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Respiratory effects of diazepam/methadone combination in rats: a study based on concentration/effect relationships

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Short title: Methadone/diazepam combination

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Abbreviations: ANOVA, analysis of variance; AUC, area under the curve; BZD, benzodiazepine; CYP, cytochrome P450; DZP, diazepam; EDDP, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; *f*, respiratory frequency; HPLC/MS, high performance liquid chromatography-mass spectrometry; LD₅₀, lethal dose 50%; NADPH, nicotinamide adenine dinucleotide phosphate; PaO₂, arterial oxygen partial pressure; PaCO₂, arterial carbon dioxide partial pressure; T_I, inspiratory time; T_E, expiratory time; T_{TOT}, total respiratory time; V_E, minute volume; V_T, tidal volume.

ABSTRACT

Methadone may cause respiratory depression and fatalities. Concomitant use of benzodiazepines in methadone-treated patients for chronic pain or as maintenance therapy for opiate abuse is common. However, the exact contribution of benzodiazepines to methadone-induced respiratory toxicity remains debatable. We investigated the respiratory effects of the combination diazepam (20 mg/kg)/methadone (5 mg/kg) in the rat, focusing on methadone concentration/effect relationships. Respiratory effects were studied using arterial blood gases and whole-body plethysmography. Plasma concentrations of both *R*- and *S*-methadone enantiomers were measured using high-performance liquid chiral chromatography coupled to mass spectrometry. To clarify mechanisms of diazepam/methadone interaction, methadone metabolism was investigated *in vitro* using rat liver microsomes. Diazepam/methadone co-administration significantly increased methadone-related effects on inspiratory time ($p < 0.001$) but did not significantly alter the other respiratory parameters when compared with methadone alone, despite significant increase in the area under the curve of plasma *R*-methadone concentrations measured during 240 min ($p < 0.05$). Diazepam/methadone co-incubation with microsomes *in vitro* resulted in a significant inhibition of methadone metabolism ($p < 0.01$), with 50%-inhibitory diazepam concentrations of 25.02 ± 0.18 $\mu\text{mol/L}$ and 25.18 ± 0.23 $\mu\text{mol/L}$ for *R*- and *S*-methadone, respectively. We concluded that co-administration of high-doses of diazepam and methadone in rats is not responsible for additional respiratory depression in comparison to methadone alone, despite significant metabolic interaction between the drugs. In humans, although our experimental data may suggest the relative safety of benzodiazepine/methadone co-prescription, physicians should remain cautious as other underlying conditions may enhance this drug-drug interaction.

Keywords: Diazepam; effect/concentration relationships; metabolism; methadone; plethysmography; respiratory effects

1. Introduction

Methadone, a full agonist opioid, is widely used as treatment for chronic pain and for maintenance therapy in heroin addicts (Cicero and Inciardi, 2005; Mattick et al., 2008). Marketed methadone is a racemic mixture, with *R*-methadone believed to account for most, if not all, of its therapeutic effects and *S*-methadone suspected of playing a significant role in its adverse reactions (Mitchell et al., 2004). In humans, methadone (6-dimethylamino-4,4-diphenyl-3-heptanone) is metabolized by multiple cytochrome P450 (CYP) isoforms including 3A4, 2B6, 2C19, 2C9, and 2D6 to 2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), an inactive primary metabolite (Iribarne et al., 1996), with the potential risk of significant drug-drug interactions.

A steep rise in recreational use of prescription opioids was observed during the past two decades, resulting in increased fatalities mainly in relation to methadone abuse (Paulozzi, 2006). Methadone is increasingly prescribed and, as a result of its increased availability to patients, adjunctively diverted to recreational or habitual opioid abusers (Cicero and Inciardi, 2005). To date, although representing less than 5% of prescribed opioids in the U.S., methadone has been implicated in one-third of opioid-related poisonings (Webster et al., 2011).

Circumstances of methadone poisonings include overdoses and drug-drug interactions. Benzodiazepines (BZDs) are commonly used by opioid abusers to reduce anxiety, reinforce opioid effects, and treat craving and withdrawal symptoms (Lintzeris and Nielsen, 2009). Prevalence of BZD use among methadone-maintained patients ranges from 50 to 70% (Jones et al., 2012). Screening for BZDs was found to be positive in more than 85% of methadone-intoxicated patients admitted to the intensive care unit (Mégarbane et al., 2010). Among BZDs, diazepam is one of the most preferred, prescribed, and thus abused molecules (Bramness and Kornor, 2007; Iguchi et al., 1993).

The real contribution of BZDs to methadone-related respiratory toxicity and fatalities remains under debate (Häkkinen et al., 2012; Jones et al., 2012; Paulozzi et al., 2009). In rats, methadone has been shown to induce a dose-dependent increase in inspiratory time (T_I) with an additional increase in expiratory time (T_E) at elevated doses, resulting in respiratory acidosis and hypoxemia (Chevallard et al.,

2010; McCormick et al., 1984; White and Zagon, 1979). In contrast, BZDs were shown to weakly alter arterial blood gases, even at elevated doses (McCormick et al., 1984; Mégarbane et al., 2005; Nielsen and Taylor, 2005), while BZD pre-treatment reduced methadone's median lethal dose (Borron et al., 2002) and potentiated methadone-related respiratory depression (McCormick et al., 1984; Nielsen and Taylor 2005). A pharmacokinetic interaction between methadone and diazepam was suggested in rats, in relation to a non-competitive inhibition of methadone metabolism by diazepam (Liu et al., 1978; Spaulding et al., 1974). However, using human microsomes, diazepam appeared to be a weak competitive CYP3A4 inhibitor *in vitro*, questioning the clinical relevance of such metabolic interaction (Iribarne et al., 1997). Consistently, clinical investigations in methadone-treated patients ruled out significant pharmacokinetic interactions between diazepam and methadone (Pond et al., 1982; Preston et al., 1986).

Given the possible role of BZDs in methadone-related toxicity and concerns regarding the *in vivo* relevance of pharmacokinetic interaction between these drugs, we designed an experimental study to better quantify the respiratory effects resulting from diazepam/methadone interaction, aiming to analyze its mechanism and clarify the exact role of each methadone enantiomer.

2. Experimental procedures

2.1. Animals

Male Sprague-Dawley rats (Janvier, France) weighing between 250 and 350 g at the time of experimentation were used. Animals were housed for 5 days before experimentation in an environment maintained at $21 \pm 0.5^{\circ}\text{C}$ with controlled humidity and a light-dark cycle (lights on between 08:00 and 20:00 h). Food and tap water were provided *ad libitum*. All experimental protocols used in this study were carried out within the ethical guidelines established by the National Institutes of Health, the French Minister of Agriculture, and the European Union Legislation. Protocols were approved by the local ethics committee for animal experimentation of Paris-Descartes University.

2.2. Chemicals and drugs

Methadone hydrochloride and diazepam were purchased from Francopia, France. Pooled male rat liver microsomes (20 mg/mL in a 0.25 mol/L sucrose solution) and nicotinamide adenine dinucleotide phosphate (NADPH) regenerating system were purchased from Becton Dickinson Gentest, Le Pont de Claix, France. All other chemicals were purchased from Sigma, Saint-Quentin Fallavier, France. Methadone was diluted in Tween-20[®] and sterile water to obtain solutions of 16 and 0.1 mmol/L, respectively. Diazepam was diluted in Tween-20[®] and methanol to obtain solutions of 70 and 10 mmol/L, respectively.

2.3. Femoral artery catheterization

The day before the study, animals were anesthetized with 70 mg/kg ketamine (Ketalar[®]) and 10 mg/kg xylazine (Rompum[®]) intraperitoneally (i.p.) then placed on a warming blanket with regulating thermostat. The femoral artery was catheterized using 30 cm-silastic tubing with external and internal diameters of 0.94 and 0.51 mm, respectively (Dow Corning Co., Midland, MI). Arterial catheters were tunneled subcutaneously (s.c.) and fixed at the back of the neck. Heparinized saline was injected into the catheter to avoid thrombosis and catheter obstruction. Rats were then returned to their individual cages for a minimum recovery period of 24 h, to allow complete anesthesia washout. Moreover, ketamine/xylazine combination, routinely used for short surgical procedures, was chosen based on drug's short elimination half-lives to avoid any further significant interaction on ventilation with the investigated drugs tested 24h later.

On the day of experimentation, rats were placed in horizontal Plexiglas cylinders (6.5 cm-internal diameter, up to 20 cm-adjustable length) (Harvard Apparatus, Inc. Holliston, MA, USA), modified by the addition of several holes at the cephalic end to avoid CO₂ rebreathing (Chevillard et al., 2010). Before drug administration, the catheter was exteriorized, purged, and its permeability verified.

2.4. Clinical findings

Sedation level based on a 4-stage scale from 0 (awake) to 3 (coma) was assessed. At stage 0, rats were completely awake and their gait and righting reflexes were intact. At stage 1, rats had reduced activity, showed light impairment of gait and an intact righting reflex with diminished muscular tonus. At stage 2, rats were asleep or static and showed a reduced righting reflex. At stage 3, rats were comatose and did not have any righting reflex. Temperature was measured using i.p. implanted temperature transmitters for the purpose of plethysmography study.

2.5. Arterial blood gas study

In catheterized rats, 100 μ L-arterial blood samples were collected before (T₀), and at 5, 10, 15, 30, 60, 120, 180, and 240 min after i.p. injection of methadone or its solvent. Samples were immediately analyzed using Rapidlab 248[®] (Siemens Healthcare Diagnostics, Saint Denis, France). Every hour, 1000 μ L heparinized 0.9% NaCl was administered to the rat via the arterial catheter to reduce the risk of catheter clotting and to compensate for volume losses. Rats were subsequently euthanized using a carbon dioxide chamber.

2.6. Whole body plethysmography

Four days before the study, temperature transmitters (DSI, Chatillon, France) were implanted in the peritoneal cavity. Rats were then returned to their individual cages for a 72 h-recovery period. Ventilatory parameters were recorded in a whole-body plethysmograph by the barometric method described and validated in the rat (Bartlett and Tenney, 1970). On the day of experimentation, animals were placed in a rectangular Plexiglas chamber with a 3 L-volume connected to a reference chamber of the same size by a high-resistance leak to minimize the effect of pressure changes in the experimentation room. The animal chamber was flushed continuously with humidified air at a rate of 5 L/min. During the recording periods, the inlet and outlet tubes were temporarily clamped and pressure changes associated with each breath were recorded using a differential pressure transducer (Validyne MP, 45 ± 3 cm H₂O, Northridge, CA), connected to the animal and reference chambers. During each measurement, calibration was performed by

three injections of 1 mL of air into the chamber and the ambient temperature was noted. The spirogram was recorded and stored on a computer with an acquisition data card (PCI-DAS 1000, Dipsi, Chatillon, France) using respiratory acquisition software (Acquis1 Software, CNRS, Gif-sur-Yvette, France) for off-line analysis.

This technique was daily validated with a series of leak tests (leak was signaled if diminution of the signal amplitude exceeded 33% in 5 s) (Bonora et al., 2004). The quantification threshold corresponded to a minimum air volume injection of 30 μ l. Within the range of tested volumes (0.1 to 3 mL), measurement was linear. The mean coefficient of intra-day variability (four series of five measurements carried out the same day) was $1.32 \pm 0.18\%$. The mean coefficient of inter-day variability (25 measurements carried out on three different days) was $1.69 \pm 0.11\%$. We checked that the mean CO₂ measured using an Ohmeda 5250 RGM capnograph (rebreathing test) during clamping periods did not exceed 0.6% of the air contained in the chamber.

Before each measurement, the temperature of the enclosure, the temperature of the animal, the volume of calibration and the atmospheric pressure were noted to permit calculation of plethysmography parameters. The first measurement was performed after a 30-60 min-period of accommodation of the animal in the plethysmography unit. During this period, the rat was quiet and not in deep or rapid eye movement sleep which could be roughly estimated from its behavior, response to noise, and breathing pattern. Then, the rat was gently removed from the chamber for i.p. injection, and replaced in the chamber for the remaining measurements. Ventilation was recorded at 5, 10, 15, 30, and then every 60 min during four hours, each record lasting about 60 s. The following parameters were determined using a specific formula (Bartlett and Tenney, 1970): tidal volume (V_T), T_I , and T_E . Additional parameters were calculated: the respiratory cycle duration ($T_{TOT} = T_I + T_E$), the respiratory frequency (f), and the minute volume ($V_E = V_T \times f$). At the end of experimentation, rats were euthanized using a carbon dioxide chamber.

2.7. In vitro study of methadone metabolism using liver microsomes

Methadone metabolism by rat liver microsomes was studied *in vitro* as previously described (Cho et al., 1990). The incubation mixture consisted in 0.98 mL of 0.1 mol/L HEPES buffer, pH 7.6 containing NADPH regenerating system (1.3 mmol/L NADP, 3.3 mmol/L glucose-6-phosphate, 3.3 mmol/L MgCl₂, 0.4 IU/mL glucose-6-phosphate dehydrogenase) and 1 mg/ml rat microsomal protein. This mixture was pre-incubated for 15 min at 37°C, and reactions were initiated by adding methadone at 1 µmol/L, the minimal concentration we previously obtained in plasma between 5 and 240 min after i.p. administration of 5 mg/kg methadone to rats (Chevallard et al., 2010). To study the inhibition of methadone metabolism by diazepam, increasing concentrations of diazepam were added. For the *in vitro* time-course of methadone metabolism, aliquots (100 µL) were removed from this mixture at 0, 15, 30, 60, and 120 min. To study the inhibition of methadone metabolism by diazepam, aliquots were removed at 0 and 120 min. The reaction was stopped by adding an equal volume of cold methanol. The reaction mixture was centrifuged at 10,000 g for 10 min, and supernatants stored at -80 °C until analyzed. Concentrations of methadone enantiomers and EDDP were measured in supernatants using liquid chromatography-mass spectrometry (HPLC/MS), without liquid phase extraction (see below). An aliquot of each supernatant (100 µL) was mixed with 20 µL internal standards (5 µmol/l [(*R,S*)-methadone-d₃ + EDDP-d₃]) and directly injected into the system. Triplicate samples were used in all the inhibition experiments.

2.8. HPLC/MS determination of *R*- and *S*-methadone and EDDP

Samples were collected in catheterized rats concomitantly to those dedicated for arterial blood gas analysis. Three hundred microliters of blood were obtained from the arterial catheter before (T₀), and at 5, 10, 15, 30, 60, 120, 180, and 240 min after i.p. administration of methadone or its solvent. Samples were placed in Eppendorf tubes and centrifuged at 3,500 g for 10 min. Subsequently, plasma (about 200 µl) was separated and refrigerated at -20°C, until liquid-liquid extraction in Tox tubes A[®] (Varian, USA) and quantification using a specific HPLC/MS assay.

Analyses were performed on a ThermoFinnigan[®] liquid chromatograph/ion trap mass spectrometer LCQ Advantage[®], including a Surveyor[®] HPLC system which consisted of a degasser, a binary pump,

and an autosampler. Chromatographic separation was carried out on a Cyclobond[®] I-2000 HP-RSP chiral column (250 mm×4.6 mm I.D., 5 μm, Thermo Electron Corporation, Waltham, MA). Single ion storage detection was performed (m/z ((*R* and *S*)-methadone) = 310.1, m/z (EDDP) = 278.2). *R*-methadone, *S*-methadone, and racemic EDDP concentrations were calculated using the peak area ratios between the compound and its corresponding internal standard, i.e. deuterated racemic methadone [(*R,S*)-methadone- d_3] or deuterated EDDP [EDDP- d_3], which was added before extraction at 5 μmol/l (20 μL). Limits of quantification were 3 nmol/L for both *R*- and *S*-methadone enantiomers and 36 nmol/L for EDDP, with coefficients of variation of 15.8%, 13.5%, and 10.8%, respectively.

2.9. Study designs:

2.9.1. *In vivo* study of diazepam/methadone combination

Rats were randomized into four groups (N=8) and treated consecutively with s.c. 20 mg/kg diazepam + i.p. 5 mg/kg methadone (diazepam/methadone group), s.c. Tween-20[®] + i.p. 5 mg/kg methadone (methadone group), s.c. 20 mg/kg diazepam + i.p. Tween-20[®] (diazepam group), and s.c. Tween-20[®] + i.p. Tween-20[®] (solvent group). Subsequently, ventilation at rest was studied using arterial blood gas analysis and plethysmography. Plasma concentrations of both methadone enantiomers were measured at each sampling time. We investigated methadone/effects relationships regarding T_I (the hallmark of opioid respiratory effects) and $PaCO_2$ (the hallmark of respiratory depression) (Chevillard et al., 2009). Methadone (5 mg/kg) and diazepam (20 mg/kg) doses and routes of administration were those previously used in rats to assess methadone/diazepam interactions (McCormick et al., 1984; Nielsen and Taylor, 2005). Both drug doses can be considered as elevated doses, taking into account the lethal doses-50% (LD_{50}), i.e. 18 mg/kg for i.p. methadone (Lynch and Meyers, 1958) and 32 mg/kg for intravenous diazepam (Pfizer, 2009).

2.9.2. *In vitro* study of diazepam-related effects on methadone metabolism

We studied the effects of 0 (control group), 25, 50, and 75 $\mu\text{mol/L}$ diazepam on methadone metabolism (1 $\mu\text{mol/L}$). The dose range of diazepam was used in accordance with the value of the inhibition constant reported with human microsomes ($K_i = 50 \mu\text{mol/L}$; Iribarne et al., 1997).

2.10. Statistical analysis

Results were expressed as mean \pm S.E.M. For plethysmography, T₀ was the mean of the three baseline measurements. To permit simultaneous analysis of the effect of time and various treatments, we calculated for each animal and for each studied parameter, the area under the curve (AUC) from T₀ to the completion of the measurement (240 min) using the trapezoid method (Tallarida and Murray, 1984). For each measurement, we compared the baseline values, the values at each sampling time, and the AUCs. Noncompartmental analysis (WinNonlin version 5.1, Pharsight Corp, Mountain View, CA) was used to calculate methadone terminal elimination half-life ($T_{1/2\beta}$) as well as the AUC from 0 to 240 ($\text{AUC}_{0\rightarrow 240}$) and the percentage of extrapolation of the AUC from 0 to infinity ($\text{AUC}_{0\rightarrow\infty}$) regarding methadone concentrations. We used Mann-Whitney *U*-tests for comparisons between two groups and two-way analyses of variance (ANOVA) followed by multiple comparison tests using Bonferroni's correction for comparisons between more than two groups. For the *in vitro* inhibition study, the 50%-inhibitory diazepam concentration (IC_{50}) was determined for each enantiomer based on the metabolism inhibition curves using nonlinear regression. Inhibition of methadone metabolism was determined for each enantiomer as the relative difference in its concentrations between 0 and 120 min in the reaction aliquot. EDDP formation was determined as the relative difference in its concentrations between 0 and 120 min in the reaction aliquot. These variables were compared using one-way ANOVA followed by multiple comparison tests with Dunnett's correction. The concentration-effect relationships were analyzed using linear regression. All tests were performed using Prism version 4.0 (GraphPad Software, Inc., San Diego, CA), and *p*-values of less than 0.05 were considered significant.

3. Results

3.1. *In vivo* study of diazepam/methadone combination's effects on ventilation

Rats were free of symptoms in the diazepam group in comparison to the solvent group. In contrast, they were significantly sedated in the methadone and diazepam/methadone groups in comparison to the solvent and diazepam groups ($p < 0.001$, Fig. 1). Interestingly, no significant differences were observed in sedation between the methadone and diazepam/methadone groups, while these groups significantly differed regarding body temperature ($p < 0.01$).

Regarding blood gases and plethysmography, no significant differences between baseline values were observed for each parameter between the groups. Methadone administration ($p < 0.05$) and diazepam/methadone co-administration ($p < 0.01$) were responsible for a significant decrease in PaO₂ when compared to the solvent and diazepam groups. Similarly, diazepam/methadone co-administration was responsible for a significant decrease in arterial pH when compared to the solvent ($p < 0.05$) and diazepam groups ($p < 0.001$). Interestingly, none of the blood gas parameters significantly differed between the methadone and diazepam/methadone groups (Fig. 2).

Using AUC measurements, only T_I was significantly increased in the diazepam/methadone group in comparison to the solvent, diazepam, and methadone groups ($p < 0.001$, Fig. 3A). No significant differences regarding f , T_E, and V_T were observed between the methadone and diazepam/methadone groups. However, in the diazepam/methadone group, T_I was significantly increased at 120 and 180 min ($p < 0.05$) and T_E at 30 ($p < 0.05$), 60 ($p < 0.01$), 120 ($p < 0.05$), and 180 min ($p < 0.01$), in comparison to the methadone group (Fig. 3B). Regarding f , a significant difference was measured at 120 min between the diazepam/methadone and the methadone group ($p < 0.05$).

3.2. *In vivo* study of diazepam/methadone combination's effects on methadone concentrations

Diazepam/methadone co-administration resulted in a significant increase in plasma *R*-methadone concentrations at 120 ($p < 0.01$), 180 ($p < 0.01$), and 240 min ($p < 0.001$), without any significant modification in *S*-methadone concentrations (Fig. 4). Using the AUC method, there was a 2.0-fold increase in plasma *R*-methadone concentrations in relation to diazepam co-administration ($p < 0.01$),

whereas plasma *S*-methadone concentrations were not significantly altered (1.5-fold increase). Regarding T_{\max} and C_{\max} of *R*-methadone, no significant differences were observed between the two groups. In rats treated with diazepam/methadone combination, neither *R*-methadone $T_{1/2\beta}$ (155.1 versus 155.5 min) nor *S*-methadone $T_{1/2\beta}$ (131.3 versus 158.3 min) was altered in comparison to rats treated with methadone alone.

3.3. Analysis of methadone-related concentration/effect relationships

We studied the relationships between methadone-induced respiratory effects (i.e. on PaCO_2 and T_I) and plasma concentrations of the active *R*-methadone enantiomer, when 5 mg/kg methadone was administered alone or in combination with 20 mg/kg diazepam (Fig. 5). A clockwise hysteresis was observed for both parameters, whether diazepam was co-administered or not.

3.4. In vitro study of diazepam-related effects on methadone metabolism

No significant differences between the kinetics of *R*- and *S*-methadone disappearance were observed. Diazepam co-incubation resulted in a dose-dependent decrease in *R*- and *S*-methadone concentrations in comparison to the control, however this was only significant with the two higher diazepam doses ($p < 0.01$) (Fig. 6A). As a consequence of the inhibitory effect of diazepam on methadone metabolism, EDDP formation was significantly decreased for all diazepam doses (Fig. 6B). IC_{50} values of diazepam on *R*- and *S*-methadone metabolism were $25.02 \pm 0.18 \mu\text{mol/L}$ and $25.18 \pm 0.23 \mu\text{mol/L}$, respectively.

4. Discussion and conclusions

In rats, the diazepam/methadone combination does not significantly worsen methadone-induced respiratory effects despite a significant increase in plasma *R*-methadone attributed to diazepam-mediated inhibition of methadone metabolism, as assessed *in vitro*.

Like any full mu-opioid receptor agonist, methadone-induced respiratory effects are characterized by a dose-related, naloxone-reversible depression of the resting total volume with a proportional reduction in V_T , decrease in PaO_2 and arterial pH, increase in $PaCO_2$, and reduction in the ventilatory drive stimulated by hypercapnia (Shook et al., 1990). Previously, we showed that methadone increases T_I in a dose-dependent manner but only increases T_E at elevated doses (~15 mg/kg) resulting in respiratory depression (Chevillard et al., 2010).

Mechanisms and consequences of BZD/methadone interaction are debated in the literature (Jones et al., 2012). In mice, neither locomotor activity nor analgesia was altered when administering the diazepam/methadone combination in comparison to methadone alone (Shannon et al., 1976). In contrast, diazepam was reported to markedly potentiate methadone respiratory depressant effects when given in combination with methadone in rats (McCormick et al., 1984). The most severe respiratory depression occurred when both drugs were used together acutely, suggesting that the maximum deleterious effect occurred when concurrently used for the first time. Here, surprisingly, and although using the same doses and administration routes, only a significant increase in T_I with a trend to a decrease in PaO_2 was observed, suggesting limited deleterious effects on rat ventilation of the diazepam/methadone combination in comparison to methadone alone. It is noteworthy that in McCormick's study, diazepam was diluted in a solution containing 10% ethyl alcohol and 1.5% benzyl alcohol, and the interaction with methadone could have been responsible for the observed significant respiratory effects. Interestingly, the second animal study attributing deleterious respiratory effects using arterial blood gases to the diazepam/methadone combination, compared rats treated with a diazepam/methadone combination to rats treated with diazepam or solvent but not to rats treated with methadone alone (Nielsen and Taylor, 2005). These concerns question the exact respiratory consequences of the diazepam/methadone combination, as shown in our study.

As previously assessed with human microsomes (Iribarne et al., 1997), we confirmed the diazepam-mediated inhibition of methadone metabolism, using rat microsomes *in vitro*. Interestingly, our calculated diazepam IC_{50} (25.02 μ mol/L) was in the same range as the value reported by Iribarne et al. (50 μ mol/L,

Iribarne et al., 1997). Moreover, no differences in either the time-course of methadone disappearance or diazepam IC_{50} values for both methadone enantiomers were observed. Surprisingly, we found marked diazepam-induced inhibition of methadone metabolism into EDDP *in vivo*: AUCs of *R*- and *S*-methadone were altered in rats treated with the diazepam/methadone combination but this was only significant for *R*-methadone, which suggested possible involvement *in vivo* of additional pathways in *S*-methadone clearance (i.e. transport or metabolic enzymes) that were not inhibited by diazepam. There are no published data regarding the stereoselectivity of metabolism enzymes for methadone in rats. In studies using human microsomes, CYP2B6 preferentially metabolizes *S*-methadone (Totah et al., 2008) and CYP2C19 *R*-methadone (Gerber et al., 2004), while CYP1A2 was not stereoselective (Eap et al., 1997). Interestingly, CYP3A4 affinity for *R*-methadone was reported to be higher than for *S*-methadone (Foster et al., 1999); however stereoselectivity of CYP3A4-mediated methadone metabolism is still debated (Gerber et al., 2004; Totah et al., 2007; Wang and Devane, 2003).

Increased AUC of methadone concentrations without alterations in its $T_{1/2\beta}$ when pre-treating rats with diazepam supported a diazepam-mediated increase in methadone i.p. bioavailability (Fig. 4). Based on the peak diazepam concentration (1.76 $\mu\text{mol/L}$) measured after intravenous administration of 1.2 mg/kg diazepam in rats (Igari et al., 1982) and assuming that diazepam pharmacokinetics are linear up to 20 mg/kg with a rapid and complete absorption by the s.c. route, we hypothesize that plasma diazepam concentrations may have reached $\sim 29.4 \mu\text{mol/L}$ in our study. Compared to diazepam IC_{50} (25.02 $\mu\text{mol/L}$), such expected concentrations supported the occurrence of significant CYP inhibition *in vivo* as reported. Additionally, the diazepam elimination half-life of 24.8 min (Igari et al., 1982) clearly explained why diazepam inhibitory effects on methadone metabolism occurred during its absorption and distribution phases (liver first-pass) rather than its elimination phase.

Here, the diazepam/methadone combination increased the mean peak *R*-methadone concentrations in plasma from 3.4 to 4.9 $\mu\text{mol/L}$. We previously studied *R*-methadone concentrations associated with the half-maximum effects (EC_{50}) on T_I (1.6 $\mu\text{mol/L}$), PaO_2 (3.7 $\mu\text{mol/L}$), $PaCO_2$ (10.8 $\mu\text{mol/L}$), and T_E (15.6 $\mu\text{mol/L}$) (Chevallard et al., 2010). These data clearly explain why alterations in ventilation resulting from

the combination of diazepam with 5 mg/kg methadone were limited to the observed significant increase in T_1 . Moreover, diazepam-mediated inhibition of methadone metabolism appears to explain the observed effects on ventilation induced by the diazepam/methadone combination, even though additional mechanisms could not be definitively ruled out, including a pharmacodynamic interaction or the alternative pharmacokinetic interaction on protein binding previously suggested to explain the significant increase in brain and liver diazepam concentrations observed when administering diazepam one hour before intravenous methadone (Liu et al., 1978).

In humans, inter-individual variability makes it impossible to anticipate, with acceptable approximation, the relationship between the dose, blood concentrations, and clinical effects of methadone (Ferrari et al., 2004). Several drugs, including BZDs, may influence CYP3A4, 2B6, 2C19, and 2D6-mediated metabolism of methadone, resulting in potential clinical respiratory toxicity (Eap et al., 2001; Foster et al., 1999; Kharasch et al., 2008; Totah et al., 2008). Methadone and diazepam are two competitive CYP3A4 substrates (Ferrari et al., 2004), leading to diazepam-mediated reduction in methadone *in vitro* metabolism (Iribarne et al., 1997) attributed to diazepam's higher affinity for CYP3A4 (Weschules et al., 2008). However, comparison of diazepam IC_{50} values (25 $\mu\text{mol/L}$) with the range of maximum diazepam plasma concentrations (7.02-8.78 $\mu\text{mol/L}$) that could be achieved seriously questions the clinical pertinence of this inhibition mechanism *in vivo* (Walker et al., 1998). Interestingly, when combining methadone with various CYP3A4-metabolised BZDs, higher *R*-methadone concentrations were achieved, supporting a mechanism of BZD-mediated competitive inhibition of methadone metabolism *in vivo* (Hallinan et al., 2009). However, in contrast to these data as well as to our findings in rats, Preston et al. found that concurrent methadone and diazepam administration did not significantly change the time-course or AUC of methadone, diazepam or N-desmethyldiazepam concentrations in plasma in comparison to concentrations measured by the administration of either drug alone (Preston et al., 1986).

In humans, several studies supported potentiating clinical effects with the BZD/methadone combination. BZD users exhibited higher levels of psychopathology and social dysfunction among

methadone maintenance clients (Drake et al., 1993). Diazepam significantly altered subjective drug responses and psychological performance in methadone-maintained subjects (Farré et al., 1998; Lintzeris et al., 2007). Interestingly, diazepam significantly increased reports of good, like, strong, and high when using methadone, resulting in a decrease in the amount of methadone consumed (Spiga et al., 2001). The diazepam/methadone combination elicited greater alterations in subjective drug effects, psychological performance, and physiologic parameters including pupil diameter and sedation in comparison to those induced by either drug alone (Lintzeris et al., 2007; Preston et al., 1984). However, none of the potentiated effects implicated respiration.

Consistently, although BZDs were hypothesized to play a significant role in methadone-related deaths by increasing upper airway obstruction (Caplehorn and Drummer, 2002), no definitive demonstration has been obtained to date. The rate of BZD detection in methadone-attributed deaths, although variable from one series to another (32% to 83%), was not significantly different from that rate in deaths not attributed to methadone. Nevertheless, in these deaths, detectable levels of methadone were found in contrast to the other prescription opioids like tramadol, codeine, and buprenorphine (Häkkinen et al., 2012; Schields et al., 2007). Fatal poisonings seldom seem to be caused by methadone, although absence of significant differences in methadone concentrations between both groups of patients as reported in forensic studies could be attributed to a multitude of factors, including tolerance, route of intake, associated central sleep apnea, and circumstantial and environmental factors (Corkery et al., 2004). Therefore, the role of BZD co-ingestion in methadone-induced toxicity appears less obvious than with buprenorphine, where poisonings and deaths were clearly related to BZD co-ingestion (Mégarbane et al., 2010; Pirnay et al., 2004). However, in methadone-related fatalities, the median concentrations of methadone decreased as the number of co-ingested drugs increased (Häkkinen et al., 2012), suggesting toxicodynamic interactions with the concomitantly used central nervous system depressant drugs.

Our study suffers from a number of limitations: 1)- We studied the diazepam combination with a moderately toxic dose of methadone (30% of its LD₅₀). Thus, our study does not rule out deleterious interactions with the higher doses that may be encountered in severe overdoses and thus lead to life-

threatening conditions. Based on the methadone EC_{50} of $PaCO_2$ increase, we can hypothesize that combining diazepam with higher methadone doses would result in significant respiratory depression in comparison to methadone alone. 2)- From a technical point of view, the reliability of plethysmography in measuring ventilation in awake animals may be questionable for the measurement of V_T which may be underestimated; however, this technique has been validated against pneumotachography and direct plethysmography with a systemic error on V_T of $< 7\%$ (Bonora et al., 2004). The more important variability we observed in V_T measurement in comparison to the other parameters could be also related to various factors that were not taken into account using Bartlett's conventional equation to determine the V_T (Bartlett and Tenney, 1970; Chaui-Berlinck and Bicudo, 1998). In addition, as significant modification of rat temperature was observed, the results of arterial blood gases were corrected using the actual rat temperatures. We chose not to correct the rat temperatures using heating blankets as this might alter methadone pharmacodynamic effects on related respiratory responses. 3)- Due to the limited sampling time, we were unable to measure methadone pharmacokinetic parameters since the extrapolated AUC represented more than 20% (27 to 39%) of the total AUC of the methadone time-course. 4)- We observed a possible enantioselectivity of methadone pharmacokinetics when diazepam was co-administered in rats; however, our experiments could not allow us to definitively conclude about its mechanism.

In conclusion, respiratory effects resulting from the diazepam/methadone combination at elevated doses are limited in comparison to those related to methadone alone despite significant pharmacokinetic interaction *in vivo* attributed to diazepam-mediated inhibition of methadone metabolism. Although our experimental findings may suggest the relative safety of BZD/methadone co-prescription, physicians should remain cautious when prescribing BZD in methadone-treated patients like drug addicts, as other underlying conditions may enhance this drug-drug interaction.

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Figure legends

Fig. 1. Effects of Tween20[®] (solvent, black), Tween20[®] + 5 mg/kg methadone (MTD, vertical line), 20 mg/kg diazepam + 5 mg/kg methadone (DZP+MTD, white), and 20 mg/kg diazepam + Tween20[®] (DZP, horizontal line) on rat body temperature (A) and sedation (B). Each group consisted of eight rats. Values represent mean \pm S.E.M of the areas under the curve (AUC) for each parameter between the time before injection and 240 min postinjection. Comparisons were performed using ANOVA followed by multiple tests using Bonferroni's correction. Comparisons versus solvent: ^{###} $p < 0.001$; versus diazepam: ^{***} $p < 0.001$; versus methadone: ^{§§§} $p < 0.001$.

Fig. 2. Effects of Tween20[®] (solvent, black), Tween20[®] + 5 mg/kg methadone (MTD, vertical line), 20 mg/kg diazepam + 5 mg/kg methadone (DZP+MTD, white), and 20 mg/kg diazepam + Tween20[®] (DZP, horizontal line) on PaCO₂, PaO₂, arterial pH, and bicarbonate concentration. Each group consisted of eight rats. Values represent mean \pm S.E.M of the areas under the curve (AUC) for each parameter between the time before injection and 240 min postinjection. Comparisons were performed using ANOVA followed by multiple tests using Bonferroni's correction. Comparisons versus solvent: [#] $p < 0.05$ and ^{##} $p < 0.01$; versus diazepam: ^{*} $p < 0.05$, ^{**} $p < 0.01$, and ^{***} $p < 0.001$.

Fig. 3. (A) Effects of Tween20[®] (solvent, black), Tween20[®] + 5 mg/kg methadone (MTD, vertical line), 20 mg/kg diazepam + 5 mg/kg methadone (DZP+MTD, white), and 20 mg/kg diazepam + Tween20[®] (DZP, horizontal line) on plethysmography parameters (respiratory frequency, inspiratory time, expiratory time, and tidal volume). **(B)** Effects of Tween20[®] (solvent, black circles), Tween20[®] + 5 mg/kg methadone (MTD, black squares), 20 mg/kg diazepam + 5 mg/kg methadone (DZP+MTD, white circles), and 20 mg/kg diazepam + Tween20[®] (DZP, black squares) on the time-course of plethysmography parameters. Each group consisted of eight rats. The time-course **(A)** and areas under the curve (AUC) between the time before injection and 240 min postinjection **(B)** for the effects on the

plethysmography parameters are expressed as mean \pm S.E.M. Comparisons were performed using ANOVA followed by multiple tests using Bonferroni's correction. Comparisons versus solvent: $^{##}p < 0.01$ and $^{###}p < 0.001$; versus diazepam: $^{***}p < 0.001$; versus methadone: $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, and $^{\$\$\$}p < 0.001$.

Fig. 4. Plasma *R*- and *S*-methadone concentrations in rats treated with Tween20[®] + 5 mg/kg methadone (MTD, white squares) or 20 mg/kg diazepam + 5 mg/kg methadone (DZP+MTD, black circles). Each group consisted of eight rats. The time-course **(A)** and areas under the curve (AUC) between the time before injection and 240 min postinjection **(B)** of methadone concentrations are expressed as mean \pm S.E.M. Comparisons were performed using Mann-Whitney *U*-test.: $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

Fig. 5. Concentration/effect relationships regarding PaCO₂ and inspiratory time in rats treated with 5 mg/kg methadone **(A)** and 20 mg/kg diazepam + 5 mg/kg methadone **(B)**.

Fig. 6. **(A)** Disappearance of *R*- and *S*-methadone enantiomers after 120 min of incubation with different concentrations of diazepam (DZP) expressed in relative percentage in comparison to the control (diazepam: 0 μ mol/L). **(B)** Formation of racemic EDDP after 120 min of incubation with different concentrations of diazepam expressed in relative percentage in comparison to the control (diazepam: 0 μ mol/L). Values are expressed as mean \pm S.E.M for three independent experiences. Comparisons were performed using one-way ANOVA followed by multiple tests using Dunett's correction. Diazepam 0 μ mol/L versus other diazepam concentrations: $^{**}p < 0.01$ and $^{***}p < 0.001$.