IN VITRO METABOLISM STUDY OF BUPRENORPHINE: EVIDENCE FOR NEW METABOLIC PATHWAYS

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ABSTRACT:

Buprenorphine (BUP) is a synthetic derivative of the morphine alkaloid thebaine. BUP is metabolized by N-dealkylation to form the active metabolite nor-buprenorphine (Nor-BUP), and both undergo subsequent glucuronidation. Although BUP has been used clinically for years, its metabolism has still not been fully elucidated. The aim of this study was to clarify the identity of the human hepatic cytochromes P450 (P450s) involved in BUP metabolism and to investigate other potential metabolites. The metabolism of BUP was examined using human liver microsomes (HLM) and Ad293 P450-transfected cell lines, as well as CYP 3A4 and 2C8 recombinant isofoms. The kinetic parameters of metabolite formation were calculated for HLM and competent isoforms. Individual contribution of P450 isoforms in BUP metabolism as well as Nor-BUP production was evaluated using chemical inhibition experiments, as well as the relative activity factor approach. The analytical method used was based on liquid chromatography-mass spectrometry. Among the 13 P450 isoforms tested, CYP 3A4, 2C8, 3A5, and 3A7 produced Nor-BUP. Based on the results of chemical inhibition, CYP 3A4 accounts for about 65% of Nor-BUP production and CYP 2C8 for about 30%. BUP utilization by either HLM or P450-transfected cells revealed that another oxidative metabolic pathway exists, which was found to involve CYP 2C9, 2C18, 2C19, and mainly CYP 3A. Incubation of BUP or Nor-BUP with HLM led to the formation of new metabolites, identified by tandem mass spectrometry as being hydroxy-BUP and hydroxy-Nor-BUP. Hydroxy-BUP was produced by the CYP 3A, but not the 2C isoforms.

Buprenorphine (BUP) is a synthetic derivative of the morphine alkaloid thebaine with partial agonist properties on the μ-opioid receptor. BUP has been used for almost two decades as an analgesic at doses ranging from 0.3 to 0.6 mg for the treatment of moderate to severe pain by intramuscular, intravenous, and sublingual routes. High-dose BUP sublingual tablets (0.4 mg, 2 mg, and 8 mg) were released in France in February 1996 as a replacement therapy for heroin addicts. Since 1996, several fatalities seemingly linked with BUP intake (cases showing common features in which no other causes of death were found) were reported by French toxicologists (Tracqui et al., 1998; Kintz 2001). In most cases, a concomitant intake of psychotropic drugs, especially benzodiazepines, was observed. It is not clear whether the mechanism of this probable drug-drug interaction is of pharmacokinetic or pharmacodynamic nature, and whether it involves BUP metabolism or not. Only a brief report described an in vitro interaction study between flunitrazepam and BUP; the authors found no effect of BUP at typical plasma concentrations on flunitrazepam metabolism (Kilicarslan and Sellers, 2000). Recently, a study investigated the potential interaction of benzodiazepines on BUP metabolism (Chang and Moody, 2005): only midazolam was found to cause a moderate inhibition of BUP elimination and nor-buprenorphine (Nor-BUP) production by human liver microsomes. Further studies would require better understanding of BUP metabolism. BUP is metabolized by N-dealkylation of its cyclopropyl group to form Nor-BUP, an active metabolite (Huang et al., 2001), and both BUP and Nor-BUP undergo subsequent glucuronidation (Cone et al., 1984). Some of the first reports on BUP metabolism described the existence of additional polar metabolites in rats (Brewster et al., 1981; Fontani et al., 1985), which have remained unidentified. Human P450 P450 3A4 was demonstrated to be the major isozyme involved in Nor-BUP formation in two reports (Irizarie et al., 1997; Kobayashi et al., 1998), but in both studies, a large part of Nor-BUP formation (about 25%) could not be attributed to CYP 3A4. Moreover, in the study by Moody et al. (2002), Nor-BUP formation was detected when BUP was incubated with CYP 2C8, 3A5, and 3A7 isoforms, and BUP metabolism (without Nor-BUP production) by other P450s was suggested. This was the first study on BUP metabolism conducted using near-therapeutic concentrations of BUP, and it pointed out the need for sensitive analytical methods for both in vivo and in vitro metabolic studies on BUP. BUP is indeed characterized by a weak oral bioavailability and low therapeutic plasma concentrations. Kuhlman et al. (1996) reported average peak plasma BUP concentrations of 3.31 ng/ml (range 1.93–7.19 ng/ml) in six subjects

ABBREVIATIONS: BUP, buprenorphine; Nor-BUP, nor-buprenorphine; OH-BUP, hydroxy-buprenorphine; OH-Nor-BUP, hydroxy-nor-buprenorphine; HLM, human liver microsomes; P450, cytochrome P450; LC-MS/MS, liquid chromatography-tandem mass spectrometry; RAF, relative activity factor; CLint, intrinsic clearance; DMSO, dimethyl sulfoxide; SIM, selective ion monitoring; EPI, enhanced product ion.
who received a single dose of 4 mg sublingually. In our experience, the steady-state 24-h serum trough level of BUP in patients given 4 to 16 mg/day sublingually ranges from 1 to 10 ng/ml.

The aim of the present study was to identify all the hepatic P450 isoforms involved in BUP metabolism, as well as to clarify the metabolic pathways for BUP and Nor-BUP, and describe other potential metabolites using complementary approaches and sensitive analytical tools.

Materials and Methods

Materials. BUP hydrochloride, NADPH, dimethyl sulfoxide, ketoconazole, and type 2 β-glucuronidase (EC 3.2.1.31) were obtained from Sigma-Aldrich (St. Louis, MO). Trimethoprim was obtained from GlaxoSmithKline (Valbonne, France). Pooled HLM as well as microsomes prepared from baculovirus-infected insect cells (Supersomes) that expressed the human CYP 3A4 and 2C8, and control microsomes were purchased from BD Gentest (Woburn, MA).

P450-Transfected Cells. Human embryonic kidney Ad293 cells were stably transfected with cDNA of the human CYP 1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 3A1, 3A4, 3A5, or 3A7 as previously described (Lacroix et al., 1997; Treluyer et al., 1997; Sonnier and Cresteil, 1998). The level of expression of P450 proteins was roughly equivalent, as demonstrated by immunoblots of cellular microsomes with appropriate antibodies and calibration with baculovirus-insect cell-expressed P450 (BD Gentest) and measurement of relevant monooxygenase activities using fluorescent substrates (methoxy-, ethoxy-, or benzyloxy-trifluoromethylcoumarin or cyanoethoxy-coumarin). All three 3A4-, 3A5-, and 3A7-expressing cell lines were cotransfected with the cDNA encoding the human NADPH-cytochrome P450 reductase as reported previously by one of us (Santos et al., 2000; Treluyer et al., 2001) and others (Ding et al., 1997). This results in a significant increased biotransformation rate for CYP 3A4 and 3A5, but has no effect on the catalytic activity of other P450 proteins. Cells were cultured at 37°C in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. Flasks (75-cm²) containing cells at near confluence were incubated with 20 μM BUP for 24 h. Control incubations were performed with no cells and with native Ad293 cells. The resulting culture medium was removed and stored at −80°C until analysis.

Incubation Procedures with Microsomes or Recombinant P450. In preliminary experiments, linearity of Nor-BUP formation was checked at 0.2 μM BUP using a range of P450 contents (0.1−1 mg/ml), and incubation time (10−60 min). The standard incubation mixture (500 μl) consisted of 0.1 M Tris-HCl buffer pH 7.4, 10 mM MgCl₂, 1 mM NADPH, HLM (0.2 mg protein/ml), CYP 3A4 (10 pmol/ml), or CYP 2C8 (25 pmol/ml) and BUP (0.2−50 μM) or Nor-BUP (0.2 μM). Substrates and microsomes were preincubated at 37°C for 5 min before starting the reaction by adding NADPH. Incubations were stopped after 15 min at 37°C by adding 10 μl of 24% v/v perchloric acid. Control incubations without microsomes, without NADPH, or without substrates were performed. All the incubations were conducted in duplicates.

Enzyme Kinetics and Relative Contribution of P450 Isoforms to BUP Metabolism. The results obtained with recombinant P450s were scaled to HLM using the previously described relative activity factor (RAF) approach (Crespi and Miller 1999; Venkatakrishnan et al., 2001). RAF values for CYP 3A4 (0.284) and CYP 2C8 (0.120) were obtained from BD Gentest. The scaled Vmax/Km ratio.

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Results were model-fitted using the Michaelis-Menten equation and the kinetic parameters calculated by nonlinear regression analysis using Winfit 3.1 (available online: http://www.unilim.fr/pages_perso/jean.debord/winreg/winnreg1.htm, accessed December 1, 2004). The intrinsic clearance (Clint) was estimated by the Vmax/Km ratio.

Following the RAF approach, the relative contribution (RCi) of CYP 3A4 and CYP 2C8 to BUP metabolism was estimated using eq. 1.

\[ \text{RCi} = \frac{V_i}{\sum V_j} \]

Chemical Inhibition Experiments. Coincubation of BUP (0.2 μM) with increasing concentrations of the selective CYP 3A4 inhibitor ketoconazole (0.25−1.5 μM) or the selective CYP 2C8 inhibitor trimethoprim (10−100 μM) (Wen et al., 2002) were performed following the same incubation protocol, except that 50 μl of the inhibitor working solution were preincubated with BUP and microsomes before starting the reaction. Stock solutions of inhibitors were prepared in DMSO and added to the incubation media so that the final amount of DMSO was less than 0.1%. Control incubations were spiked with the same amount of DMSO. BUP and Nor-BUP concentrations were measured in the incubation medium after the reaction had been stopped, and the percentage inhibition of BUP metabolism, as well as of Nor-BUP production, was calculated with respect to the control.

Urine Samples. BUP metabolites were investigated in urine samples from two patients given high-dose BUP as opiate replacement therapy. These investigations are strictly in accordance with French laws. Urine samples were analyzed before and after incubation with β-glucuronidase (1200 IU/ml) in 1 M acetate buffer (pH 4.8) during 2 h at 56°C.

Analytical Methods. Identification Experiments. Incubation supernatants, culture media, and urine samples were extracted using 60-mg Oasis MCX solid-phase extraction cartridges (Waters, Milford, MA). Samples diluted half with water were loaded on the cartridge, which had been previously activated with 1 ml of methanol followed by 1 ml of H₂O. The cartridge was rinsed with 1 ml of 0.1 N HCl. BUP and metabolites were then eluted with 1 ml of methanol containing 2% ammonium hydroxide. The extracts were evaporated to dryness and reconstituted in 50 μl of acetonitrile/2 mM ammonium formate, pH 3.0 (30:70 v/v).

For metabolite identification, a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was used. Extract (5 μl) was injected on an X-Terra MS C18, 3.5-μm reversed-phase column (Waters). The mobile phase, delivered at a constant flow rate of 50 μl/min, consisted of a gradient of solution A (2 mM ammonium formate, pH 3.0/acetoniitrile, 10:90 v/v) in solution B (2 mM ammonium formate, pH 3.0), as follows: 0 to 2 min, 5% A; 20 min, 90% A; 21 min, 90% A; 21 to 23 min, 5% A; 23 min, 5% A. Detection was performed using a QTRAP tandem mass spectrometer with linear ion-trapping capabilities in the third quadrupole (Applied Biosystems/MDS Sciex, Foster City, CA). A Turbo-Ionspray source was used in the positive ionization mode with a spray voltage of 4500 V. Ion fragmentation in the collision cell involved variable collision energy, depending on the compounds (between 35 and 50 V), and nitrogen as collision gas. Screening of BUP metabolites was performed in two steps. First, acquisitions were made in the single-quadrupole, full-scan mode as well as in the single-quadrupole selective ion monitoring (SIM) mode using the hypothetical m/z ratios of the most probable metabolites. Second, the nature of the metabolites detected was confirmed using the enhanced product ion (EPI) scan mode, in which parent ions are selected in the first quadrupole, fragmented in the collision cell, and the resulting fragments accumulated in the linear ion trap before being scanned.

BUP and Nor-BUP Determination. BUP and Nor-BUP were quantitated by LC-MS using an API 100 single quadrupole mass spectrometer, according to a previously described method (Hoja et al., 1997) used routinely in the laboratory, with minor modifications. Indeed, BUP and Nor-BUP are very difficult to fragment, so that very low analytical sensitivity is obtained in the MS/MS mode. Briefly, seven calibrators (from 0.5 to 100 ng/ml BUP or Nor-BUP) were prepared in water, and incubation samples were diluted (1:2 to 1:40) with water. The determination procedure involved solid-phase extraction with Extrelut-3 cartridges (Merck, Darmstadt, Germany), chromatographic separation using the column and mobile phase described above, fragmentation-induced dissociation in the pneumatically assisted electrospray source, and acquisition in the positive ion mode using m/z 468 and 396 for BUP and m/z 414 and 396 for Nor-BUP. All samples were analyzed in duplicate, in separate runs. Internal quality controls at 1 and 50 ng/ml in water, prepared from different stock solutions by a different technician, were analyzed with each run.
Results

Characterization of BUP Metabolic Pathways Using P450-Transfected Cell Lines. Incubation of BUP with Ad cells transfected with CYP 1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 3A4, 3A5, and 3A7 showed that only CYP 3A4, 2C8, and, to a lesser extent, 3A5 and 3A7 were able to produce Nor-BUP in a significant amount (Fig. 1). The amount of Nor-BUP produced by CYP 2C8 was almost similar to the amount of BUP metabolized by this isoform. In contrast, the amount of BUP metabolized by the CYP 3A isoforms largely exceeded the amount of Nor-BUP produced (Fig. 1), and there was an important BUP utilization without any Nor-BUP production with CYP 2C9, 2C18, and 2C19.

Nor-BUP Production by Human Liver Microsomes. Pooled HLM produced Nor-BUP with an apparent $K_m$ of 15.0 ± 3.4 μM and a $V_{max}$ of $1224.7 ± 110.0$ pmol/mg protein/min (Fig. 2). The amount of Nor-BUP produced after 15 min of incubation with HLM (0.2 mg/ml; $n = 3$) accounted for only $36.3 ± 3.1\%$ of the amount of BUP metabolized, confirming the existence of parallel or subsequent metabolic pathways.

Kinetics of Nor-BUP Production by CYP 3A4 and CYP 2C8. Nor-BUP production kinetic constants were calculated using recombinant CYP 3A4 and CYP 2C8. Velocity values were scaled to HLM using the RAF approach. Although CYP 3A4 and 2C8 have similar affinity for BUP, CYP 3A4 produced Nor-BUP more efficiently, as demonstrated by a 2.7-fold higher scaled CLint (Table 1).

Individual Contribution of P450 Isoforms to BUP Metabolism. Chemical Inhibition. The respective contribution of CYP 3A4 and 2C8 to Nor-BUP production as well as to BUP metabolism was evaluated using selective chemical inhibitors. Ketoconazole (0.25–1.5 μM) decreased Nor-BUP formation in a concentration-dependent manner up to 1.5 μM (corresponding to approximately 65% inhibition with respect to the control). Trimethoprim, described as a selective inhibitor of CYP 2C8 between 5 and 100 μM (Wen et al., 2002), also inhibited Nor-BUP formation in a concentration-dependent manner between 10 and 100 μM (maximum inhibition, 30.8%) (Table 2).

The effect of ketoconazole at each tested concentration on BUP consumption (with respect to the control experiment without microsomes, where no BUP was metabolized) was greater than inhibition of Nor-BUP production. At the highest concentration tested (1.5 μM), ketoconazole inhibited BUP consumption by 88.5%. The maximal inhibition of BUP consumption by trimethoprim was low, approximately 5% (Table 2).

RAF Approach. The relative contribution of microsomal CYP 3A4 and 2C8 to Nor-BUP production was confirmed by means of the enzyme kinetic parameters estimated using recombinant human P450 and the RAF approach. According to this approach, CYP 3A4 was found to play the main role in Nor-BUP production with a constant contribution of 73%, independent of BUP concentrations in the range 0.2 to 50 μM.

![Graph](image1.png)

![Graph](image2.png)

**TABLE 1**

<table>
<thead>
<tr>
<th>Isoform</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (pmol/min/mg protein)</th>
<th>Predicted CLint (μl/min/mg protein)</th>
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<tr>
<td>CYP 3A4</td>
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<td>520.0 ± 45.3</td>
<td>38.3</td>
</tr>
<tr>
<td>CYP 2C8</td>
<td>12.4 ± 1.5</td>
<td>176.3 ± 8.4</td>
<td>14.2</td>
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</table>
Characterization of New Oxidative Pathways in BUP Metabolism. Analyzing by LC-MS/MS both supernatants from in vitro incubation with HLM and urine samples from treated patients, hydroxylated metabolites of BUP and Nor-BUP could be identified. HLM produced hydroxy-buprenorphine (OH-BUP) when incubated with BUP and hydroxy-nor-buprenorphine (OH-Nor-BUP) when incubated with Nor-BUP (Fig. 3). OH-BUP and OH-Nor-BUP showed similar fragmentation patterns. [OH-BUP + H]+ (m/z 484) produced [OH-Nor-BUP + H]^+ (m/z 430) when fragmented. Both species produced a characteristic fragment at m/z 412 resulting from dehydration of [OH-Nor-BUP + H]^+ and corresponding to the hydroxylated form of a fragment ion of both BUP and Nor-BUP (m/z 396) (Hoja et al., 1997; Moody et al., 2002). The mass spectra of OH-BUP and OH-Nor-BUP did not allow clear assignment of the site of hydroxylation but suggest that it might occur on the phenyl-ring (Fig. 4). The amounts of hydroxylated metabolites produced after incubation of BUP or Nor-BUP (0.2 μM) with HLM were low, as suggested by the LC-MS response observed (Fig. 3). In addition, two compounds with a mass corresponding to OH-BUP-glucuronide (m/z 660) (Fig. 5A; retention time = 9.1 and 9.6 min), as well as two others with a mass compatible with OH-Nor-BUP-glucuronides (m/z 606), were detected in urine samples from patients treated with BUP. Fragmentation of these compounds led to a major fragment ion corresponding to OH-BUP and OH-Nor-BUP and their corresponding secondary fragment ions, respectively, strongly suggesting that these compounds were actually OH-BUP- and OH-Nor-BUP-glucuronides. Figure 4 (C and D) shows the EPI mass spectra of the major OH-BUP- and OH-Nor-BUP-glucuronides. The glucuronidation sites could not be deduced from the mass spectra, but they are presumably the two phenolic hydroxy groups of OH-BUP (or OH-Nor-BUP). After incubation of the patients’ urine samples with β-glucuronidase, OH-BUP or OH-Nor-BUP conjugates were completely degraded, leading to increased OH-BUP (Fig. 5) or OH-Nor-BUP chromatographic peaks (data not shown).

OH-BUP was only detected in minute amounts in supernatants from incubation experiments with CYP 3A4-, CYP 3A5-, and CYP 3A7-transfected cells, precluding its quantitative determination.

Other metabolites were investigated in vitro, with or without solid-phase extraction (direct injection of microsomal incubation supernatant diluted by half in acetonitrile, to avoid excessive extraction selectivity), and in vivo using LC-MS/MS in the single-quadrupole full-scan mode or SIM, or in the EPI mode with selection of the m/z ratios of these hypothetical metabolites (i.e., desmethyl-BUP, OH-desmethyl-BUP, demethyl-Nor-BUP, etc.). None could be detected.

Discussion

It has been known for a long time that BUP is extensively metabolized in the liver by dealkylation and subsequent glucuronidation (Cone et al., 1984). Previous in vitro studies on BUP oxidative metabolism were performed using individual microsomal preparations and suggested a high interindividual variability (Iribarne et al., 1997). Consequently, we used a commercial pool of human liver microsomes (prepared from 29 different livers), which would be more representative of an average of many individuals. This might partly explain the difference in the affinity of human liver microsomes for Nor-BUP production described here (Km = 15 μM), compared with those reported by Iribarne et al. (1997) (Km = 89 ± 45 μM) or Kobayashi et al. (1998) (Km = 39 ± 9 μM). Part of this difference might also be

![Fig. 3. Chromatograms of BUP- and Nor-BUP-hydroxylated metabolites in the single quadrupole (Q1) SIM mode corresponding to incubation of 0.2 μM BUP (A) or Nor-BUP (B) with human liver microsomes.](image_url)
linked to the difference in buprenorphine concentration and experimental procedures between these studies.

CYP 3A4 was described to be largely involved in Nor-BUP production, but at least 25% of this oxidative metabolism could not be attributed to CYP 3A4 (Iribarne et al., 1997; Kobayashi et al., 1998), since chemical inhibition of CYP 3A4 incompletely inhibited Nor-BUP production. In these two studies, the BUP concentrations used in vitro were far above the usual range of plasma concentrations found in patients treated with high-dose BUP (Kuhlman et al., 1996). The study by Moody et al. (2002), conducted with BUP concentrations closer to this clinical range, suggested the involvement of other P450s (i.e., CYP 3A5, 3A7, 2C8) in BUP metabolism and showed that alternate metabolic pathways probably exist. The present study confirms, using three different approaches (i.e., P450-transfected cell lines, recombinant enzymes, and chemical inhibition experiments) that CYP 3A4, 2C8, and, to a minor extent, 3A5 and 3A7 are involved in Nor-BUP formation. The relative contribution of CYP 3A4 and CYP 2C8 to Nor-BUP production was estimated using two different methods (i.e., chemical inhibition and the RAF approach). The RAF approach suggests that CYP 3A4 is responsible for approximately three-fourths of Nor-BUP production. According to chemical inhibition experiments, CYP 3A4 would account for approximately 65% and CYP 2C8 for approximately 30% of Nor-BUP production, which is in agreement with the inhibition experiments conducted by Iribarne et al. (1997) using ketoconazole (about 75% inhibition) or the 3A mechanism-based inhibitors troleandomycin, gestodene, and erythral (about 70% inhibition). Here, the unexplained part of Nor-BUP production could be clearly attributed to CYP 2C8. As previously described by Moody et al. (2002), BUP utilization and Nor-BUP production were similar for this isoform. This finding suggests that CYP 2C8 is not involved in an alternate metabolic pathway. Since few drugs have been identified as CYP 2C8 substrates, there is no clue for drug-drug interactions involving CYP 2C8 so far.

Moody et al. (2002) reported a high activity of CYP 3A5 recombinant enzyme in BUP metabolism. We also found that this isoform was active, but the method used in the present study (i.e., chemical inhibition and RAF approach) could not provide a quantitative estimate of its involvement in BUP metabolism. CYP 3A5 is polymorphically expressed in humans and could significantly contribute to the metabolic clearance of CYP 3A substrates and, thus, could be a source of interindividual exposure variability (Huang et al., 2004). Further studies, comparing the activity of CYP 3A5-genotyped or -immunoquantified microsomes, are required to clearly measure CYP 3A5-mediated BUP metabolism in humans.

Our results also show that Nor-BUP production is not the only phase 1 metabolic pathway for BUP and that other or subsequent pathways and metabolites exist: HLM metabolized BUP more efficiently than they produced Nor-BUP; ketoconazole inhibited BUP utilization more efficiently than Nor-BUP production by HLM; and BUP in vitro metabolism using Ad293 P450-transfected cells showed that BUP consumption exceeded Nor-BUP production when incubated with CYP 3A, and that BUP was consumed without Nor-BUP production when incubated with CYP 2C18, 2C19, and 2C9. A hypothetical residual effect of UDP-glucuronosyltransferase in Ad293-transfected cells, which could explain such findings, could be ruled out using BUP and Nor-BUP determination before and after hydrolysis by β-glucuronidase (data not shown). The involvement of CYP 2C18 and 2C19 in BUP metabolism as well as the high activity of CYP 3A was previously reported by Moody et al. (2002) using P450 Supersomes and BUP degradation monitoring. However, contrary to their findings, CYP 2D6 and 2E1 did not metabolize BUP in the present study.
When considering BUP consumption, chemical inhibition of CYP 3A4 by ketoconazole or of CYP 2C8 by trimethoprim showed that approximately 90% of BUP was metabolized by CYP 3A and approximately 5% by CYP 2C8. Thus, other P450s (i.e., CYP 2C18, 2C19, and 2C9) apparently play a minor role in BUP metabolism (approximately 5%). Consequently, CYP 3A4 would be involved in an alternative, unknown BUP metabolic pathway. Using a highly sensitive LC-MS/MS procedure, hydroxylated metabolites were detected in vitro for BUP and Nor-BUP and further evidenced in vivo. These new BUP metabolites are subsequently glucuronidated, since glucuro-OH-BUP and glucuro-OH-Nor-BUP were identified in patients’ urine samples. OH-BUP was only produced by CYP 3A isoenzymes in low amounts, which probably cannot account for the whole alternate activity of CYP 3A4 that seems to represent a large part of BUP utilization. No adjustment of the MS parameters was possible for these new metabolites and, thus, the MS response might not exactly reflect the amounts produced; but, since the structural modification of BUP is minor, the MS response should not largely underestimate OH-BUP and OH-Nor-BUP. As a consequence, it can be inferred that not all BUP metabolites have presumably been identified yet, despite the many different extraction, separation, and detection procedures tested here (data not shown).

In conclusion, the present study gave clear evidence of alternate metabolic pathways involving to a minor extent CYP 2C9, 2C19, and 2C18 and to a major extent CYP 3A, and allowed us to identify two new BUP metabolites and their respective glucuronides (Fig. 6). However, not all BUP phase 1 metabolites have presumably been identified so far, and this warrants further investigations. Since interaction of BUP with benzodiazepines is probably involved in numerous fatalities in patients treated with high-dose BUP and is still poorly

![Fig. 5. Chromatogram in the EPI mode of OH-BUP (parent ion m/z 484; dotted line) and OH-BUP-glucuronide (m/z 660; solid line) corresponding to a urine sample analyzed before (A) and after (B) incubation with β-glucuronidase.](image)

![Fig. 6. Buprenorphine metabolic pathways in humans.](image)
understood, the results of this study might help understand their interaction mechanism.

References


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