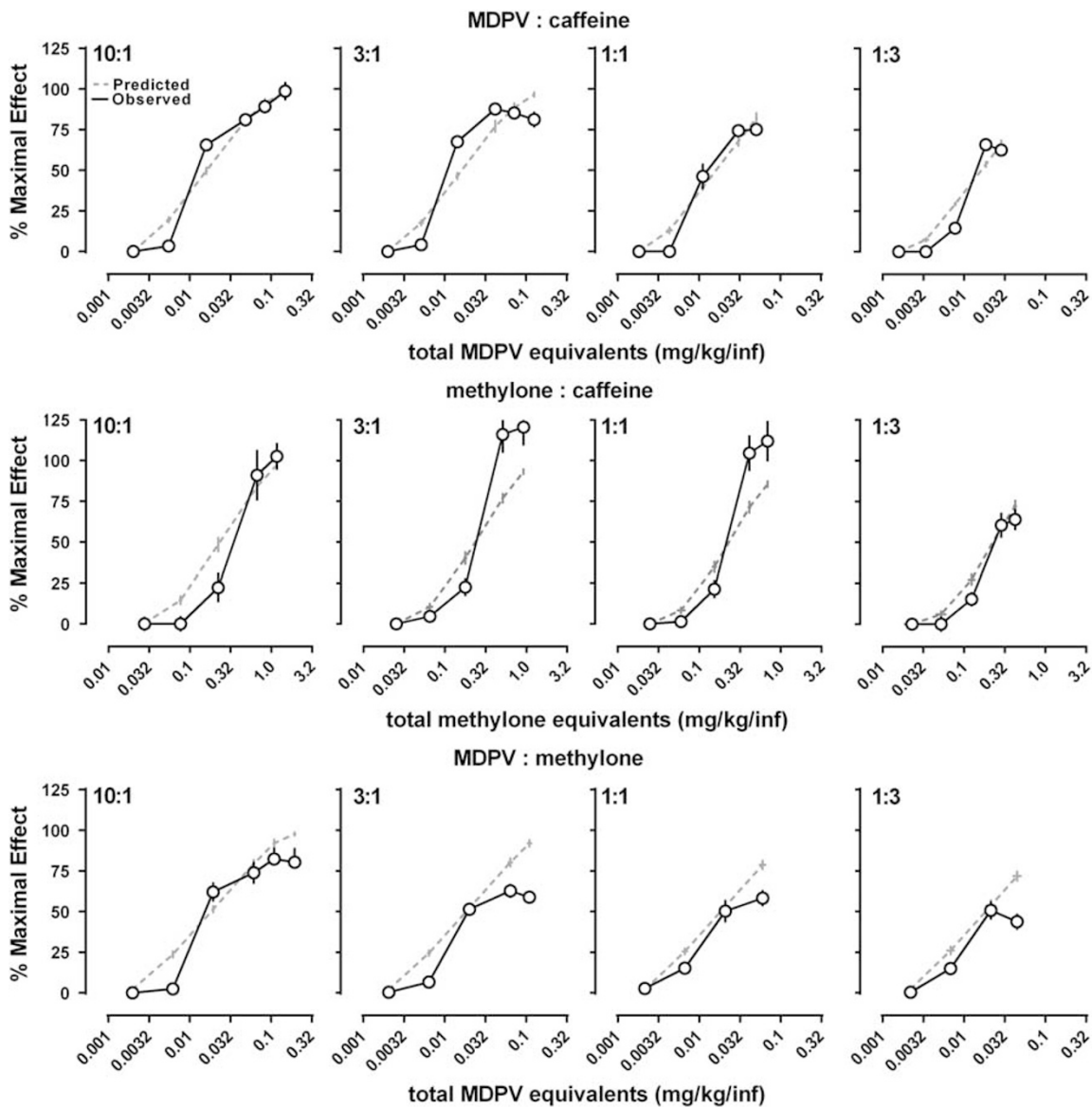


Figure 2 Dose–response curves for the self-administration of binary mixtures of MDPV+caffeine (top row), methylo+caffeine (middle row), or MDPV +methylo (bottom row; $n = 12$ per group). Each mixture was tested at four fixed-dose ratios (10 : 1 (left column), 3 : 1 (left-center column), 1 : 1 (right-center column), 1 : 3 (right column)) relative to the ED_{50} of the drug in each pair that maintained the most responding. Experimentally determined dose–response curves (white circles) represent the mean (\pm SEM) for 12 rats. Predicted, additive dose–response curves (gray, dashed lines) represent the mean (\pm SEM) for 12 rats. Abscissa: doses refer to total MDPV (top and bottom rows) or methylo (middle row) equivalents available for infusion expressed as mg/kg/inf on a log scale. Ordinate: percent of the maximal effect, normalized to the drug in each pair that maintained the most responding (100%), and saline (0%) as shown in Figure 1.

combined at dose ratios of 10:1, 3:1, 1:1, and 1:3, mixtures of MDPV+caffeine maintained responding in dose-dependent manner. With the exception of the 3:1 MDPV : caffeine ratio, the dose-response curves obtained for mixtures of MDPV+caffeine did not depart from the predicted additive dose-response curves. Although the 3:1 mixture of MDPV+caffeine was significantly more potent than predicted (Figure 3; top row, left panel), none of the mixtures of MDPV and caffeine differed from predictions for a strictly additive interaction with regard to their reinforcing effectiveness (Figure 3; top row, right panel). The $E_{max} \pm SEM$ and the final ratio completed $\pm SEM$ for each mixture of MDPV+caffeine are reported in Table 1.

Reinforcing Effects of Binary Mixtures of Methylone and Caffeine

Predicted and observed dose-response curves for mixtures of methylone and caffeine are also shown in Figure 2 (middle row). As with mixtures of MDPV+caffeine, dose-dependent increases in responding were observed for all fixed-dose ratios of methylone+caffeine. Although each of the mixtures exhibited additive interactions with respect to potency (Figure 3; middle row, left panel), when evaluated at 3:1 and 1:1 ratios of methylone : caffeine, the two largest dose pairs of each mixture consistently maintained more responding than predicted for an additive interaction. Indeed, the effectiveness ratios for the 3:1 and 1:1 mixtures of methylone+caffeine were significantly smaller than 1, indicating a supra-additive interaction between methylone and caffeine with regard to their reinforcing effectiveness (Figure 3; middle row, right panel). The $E_{max} \pm SEM$ and the final ratio completed $\pm SEM$ for each mixture of methylone+caffeine are reported in Table 1.

Self-administration of the largest dose pair of the 3:1 mixture of methylone+caffeine (0.80 mg/kg/inf methylone +0.79 mg/kg/inf caffeine) was lethal in 2 out of 14 rats.

Reinforcing Effects of Binary Mixtures of MDPV and Methylone

As observed with other binary bath salts mixtures, mixtures of MDPV and methylone maintained dose-dependent increases in self-administration, regardless of the ratio at which the cathinones were mixed (Figure 2; bottom row). Although the reinforcing effects of small dose pairs of MDPV+methylone appeared to be additive in nature, larger dose pairs at the 3:1, 1:1, and 1:3 ratios of MDPV +methylone tended to maintain less responding than predicted for an additive interaction. Indeed, although substantial variability was observed among the potency ratios for the MDPV+methylone group, the 3:1 mixture of MDPV+methylone exhibited a sub-additive interaction with regard to potency; additive interactions were observed for all other mixtures (Figure 3; bottom row, left panel). With regard to reinforcing effectiveness, an additive interaction was observed for the 10:1 ratio, whereas the effectiveness ratios for the 3:1, 1:1, and 1:3 mixtures of MDPV+methylone were all significantly greater than 1, indicative of a sub-additive interaction (Figure 3; bottom row, right panel). The $E_{max} \pm SEM$ and the final ratio completed $\pm SEM$ for each mixture of MDPV+methylone are reported in Table 1.

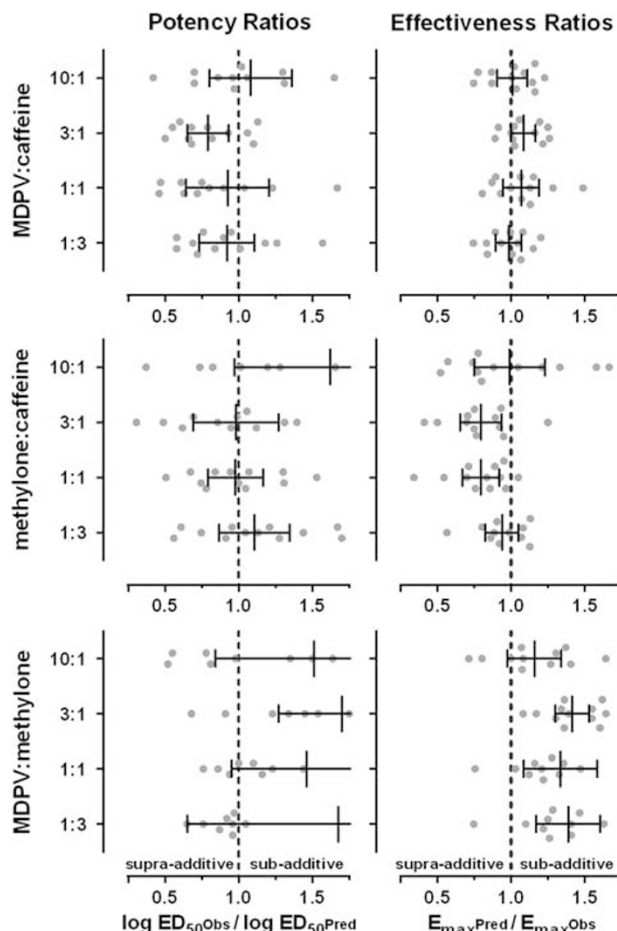


Figure 3 Potency ratios (observed ED_{50} /predicted additive ED_{50} , left column) and effectiveness ratios (predicted additive E_{max} /observed E_{max} , right column) for binary mixtures of MDPV+caffeine (top row), methylone +caffeine (middle row), and MDPV+methylone (bottom row; $n = 12$ per group). Ratios for individual subjects are depicted by the gray dots. The group means and 95% confidence intervals are depicted by the vertical black line and error bars, respectively.

The self-administration of MDPV+methylone mixtures was associated with lethality in 5 of 17 rats. For the 1:1 mixture, 1 of 14 rats died at the fourth dose pair (0.03 mg/kg/inf MDPV+1.23 mg/kg/inf methylone), and 2 of 4 rats died at the fifth dose pair (0.05 mg/kg/inf MDPV+2.19 mg/kg/inf methylone). At the 1:3 mixture, 1 of 14 rats died at the fourth dose pair (0.015 mg/kg/inf MDPV+1.85 mg/kg/inf methylone), and 1 of 2 rats died at the fifth dose pair (0.03 mg/kg/inf MDPV, 3.3 mg/kg/inf methylone). Because of the high incidence of lethality (50%), the largest dose pairs of the 1:1 and 1:3 mixtures of MDPV+methylone were not evaluated in all subjects.

DISCUSSION

bath salts preparations typically contain mixtures of drugs including multiple synthetic cathinones or synthetic cathinones and caffeine, and use of these preparations is associated with high rates of abuse, toxicity, and death. Despite this, little is known about how the composition of these bath salts preparations has an impact on their abuse-

related and toxic effects. As such, the current study evaluated the reinforcing effects of three common bath salts constituents (ie, MDPV, methylone, and caffeine) and used dose-addition analyses to characterize the nature of the interaction(s) between binary bath salts mixtures comprising two synthetic cathinones (ie, MDPV+methylone) or a synthetic cathinone and caffeine (ie, MDPV+caffeine and methylone+caffeine). The present study provides evidence that the composition of bath salts preparations can have a significant impact on both their reinforcing potency and effectiveness, with mixtures of MDPV+caffeine (3:1) being more potent than predicted for an additive interaction, mixtures of methylone+caffeine (3:1 and 1:1) being more effective than predicted for an additive interaction, and mixtures of MDPV+methylone (3:1, 1:1, and 1:3) being less effective and less potent (3:1) than predicted for an additive interaction.

Consistent with literature describing the reinforcing effects of MDPV (Aarde *et al*, 2013; Watterson *et al*, 2014; Schindler *et al*, 2016; Gannon *et al*, 2017a; Gannon *et al*, 2017b), methylone (Watterson *et al*, 2013; Creehan *et al*, 2015; Vandewater *et al*, 2015; Nguyen *et al*, 2016), and caffeine alone (Collins *et al*, 1984; Briscoe *et al*, 1998), MDPV was the most effective, whereas caffeine was the least effective of the bath salts constituents. Importantly, although numerous studies have used PR schedules to compare relative reinforcing effectiveness of MDPV and methylone to other stimulants (eg, cocaine, methamphetamine, and MDMA), this is the first to directly compare these two common bath salts constituents. Using the final ratio completed as an index of relative reinforcing effectiveness, MDPV (1123.2 ± 114.1) was an ~6-fold more effective reinforcer than methylone (190.4 ± 39.4) and an ~45-fold more effective reinforcer than caffeine (24.7 ± 1.9). Although previous reports suggest that the reinforcing effects of IV caffeine are dubious and short-lived (Atkinson and Enslin, 1976; Briscoe *et al*, 1998), in the present study, caffeine maintained low but consistent levels of responding when substituted from a MDPV or methylone baseline, regardless of the order that the doses were evaluated. However, upon re-evaluation at the end of the study, caffeine appeared to be less effective in the MDPV-trained rats.

Even though caffeine was found to be a weak reinforcer, supra-additive interactions were observed when caffeine was self-administered in combination with more effective reinforcers. For instance, when mixed at a 3:1 ratio with MDPV, a highly effective reinforcer, caffeine produced a supra-additive interaction with regard to reinforcing potency, suggesting that bath salts preparations containing MDPV and caffeine can produce a full MDPV-like effect with significantly less MDPV. Conversely, when mixed at a 3:1 or 1:1 ratio with methylone, a moderately effective reinforcer, caffeine produced a supra-additive interaction with regard to reinforcing effectiveness, suggesting that the reinforcing effects of bath salts preparations containing methylone and caffeine would be significantly greater than bath salts preparations containing only methylone. Although these studies were not designed to identify the mechanism(s) that underlie interactions between caffeine and the synthetic cathinones, caffeine (through its antagonism of adenosine A_{2A} receptors) can increase dopamine D_2 receptor signaling, which has been hypothesized to have a role in caffeine's

ability to potentiate the psychostimulant effects of other indirect dopamine receptor agonists (eg, cocaine; Ferré, 2016). Although it is unclear why the effects of caffeine differed in bath salts mixtures containing caffeine+MDPV (increased potency) and caffeine+methylone (increased effectiveness), it is a possibility that a ceiling effect limited our ability to detect enhancements in the effectiveness for mixtures of MDPV+caffeine, and that supra-additive interactions would have been observed if different methods for quantifying reinforcing effectiveness had been employed (eg, demand curve analyses). Alternatively, it is also possible that differences in the types of supra-additive interactions are related to differences in the mechanism of action for MDPV (highly selective inhibitor of DAT) and methylone (non-selective substrate at DAT, NET, and SERT; Baumann *et al*, 2013; Simmler *et al*, 2013). Clearly, more in-depth investigations into the mechanisms that underlie these interactions are warranted.

Whereas supra-additive interactions were observed for bath salts mixtures containing caffeine, sub-additive interactions were observed when MDPV and methylone were combined. Although the 3:1 mixture of MDPV+methylone was found to be less potent than predicted for an additive interaction, 3:1, 1:1, and 1:3 mixtures of MDPV+methylone were all found to exhibit sub-additive interactions with regard to reinforcing effectiveness. This finding contrasts a report that identified a supra-additive interaction between mephedrone (a monoamine transporter substrate) and MDPV with regard to excitatory hDAT currents, and proposed that this could result in mixtures of substrates and inhibitors producing effects greater than either drug alone (Cameron *et al*, 2013). However, it is important to note that methylone, and MDPV to a lesser extent, also has actions at NET and SERT that likely have an impact on the reinforcing effects of these drugs/drug mixtures that are not captured in an isolated *in vitro* system. Interestingly, as the proportion of methylone in the MDPV+methylone mixtures increased, the maximal effect observed became more similar to the maximal effect produced by methylone alone (see Figure 1, bottom right panel). Thus, one interpretation of these findings is that the actions of methylone at SERT may be decreasing the reinforcing effectiveness of the MDPV+methylone mixture, a notion that is supported by the demonstration that increases in serotonergic activity are associated with decreases in stimulant self-administration by nonhuman primates (Wee *et al*, 2005; Wee and Woolverton, 2006).

However, because these sub-additive interactions were observed at larger dose pairs, it is also possible that decreases in reinforcing effectiveness resulted from the onset of other/adverse effects that limited the ability of the rats to respond at high rates. Indeed, the self-administration of these large dose pairs was lethal in five rats. Although the mechanism of this toxicity is unclear, it is likely related to the fact that methylone is a mechanism-based inhibitor of CYP2D6, the enzyme primarily responsible for the metabolism of methylone (Pederson *et al*, 2013; Elmore *et al*, 2017). However, because lethality has not previously been reported for rats self-administering methylone (eg, Watterson *et al*, 2013; Creehan *et al*, 2015; Vandewater *et al*, 2015; Nguyen *et al*, 2016), and because the majority of the deaths (7 of 10) were associated with the self-administration of bath salts mixtures containing methylone (five with methylone+

MDPV and two with methylone+caffeine), pharmacodynamic interactions between the toxic effects of bath salts constituents cannot be ruled out. Importantly, methylone has also been associated with toxicity and death in human bath salts users, particularly when used in preparations that also contain MDPV (eg, Spiller *et al*, 2011; Pearson *et al*, 2012). Since MDPV maintains very high levels of responding without overt signs of toxicity, and toxicity was only observed in the presence of methylone, it is likely that the toxicity observed with mixtures of MDPV+methylone was the result of MDPV maintaining high rates of responding that also resulted in large cumulative doses of methylone.

Abuse and toxicity associated with the use of bath salts has become a major public concern in the past decade. While it has been demonstrated that some common constituents of these preparations (eg, MDPV) are more effective than other drugs of abuse when self-administered alone (eg, Gannon *et al*, 2017a), bath salts preparations are often mixtures of multiple drugs. The present study is the first to directly assess the reinforcing effects of mixtures of common bath salts constituents. There were four main findings: (1) MDPV is a more potent and effective reinforcer than methylone, which is equipotent but more effective than caffeine; (2) bath salts mixtures containing caffeine can function as more potent (MDPV+caffeine) and more effective (methylone+caffeine) reinforcers than expected based on the effects of the constituents alone; (3) bath salts mixtures containing MDPV+methylone are less effective reinforcers than expected; and (4) bath salts mixtures containing methylone appear to be more toxic than methylone alone. Although most bath salts mixtures exhibited strictly additive interactions, these findings indicate that supra-additive interactions can occur between drugs with different mechanisms of action (ie, MDPV+caffeine and methylone+caffeine) and sub-additive interactions can occur between drugs that act primarily at monoamine transporters but differ in transporter selectivity (ie, MDPV+methylone). Taken together, these studies demonstrate that the composition of bath salts preparations can have a significant impact on their abuse-related and toxic effects, and suggest that such interactions could contribute to the patterns of use and/or the adverse effects reported by human bath salts users.

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