Buprenorphine Alters Desmethylflunitrazepam Disposition and Flunitrazepam Toxicity in Rats

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High-dosage buprenorphine (BUP) consumed concomitantly with benzodiazepines (BZDs) including flunitrazepam (FZ) may cause life-threatening respiratory depression despite a BUP ceiling effect and BZDs' limited effects on ventilation. However, the mechanism of BUP/FZ interaction remains unknown. We hypothesized that BUP may alter the disposition of FZ active metabolites in vivo, contributing to respiratory toxicity. Plasma FZ, desmethylflunitrazepam (DMFZ), and 7-aminoflunitrazepam (7-AFZ) concentrations were measured using gas chromatography–mass spectrometry. Intravenous BUP 30 mg/kg pretreatment did not alter plasma FZ and 7-AFZ kinetics in Sprague-Dawley rats infused with 40 mg/kg FZ over 30 min, whereas resulting in a three-fold increase in the area under the curve (AUC) of DMFZ concentrations compared with control (p < 0.01). In contrast, BUP did not significantly modify plasma DMFZ concentrations after intravenous infusion of 7 mg/kg DMFZ, whereas resulting in a similar peak concentration to that generated from 40 mg/kg FZ administration. Regarding the effects on ventilation, BUP (30 mg/kg) as well as its combination with FZ (0.3 mg/kg) significantly increased PaCO₂, whereas only BUP/FZ combination decreased PaO₂ (p < 0.001). Interestingly, FZ (40 mg/kg) but not DMFZ (40 mg/kg) significantly increased PaCO₂ (p < 0.05), whereas DMFZ but not FZ decreased PaO₂ (p < 0.05). Thus, decrease in PaO₂ appears related to BUP-mediated effects on DMFZ disposition, although increases in PaCO₂ relate to direct BUP/FZ additive or synergistic dynamic interactions. We conclude that combined high-dosage BUP and FZ is responsible for increased respiratory toxicity in which BUP-mediated alteration in DMFZ disposition may play a significant role.

Key Words: buprenorphine; desmethylflunitrazepam; flunitrazepam; interaction; metabolism; pharmacokinetics.

Buprenorphine (BUP), an hemisynthetic opioid, exerts agonist properties on μ opioid-receptors and antagonist properties on κ opioid-receptors, with an elevated affinity for both types of receptors (Cowan, 1995, 2003). BUP demonstrates a very slow dissociation from opioid-receptors resulting in a long duration of action. Interestingly, in contrast with morphine and methadone (Cowan et al., 1977; McCormick et al., 1984), a plateau of respiratory depressive effects has been consistently reported both in animals and humans (Cowan et al., 1977; Dahan et al., 2005, 2006; Nielsen and Taylor, 2005; Walsh et al., 1994).

Due to its duration of action and ceiling effects supporting its safety, high-dosage BUP has been marketed as a maintenance therapy for heroin addiction. Consequently, a reduction in lethal overdoses and improvement in patients’ social functioning has been observed (Emmanuelli and Desenclos, 2005). However, forensic studies have reported several fatalities involving BUP (Kintz, 2001; Pirnay et al., 2004a; Reynaud et al., 1998; Traquici et al., 1998). The observed asphyxial deaths were attributed to either BUP misuse consisting of i.v. self-injection of crushed tablets or concomitant intake of psychotropic drugs including benzodiazepines (BZDs) (Kintz, 2001; Lai et al., 2006; Pirnay et al., 2004a; Reynaud et al., 1998; Traquici et al., 1998). Consistent with the latter, BZDs have been detected in 91 among 117 fatalities with BUP intoxication (Kintz, 2001), suggesting a major role for BZDs in the occurrence of BUP-induced respiratory toxicity.

Widespread prescription of BZDs has been attributed to their wide therapeutic index and minimal adverse side effects. However, these drugs have been subject to increasing misuse and abuse (Garretty et al., 1997; Hojer et al., 1989; Verwey et al., 2000). While deaths involving BZD alone appear uncommon in the absence of underlying pathology (Buckley and McManus, 2004; Drummer et al., 1993), BUP/BZD deleterious interactions were hypothesized in BUP-associated fatalities. The likelihood of such a drug-drug interaction was further supported by blood BUP concentrations within the therapeutic range in the majority of deaths (Kintz, 2001; Lai et al., 2006; Pirnay et al., 2004a; Traquici et al., 1998). Although rarely detected due to rapid in vitro degradation,
Flunitrazepam (FZ) was suspected to be involved in a large number of both BUP nonfatal (Gueye et al., 2002b) as well as fatal intoxications (Druid et al., 2001; Kintz, 2001; Pirnay et al., 2004a). However, the mechanism of BUP/FZ toxicity including any interaction regarding FZ disposition has not been worked out in humans.

In rats, we previously showed that the combination of 40 mg/kg FZ and 30 mg/kg BUP induced the rapid onset of respiratory depression (Mégarbane et al., 2005a). However, the mechanism of BUP/FZ interaction remained poorly understood. We demonstrated that rat pretreatment with FZ altered neither plasma nor striatal BUP kinetics (Mégarbane et al., 2005a). Moreover, in vitro studies using rat as well as human liver microsomes predicted no significant metabolic BUP/FZ interaction in vivo (Ibrahim et al., 2000; Kilicaslan and Sellers, 2000; Umehara et al., 2002). However, to our knowledge, BUP effects on FZ pharmacokinetics have not been previously addressed in vivo. Because many BZDs have active metabolites, we hypothesized that BUP may alter in vivo the disposition of active FZ metabolites, thus increasing FZ toxicity. Therefore, we studied in the rat the respiratory effects of a BUP/FZ combination, investigating BUP effects on the kinetics of FZ and its main active metabolites, desmethyl-flunitrazepam (DMFZ), and 7-aminoflunitrazepam (7-AFZ). We choose to study elevated doses of both drugs in rats in order to mimic clinical exposures in drug addicts, as reported in the literature on severe or fatal poisonings (Kintz, 2001; Pirnay et al., 2004a) and help appropriate extrapolation from rats to humans.

MATERIALS AND METHODS

All experiments were carried out within the ethical guidelines established by the National Institute of Health and the French Minister of Agriculture.

Drugs

BUP was generously supplied by Schering-Plough, SA. It was subsequently dissolved in a mixture of sterile water (4.7 ml), absolute ethanol (400 µl), and hydrochloric acid 0.1 N (300 µl) at a concentration of 18.5 mg/ml. d6-BUP was purchased from Radian Inc. FZ was generously provided by Hoffman-La Roche, Inc. A FZ solution was prepared in propylene glycol at a concentration of 10 mg/ml. Both FZ metabolites, DMFZ and 7-AFZ, as well as d6-DMFZ were purchased from Radian Inc. A mixture of 99% N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) was purchased from Sigma Chemical Co.

Animals

Male Sprague-Dawley rats (Iffa-Credo, France) weighing 250–300 g at the time of experimentation were used. They were housed for 8 days before experimentation in a temperature- and light-controlled animal care unit. They were allowed food and water ad libitum until one day prior to experimentation. The day before the study, animals were anesthetized with i.p. 70 mg/kg ketamine (Ketalar, Parke Davis) and 10 mg/kg xylazine (Rompun, Bayer), then placed on a warming blanket with a regulating thermostat. A rectal probe permitted feedback temperature control. The femoral vein and artery were catheterized with 30-cm-long silastic tubes (Dow Corning Co, Midland, MI) with external and internal diameters of 0.94 and 0.51 mm, respectively (Gueye et al., 2001, 2002a). Arterial and venous catheters were then tunneled subcutaneously and fixed at the back of the neck. Heparinized saline was injected into each catheter to avoid thrombosis and catheter obstruction. Rats were then returned to their individual cages for a minimum 24-h recovery period to allow complete washout of anesthesia. On the experiment day, rats were placed in a restraining chamber (Harvard Apparatus). Drugs were administered into the venous catheter via an infusion pump (Harvard Instruments-PHD 2000). The final injected volumes were constant and adjusted with 0.9% NaCl to 1.3 ml. The arterial catheter allowed blood sampling for the kinetic and arterial blood-gases studies. Blood samples were obtained in restrained rats and collected in heparinized syringes.

Gas Chromatography–Mass Spectrometry

A general gas chromatography–mass spectrometry (GC-MS) method for detection and quantification of FZ, BUP, and their main metabolites was validated according to internationally accepted criteria (Shah et al., 2000). The method was set up and adapted for analysis of small rat plasma samples as previously reported (Pirnay et al., 2004b). Extraction involved a clean-up procedure using liquid-liquid Toxi-tubes A cartridges followed by derivatization with N,O-bis(trimethylsilyl)trifluoroacetamide, according to previously reported methods (Libong et al., 2003; Pirnay et al., 2002). The percentages of recovery were 71, 67, 51, and 81%, for BUP, FZ, 7-AFZ, and DMFZ, respectively, with coefficient of variation (CV) ranging from 5.4 to 13.9%. They were concentration independent.

All analyses were performed on a Hewlett Packard 5973 apparatus including a gas chromatograph coupled with a quadruple mass spectrometer and fitted with an autosampler. Chromatographic separation was carried out on a HP-5 MS capillary column (30 m × 0.25 mm I.D., 0.25 µm film thickness). All chromatographic and spectrometric parameters were established according to a previously reported validation (Pirnay et al., 2004b). Detection was operated under selected ion monitoring mode. The ions: m/z 285.1 for FZ, m/z 355.2 for 7-AFZ, m/z 370.1 for DMFZ, m/z 450.2 for BUP, m/z 375.1 for DMFZ-d4, m/z 454.3 for BUP-d4 were the most abundant and used for quantification (Pirnay et al., 2004b).

Limits of Quantification. The limits of quantification in plasma were 25 ng/ml for 7-AFZ and 125 ng/ml for DMFZ, FZ, and BUP. The CVs varied between 3.85 (BUP) and 13.45% (FZ); accuracies between 10.09 (BUP) and 14.54% (7-AFZ) (Pirnay et al., 2004b).

Linearity Range. Linearity was found in plasma between 125 and 25,000 ng/ml for BUP, 125 and 5000 ng/ml for DMFZ, 125 and 5000 ng/ml for FZ, and between 25 and 50,000 ng/ml for 7-AFZ. Coefficients of correlation (R²) were 0.999 for DMFZ, FZ, and BUP and 0.984 for 7-AFZ (Pirnay et al., 2004b). For all plasma concentrations above the upper limit of linearity range, plasma was diluted as required before quantification.

Intra- and Interassay Precision and Accuracy. Good reproducibility (intra-assay CV = 0.32–11.69%; interassay CV = 0.63–9.55%) and accuracy (intra-assay error = 2.58–12.73%; interassay error = 0.83–11.07%) were obtained (Pirnay et al., 2004b).

Sample Preparation. Eighty microliters of DMFZ-d4 and 80 µl of BUP-d4 at 1 µg/ml in acetonitrile and methanol, respectively, were added to 40 µl of plasma. The final volume was adjusted to 1 ml by addition of deionized water. The mixture was vortexed and transferred into a toxi-tube A (Ansyl Diagnostics, Inc.) which had been previously conditioned by 2 ml of deionized water. The tube was mechanically agitated before centrifugation for 5 min at 3000 rpm. The organic phase was transferred into a clean tube and evaporated to dryness under a nitrogen stream at 25°C. After complete evaporation of the solvent, the residue was heated at 80°C for 5 min in order to eliminate any trace of water. Forty microliters of BSTFA/TMCS (99:1%) were added to the residue for trimethylsilylation. The sample was then heated at 80°C for 20 min and cooled down to an ambient temperature prior to GC-MS analysis.

To separate free from bound plasma proteins by ultrafiltration, 200 µl of plasma were added on a Centrifree Micropartition Device (Millipore, Inc), with a cut of 30,000 Da. The cartridges were centrifuged for 30 min at 2000 rpm.
Eighty microliters of d4-DMFZ and 80 µl of d4-BUP at 1 µg/ml in acetonitrile and methanol, respectively, were added to 40 µl of the ultrafiltrate. The mixture was vortexed and transferred into a toxi-tube A (Ansys Diagnostics, Inc.) which had been previously conditioned with 2 ml of deionized water. The protocol as described above was used for the extraction and silylation prior to GC-MS analysis.

### Study Designs and Dosage Justification

#### Study 1. Study of plasma FZ kinetics at four dosages (3, 10, 30, and 40 mg/kg) in order to test its linearity
Catheterized rats were randomized into four groups of five animals. Rats received i.v. infusion of either 3, 10, 30, or 40 mg/kg FZ over 30 min. Plasma FZ kinetics were studied over 240 min. One-hundred microliters of blood samples was collected at −30, −25, −20, −15, −10, and at 0, 5, 10, 20, 30, 60, 120, and 240 min following FZ injection.

#### Study 2. Effect of 30 mg/kg BUP pretreatment on the time course of plasma FZ, DMFZ, and 7-AFZ concentrations after 40 mg/kg FZ infusion
Dosages were chosen in accordance with those previously used to describe the respiratory depressive effects of BUP/FZ combinations (Mégarbane et al., 2005a). These high doses of both molecules were reported to be deprived of significant effects on rat arterial blood gases in comparison to solvent. Catheterized rats were randomized into two groups of five animals. Rats in the control group received i.v. infusion of BUP solvent over 3 min followed by 40 mg/kg FZ i.v. over 30 min. Rats in the study group received 30 mg/kg BUP i.v. over 3 min followed by 40 mg/kg FZ i.v. over 30 min. Plasma kinetics of FZ and its metabolites DMFZ and 7-AFZ were studied over 180 min. One-hundred microliters of blood samples was collected before and after BUP (or solvent) infusion at −33 and −30 min, and during FZ infusion at −25, −20, −15, −10, and at 0, 5, 10, 20, 60, 120, and 180 min following FZ injection.

#### Study 3. Effect of 40 mg/kg FZ on the time course of plasma BUP concentrations after 30 mg/kg BUP administration
Catheterized rats were randomized into two groups of five animals. Rats in the control group received i.v. infusion of 30 mg/kg BUP over 3 min followed by FZ solvent i.v. over 30 min. Rats in the study group received 30 mg/kg BUP over 3 min followed by 40 mg/kg FZ i.v. over 30 min. Plasma kinetics of BUP were studied over 180 min. One-hundred microliters of blood samples was collected before and after BUP infusion at −33 and −30 min, and during FZ infusion at −25, −20, −15, −10, and at 0, 5, 10, 20, 60, 120, and 180 min following FZ (or solvent) injection.

#### Study 4. Effect of 30 mg/kg BUP pretreatment on the time course of plasma DMFZ after 7 mg/kg DMFZ infusion
The DMFZ dosage was chosen as it was estimated to give a similar peak concentration ($C_{\text{max}}$) to the one observed with 40 mg/kg FZ. Catheterized rats were randomized into two groups of five animals. Rats in the control group received i.v. infusion of BUP solvent over 3 min followed by 7 mg/kg DMFZ i.v. over 30 min. Rats in the study group received 30 mg/kg BUP over 3 min followed by 7 mg/kg DMFZ i.v. over 30 min. Plasma kinetics of total DMFZ were studied over 240 min. One-hundred microliters of blood samples was collected before and after BUP (or solvent) infusion at −33 and −30 min, and during DMFZ infusion at −25, −20, −15, −10, and at 0, 5, 10, 20, 30, 60, 120, 180, and 240 min following DMFZ injection.

#### Study 5. Effect of 30 mg/kg BUP pretreatment on the total and free plasma concentrations of FZ and DMFZ
Catheterized rats were randomized into two groups of five animals. Rats in the control group received i.v. infusion of BUP solvent over 3 min followed by 40 mg/kg FZ i.v. over 30 min. Rats in the study group received 30 mg/kg BUP over 3 min followed by 40 mg/kg FZ i.v. over 30 min. Plasma concentrations of total FZ and DMFZ as well as free FZ and DMFZ were studied during FZ infusion at −20 min and at the end of FZ infusion. Five-hundred microliters of blood samples was collected at each sampling point.

#### Study 6. Effect of 40 mg/kg FZ and 40 mg/kg DMFZ on arterial blood gases
We chose to test elevated dosages of both FZ and DMFZ in order to maximize their potential effects on ventilation. We used 40 mg/kg FZ, based on previous data showing that this dose administered i.p. induced a long-lasting coma without mortality (Borron et al., 2002). Catheterized rats were randomized into three groups of four animals. Rats in the control group received BUP i.v. solvent over 3 min, followed by FZ solvent i.v. over 30 min. Rats in the FZ group received BUP solvent i.v. over 3 min followed by 40 mg/kg FZ i.v. over 30 min. Rats in the DMFZ group received BUP solvent i.v. over 3 min, followed by 40 mg/kg DMFZ i.v. over 30 min. Three-hundred microliters of arterial blood samples was collected at 5, 20, 40, 60, 90, 120, and 180 min after FZ, DMFZ or solvent infusion. Blood gases were immediately measured using a Rapid Lab 248 (Bayer corporation, East Walpole, MA) blood-gas analyzer.

#### Study 7: Effect of 30 mg/kg BUP, 0.3 mg/kg FZ, and their combination on arterial blood gases
We chose a pharmacological range FZ dosage in the BUP/FZ combination in order to better substantiate the effects of kinetic drug-drug interaction. We chose 0.3 mg/kg FZ as previously shown to represent the median effective dose to significantly modify rat behavior in continuous conditioned avoidance tests without incapacitation (Randall and Kappell, 1973; Zbinden and Randall, 1967). Nevertheless, pharmacokinetics of FZ were not determined at this dosage, because plasma concentrations were anticipated to be lower than the limit of quantification of our GC-MS method. Catheterized rats were randomized into four groups of seven animals. Rats in the control group received BUP solvent i.v. over 3 min, followed by FZ solvent i.v. over 30 min. Rats in the BUP group received 30 mg/kg BUP i.v. over 3 min followed by FZ solvent i.v. over 30 min. Rats in the FZ group received BUP solvent i.v. over 3 min followed by 0.3 mg/kg FZ i.v. over 30 min. Rats in the BUP/FZ combination group received 30 mg/kg BUP i.v. over 3 min followed by 0.3 mg/kg FZ i.v. over 30 min. Three-hundred microliters of blood samples was collected at 5, 20, 40, 60, 90, 120, and 180 min after FZ or solvent infusion. Blood gases were immediately determined.

### Pharmacokinetic Analysis
Noncompartmental analysis (WinNonlin v 4.1, Pharsight Corp, Mountain View, CA) was used to calculate the terminal elimination half-lives in plasma, the AUC from 0 to infinity ($\text{AUC}_{0\rightarrow\infty}$), the total plasma clearance

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**Note.** Values represent mean ± SEM.

**TABLE 1**

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>$C_{\text{max}}$ (ng/ml)</th>
<th>AUC$_{0\rightarrow\infty}$ (ng/min/ml)</th>
<th>$C_l$ (ml/min/kg)</th>
<th>$V_s$ (ml/kg)</th>
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<tr>
<td>3</td>
<td>2771 ± 980</td>
<td>66,362 ± 15,994</td>
<td>48 ± 12</td>
<td>1370 ± 288</td>
</tr>
<tr>
<td>10</td>
<td>6366 ± 1604</td>
<td>238,916 ± 77,750</td>
<td>51 ± 21</td>
<td>1444 ± 540</td>
</tr>
<tr>
<td>30</td>
<td>20,163 ± 1980</td>
<td>799,860 ± 49,144</td>
<td>38 ± 3</td>
<td>1574 ± 144</td>
</tr>
<tr>
<td>40</td>
<td>28,091 ± 3676</td>
<td>1,137,756 ± 197,847</td>
<td>40 ± 6</td>
<td>1697 ± 295</td>
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**Noncompartmental analysis (WinNonlin v 4.1, Pharsight Corp, Mountain View, CA)** was used to calculate the $C_{\text{max}}$, the terminal elimination half-lives in plasma, the AUC from 0 to infinity ($\text{AUC}_{0\rightarrow\infty}$), the total plasma clearance.
Pharmacokinetic Studies (Studies 1–5)

We first demonstrated that FZ kinetics were linear in the 3–40 mg/kg dosage range as AUC/dosage did not significantly differ with respect to FZ dosage (Study 1, Table 1). This study showed that FZ kinetics were of first-order in this range, allowing an adequate assessment of BUP/FZ drug-drug interaction.

Then, we studied 40 mg/kg BUP effects on the time course of FZ, DMFZ, and 7-AFZ concentrations in plasma after 30 mg/kg i.v. FZ administration (Study 2, Fig. 1). None of the parameters of plasma FZ kinetics, including the C\text{max}, the peak time (T\text{max}), the AUC\text{0—\text{\infty}}, the Cl, and the V\text{SS} were significantly altered by BUP pretreatment (Fig. 1A; Table 2A). By contrast, significant differences were observed in the time course of plasma DMFZ concentrations (Fig. 1B; Table 2B). DMFZ C\text{max} was 5592 ± 1312 ng/ml in the BUP + FZ group versus 2708 ± 588 ng/ml in the FZ group (p = 0.03). Similarly, the AUC\text{0—\text{\infty}} values were significantly different (p = 0.009), whereas semilog representation of the terminal phases of DMFZ and FZ kinetics showed parallel apparent declines irrespective of BUP pretreatment (Fig. 2). In contrast to DMFZ, no significant alteration was observed in the time course of 7-AFZ concentrations in plasma following BUP pretreatment (Fig. 1C; Table 2B). In addition, FZ infusion did not alter BUP kinetics in plasma (Study 3, Fig. 3).

**RESULTS**

Pharmacokinetic Studies (Studies 1–5)

We first demonstrated that FZ kinetics were linear in the 3–40 mg/kg dosage range as AUC/dosage did not significantly differ with respect to FZ dosage (Study 1, Table 1). This study showed that FZ kinetics were of first-order in this range, allowing an adequate assessment of BUP/FZ drug-drug interaction.

Then, we studied 40 mg/kg BUP effects on the time course of FZ, DMFZ, and 7-AFZ concentrations in plasma after 30 mg/kg i.v. FZ administration (Study 2, Fig. 1). None of the parameters of plasma FZ kinetics, including the C\text{max}, the peak time (T\text{max}), the AUC\text{0—\text{\infty}}, the Cl, and the V\text{SS} were significantly altered by BUP pretreatment (Fig. 1A; Table 2A). By contrast, significant differences were observed in the time course of plasma DMFZ concentrations (Fig. 1B; Table 2B). DMFZ C\text{max} was 5592 ± 1312 ng/ml in the BUP + FZ group versus 2708 ± 588 ng/ml in the FZ group (p = 0.03). Similarly, the AUC\text{0—\text{\infty}} values were significantly different (p = 0.009), whereas semilog representation of the terminal phases of DMFZ and FZ kinetics showed parallel apparent declines irrespective of BUP pretreatment (Fig. 2). In contrast to DMFZ, no significant alteration was observed in the time course of 7-AFZ concentrations in plasma following BUP pretreatment (Fig. 1C; Table 2B). In addition, FZ infusion did not alter BUP kinetics in plasma (Study 3, Fig. 3).
To test the hypothesis of a direct interaction of BUP on DMFZ distribution in plasma, we studied the effects of 40 mg/kg BUP i.v. on DMFZ kinetics after 7 mg/kg DMFZ infusion (Study 4). The $C_{\text{max}}$ generated by 7 mg/kg DMFZ (2024 ± 204 ng/ml) was similar to that observed when 30 mg/kg FZ was given (2708 ± 588 ng/ml). The time course of DMFZ concentrations in plasma as well as the various parameters of plasma DMFZ kinetics were not significantly altered by BUP pretreatment (Fig. 4, Table 3). Semilog representation of the terminal phases of DMFZ kinetics showed parallel apparent declines whether DMFZ was directly administered or resulted from FZ administration (Fig. 2). DMFZ elimination half-lives were not significantly different in either situations, that is, in the absence (62 ± 14 vs. 41 ± 7 min) or presence of BUP (56 ± 9 vs. 64 ± 22 min). In addition, to test the hypothesis of BUP effect on FZ or DMFZ protein binding in plasma, we measured the free/total ratios of FZ and DMFZ in rats treated with 40 mg/kg FZ i.v. (Study 5). There was no significant alteration of these ratios in relation to 30 mg/kg BUP i.v. pretreatment (Table 4).

### Pharmacodynamic Studies (Studies 6 and 7)

We studied the effects on arterial blood gases of 40 mg/kg FZ and 40 mg/kg DMFZ in comparison to FZ solvent (Study 6). Arterial pH did not significantly differ between the three groups (Fig. 5A). PaCO$_2$ was significantly more elevated in the FZ than in DMFZ or solvent treated rats ($p = 0.03$, Fig. 5B).

### Table 2

<table>
<thead>
<tr>
<th>(A) FZ</th>
<th>Half-life (min)</th>
<th>$C_{\text{max}}$ (ng/ml)</th>
<th>$\text{AUC}_{0-\infty}$ (ng/min/ml)</th>
<th>$\text{Cl}_f$ (ml/min/kg)</th>
<th>$V_{ss}$ (ml/kg)</th>
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<tr>
<td>FZ group</td>
<td>48 ± 6</td>
<td>26,188 ± 6117</td>
<td>1,053,796 ± 327,025</td>
<td>43 ± 10</td>
<td>1802 ± 496</td>
</tr>
<tr>
<td>BUP + FZ group</td>
<td>45 ± 6</td>
<td>34,824 ± 13,739</td>
<td>1,279,224 ± 517,192</td>
<td>37 ± 13</td>
<td>1539 ± 824</td>
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**FIG. 2.** Log $y$ axis scale representation of the time courses of plasma FZ (open circles) and DMFZ (open squares) concentrations after rat treatment with either BUP solvent + 40 mg/kg FZ (A) or 30 mg/kg BUP + 40 mg/kg FZ (B). Time courses of plasma DMFZ (filled squares) after treatment with either BUP solvent + 7 mg/kg DMFZ (A) or 30 mg/kg BUP + 40 mg/kg DMFZ (B) were added. In each group, five rats were used. Values represent mean ± SEM at each time.

**FIG. 3.** Kinetics of BUP in plasma after rat treatment with either 30 mg/kg BUP + 40 mg/kg FZ (filled squares) or 30 mg/kg BUP + FZ solvent (open squares). In each group, five rats were used. Values represent mean ± SEM at each time. No significant differences were shown using Mann-Whitney tests.
Interestingly, there was no significant difference in PaO₂ between the FZ and solvent treated rats, whereas PaO₂ was significantly lower with DMFZ treatment in comparison to the solvent (p = 0.03, Fig. 5C). The lowest PaO₂ value was observed in the DMFZ group at 20 min and significantly differed from the solvent (9.36 ± 0.23 vs. 12.58 ± 0.28 kPa, p = 0.03).

Then, we investigated the effects on arterial blood gases of 30 mg/kg BUP as well as the combination of 30 mg/kg BUP + 0.3 mg/kg FZ significantly increased PaCO₂ in comparison with the solvents (p = 0.03 and 0.001, respectively). The highest PaCO₂ value was observed in the combination group at the end of FZ infusion and significantly differed from the solvents (7.17 ± 0.14 vs. 5.56 ± 0.19 kPa, p = 0.001).

PaO₂ did not significantly differ between 30 mg/kg BUP, 0.3 mg/kg FZ, and the solvent groups, whereas PaO₂ was significantly lower in the BUP/FZ combination group (p = 0.002) (Fig. 6C). The lowest PaO₂ value was observed in the combination group at 20 min and significantly differed from the solvents (8.68 ± 0.29 vs. 10.38 ± 0.45 kPa, p = 0.004).

**DISCUSSION**

In this study, we investigated the respiratory effects of a BUP/FZ combination and demonstrated the existence of a significant BUP-mediated alteration of DMFZ disposition that may have contributed to increase FZ toxicity. To our knowledge, we report here for the first time plasma kinetics of FZ and its major metabolites in rat at a toxic dosage (40 mg/kg). Previous studies described kinetics after intravenous administration of 1 mg/kg (Becherucci et al., 1985) and 2.5 mg/kg (Mandema et al., 1991) FZ. We demonstrated that FZ kinetics were linear in the 3–40 mg/kg dosage range obviating any saturation in distribution or elimination processes. We found comparable kinetic parameters with those published at 2.5 mg/kg (half-life: 76 ± 10 min; V₅₀: 3800 ± 300 ml/kg; Cl: 80 ± 5 ml/min/kg) (Mandema et al., 1991). Thus, our experimental conditions were considered as favorable to allow pertinent analysis of FZ pharmacokinetic/pharmacodynamic relationships.

We first studied the respiratory effects of an elevated BUP dosage (30 mg/kg). As previously demonstrated (Gueye et al., 2001, 2002a; Ohtani et al., 1997), we found only a mild and transient effect on PaCO₂ with no significant consequence on pH or PaO₂ in comparison with the solvent. Similarly, we showed that 40 but not 0.3 mg/kg FZ increased PaCO₂, whereas neither dosage resulted in significant changes in arterial pH and PaO₂. BZD effects on ventilation are known to be rather limited. Consistently, a massive dose of i.p. midalozam (160 mg/kg) reproducibly induced deep coma and moderate respiratory acidosis, without significant change in PaO₂ (Gueye et al., 2002a; Mégarbane et al., 2005b). Moreover, even an increase in PaO₂ has been reported following i.p. administration of 20 mg/kg diazepam (McCormick et al., 1984) or subcutaneous administration of 10 mg/kg chloralazine (Verborgh et al., 1998).

In stark contrast to the limited effects on resting ventilation resulting from separate administration of BUP or BZDs, the combination of both drugs has been reported to induce significant toxicity. Pretreatment with 40 mg/kg FZ induced a sixfold decrease in the BUP median lethal dose and significantly prolonged time to death in BUP-treated animals (Borron et al., 2002). In this study, BUP/FZ combination

**TABLE 3**

<table>
<thead>
<tr>
<th>Group</th>
<th>Half-life (min)</th>
<th>Cₘₛ (ng/ml)</th>
<th>AUC₀→∞ (ng/min/ml)</th>
<th>Clᵢ (ml/min/kg)</th>
<th>Vᵢₘ (ml/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMFZ group</td>
<td>62 ± 14</td>
<td>2024 ± 204</td>
<td>53,085 ± 9844</td>
<td>144 ± 23</td>
<td>3995 ± 1315</td>
</tr>
<tr>
<td>BUP + DMFZ group</td>
<td>56 ± 9</td>
<td>2431 ± 197</td>
<td>66,099 ± 5036</td>
<td>108 ± 8</td>
<td>4053 ± 451</td>
</tr>
</tbody>
</table>

*Note. Values represent mean ± SEM.*
induced hypoxia and hypercapnia, whereas 0.3 mg/kg FZ alone induced neither hypercapnia nor hypoxia and 40 mg/kg FZ induced only significant hypercapnia.

Interestingly, we observed that 40 mg/kg DMFZ, the major FZ metabolite induced hypoxia like the BUP/FZ combination, whereas FZ did not. Thus, whereas the BZD relationship to opioids in augmenting respiratory depression has been qualified as simply additive (Henry, 1999), experimental data suggest that the BUP/BZD interaction appears more complex than previously believed, enhancing the interest of mechanistic studies.

Drug-drug interactions may result from either a pharmacokinetic or a pharmacodynamic process. Regarding BUP/FZ kinetic interactions, previous in vitro studies performed on rat as well as human microsome preparations predicted the absence of in vivo metabolic interactions between both drugs at therapeutic concentrations (Ibrahim et al., 2000; Kilicarslan and Sellers, 2000; Umehara et al., 2002). In addition, we previously demonstrated that rat pretreatment with FZ alter neither plasma nor striatal BUP distribution (Mégarbane et al., 2005a). Here we confirmed that 30 mg/kg BUP had no significant impact on plasma FZ kinetics nor did 40 mg/kg FZ have an impact on plasma BUP kinetics. In contrast, we clearly showed that BUP pretreatment had a significant impact on the time course of DMFZ concentrations, tripling its AUC0→∞ in comparison to control (p = 0.009). Moreover, although DMFZ Cmax values were equivalent whether 40 mg/kg FZ (2708 ± 588 ng/ml) or 7 mg/kg DMFZ (2024 ± 204 ng/ml) was administered to rats in the absence of BUP, DMFZ Cmax was significantly higher in the presence of 30 mg/kg BUP, when 40 mg/kg FZ (5592 ± 1312 ng/ml) rather than 7 mg/kg DMFZ (2431 ± 197 ng/ml) was administered (p = 0.009, Tables 2B and 3). The significant increase in PaCO2 with BUP/FZ combination in comparison to FZ did not appear to be related to BUP-mediated increase in DMFZ AUC0→∞ as DMFZ was not responsible of any alteration in PaCO2. This observation thus suggested that effect of BUP/FZ combination on PaCO2 may be the consequence of additive or synergic direct pharmacodynamic interactions of high dosages of both drugs. In contrast, as DMFZ induced a significant decrease in PaO2, the decrease in PaO2 with BUP/FZ combination in comparison to FZ or BUP may be attributed at least in part to a BUP-mediated increase in DMFZ AUC0→∞. This effect was more clearly established in this study at pharmacological (0.3 mg/kg) than in our previous study at toxic dosage (40 mg/kg) of FZ (Mégarbane et al., 2005a), supporting the hypothesis of a possible BUP/FZ kinetic interaction evidenced at a lower dosage.

We considered various possible mechanisms of BUP/FZ kinetic interaction that may result in the increase of DMFZ concentrations. From a theoretical point of view, BUP effects

### Table 4

Comparison of the Free/Total Ratios of Plasma FZ (A) and DMFZ (B) in Rats Treated with either 30 mg/kg BUP + 40 mg/kg FZ (BUP + FZ Group) or BUP Solvent + 40 mg/kg FZ (FZ Group)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BUP + FZ group</th>
<th>FZ group</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Free/total ratios of plasma FZ concentrations (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-20</td>
<td>22.5 ± 1.5</td>
<td>16.9 ± 2.8</td>
<td>0.13</td>
</tr>
<tr>
<td>T0</td>
<td>20.5 ± 1.4</td>
<td>26.9 ± 5.6</td>
<td>0.94</td>
</tr>
<tr>
<td>(B) Free/total ratios of plasma DMFZ concentrations (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-20</td>
<td>29.4 ± 4.2</td>
<td>29.7 ± 2.9</td>
<td>0.66</td>
</tr>
<tr>
<td>T0</td>
<td>21.4 ± 1.6</td>
<td>21.5 ± 4.0</td>
<td>1</td>
</tr>
</tbody>
</table>

Note. Measurements were performed at two times: 20 min after the initiation of FZ infusion (T-20) and at the end of FZ infusion (T0). Values represent mean ± SEM. No significant differences were shown using Mann-Whitney tests.
on DMFZ disposition may be the consequence of either a formation-dependent or an elimination-dependent process. BUP did not appear to interact with DMFZ elimination process as there was no significant increase in DMFZ elimination half-life when DMFZ was directly administered to the rats. Interestingly, we observed parallel terminal slopes of both FZ and DMFZ in plasma, irrespective of BUP administration (Fig. 2). Moreover, we did not observe any significant BUP effect on FZ or on DMFZ free concentrations in plasma (Table 4). FZ binds to human serum albumin and to alpha 1-acid glycoprotein (Maruyama et al., 1992). Heel et al. (1979) reported unpublished human data from Reckitt & Colman, indicating that BUP is highly protein-bound (95–98%), primarily to alpha and beta globulin fractions. However, to our knowledge, FZ and BUP protein binding has never been described in rats.

On the other hand, as we used a single BUP dose and as BUP effect on FZ kinetics was rapidly observed as soon as 10 min after the start of FZ infusion, we assumed that we were unable to assess a formation-dependent mechanism such as an enzymatic induction. However, using the same calculation previously performed in humans (Drouet-Coassolo et al., 1990), we showed that the DMFZ $AUC_{0-\infty}/FZ\ AUC_{0-\infty}$ ratio named “metabolism index” was significantly increased in BUP-pretreated rats (41%) in comparison to controls (15%).

In humans, both cytochrome (CYP)3A4 and 2C19 are involved in the metabolic pathways of FZ to DMFZ and to the third FZ metabolite, 3-OHFZ (Coller et al., 1998; Hesse et al., 2001). Although not firmly established, CYP2C19 appears to be the primary route for DMFZ formation, whereas CYP3A4 is the primary route for 3-OHFZ formation (Kilicarslan et al., 2001). In contrast in rats, the metabolic pathways of FZ are unknown, rendering it impossible to establish any definitive hypothesis. However, we showed that administration of massive doses of BUP resulted in significant DMFZ production while not altering FZ and 7-AF concentrations, thus raising the question of a possible substrate competition for FZ metabolism, downshifting the route producing DMFZ relative to the routes producing other metabolites, including 3-OHFZ. This hypothesis may be supported by in vitro studies on human microsomes showing significant inhibition of CYP3A4-mediated pathways of 3-OHFZ (Ibrahim et al., 2000; Kilicarslan and Sellers, 2000; Umehara et al., 2002). However, apparent $K_{i}$ values of CYP3A4-mediated 3-OHFZ formation were shown to be at least 120 times more elevated than the expected plasma BUP concentrations in treated patients (Umehara et al., 2002).

Thus, this kinetic mechanism of BUP/FZ interaction was considered as clinically nonsignificant. Projected in vivo inhibition of CYP3A4-mediated FZ metabolism by BUP was even estimated at 0.1–2.5% under pharmacological conditions (Kilicarslan and Sellers, 2000). Our results were consistent with these conclusions, as plasma BUP concentrations ($C_{max}$: $8360 \pm 2553 \text{ ng/ml} = 17.9 \pm 5.5 \mu\text{mol/l}$) was significantly lower than the reported concentration (>150 $\mu$mol/l) required for a 50% reduction in CYP3A activity in rat liver microsomes (Ibrahim et al., 2000). Furthermore, under the hypothesis of BUP inhibition of CYP3A-mediated pathway of FZ metabolism, an increase in FZ concentrations would be expected, contrasting with our observations. Thus, the exact mechanism of BUP-mediated increase of DMFZ concentrations would be expected, contrasting with our observations. This, the exact mechanism of BUP-mediated increase of DMFZ concentrations in vivo remains to be determined. However, based on our results, we may clearly question the supposition that FZ/BUP interaction is unrelated to a pharmacokinetic mechanism in the situation of drug abusers with combination of elevated doses of both drugs, resulting in severe or even fatal poisonings (Kintz, 2001;
Pirnay et al., 2004a; Reynaud et al., 1998; Tracqui et al., 1998). To our best knowledge, no study including FZ and DMFZ kinetics was performed in such situation in humans. Acute and prolonged multidrug exposures in drug addicts make drug-drug kinetic interactions highly plausible. It would be of interest, based on our data, to investigate BUP alteration of DMFZ disposition in patients with acute respiratory depression resulting from self coadministration of BUP and FZ.

One important limitation of this work was that 3-OHFZ, including its possible contribution to toxicity, was not studied. However, it is generally believed that the parent drug FZ is principally responsible for the sleep-inducing effect, whereas the relative activities of metabolites are unclear (Kilicarslan et al., 2001). The pharmacological activity of 3-OHFZ has not been established to our knowledge, whereas it has been suggested that DMFZ is active (Berthault et al., 1996) and that 7-ÅFZ can have anesthetic activity in animals (Korttila and Linnoila, 1976). Thus we chose to limit our study to the main active metabolites of FZ.

In conclusion, our study showed that BUP/FZ combination induced significant toxic effects on rat ventilation. Pretreatment with BUP had no effects on FZ disposition while inducing a three-fold increase in plasma DMFZ concentrations. When analyzing the respiratory effects of this BUP/FZ combination, the decrease in PaO2 appears to be rather related to BUP-mediated effects on DMFZ disposition, whereas increase in PaCO2 to a direct BUP/FZ additive or synergic dynamic interaction. However, the exact mechanisms of each of these drug/drug interactions remain to be determined.

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