IN VITRO METABOLISM STUDY OF BUPRENORPHINE:

EVIDENCE FOR NEW METABOLIC PATHWAYS

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Buprenorphine oxidative metabolism

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Abbreviations: BUP: buprenorphine; Nor-BUP: nor-buprenorphine; OH-BUP: hydroxy-buprenorphine; OH-Nor-BUP: hydroxy-nor-buprenorphine; HLM: human liver microsomes; CYP: cytochrome P450; LC-MS/MS: liquid chromatography-tandem mass spectrometry; RAF: relative activity factor; Cl_{int}: intrinsic clearance; RT: retention time.
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 (CYP) involved in BUP metabolism and to investigate other potential metabolites. The metabolism of BUP was examined using human liver microsomes (HLM), Ad 293 CYP transfected cell lines as well as CYP 3A4 and 2C8 recombinant isoforms. The kinetic parameters of metabolites formation were calculated for HLM and competent isoforms. Individual contribution of CYP isoforms in BUP metabolism as well as Nor-BUP production was evaluated using chemical inhibition experiments, as well as the relative activity factor (RAF) approach. The analytical method used was based on liquid chromatography-mass spectrometry. Among the 13 CYP 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 CYP 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 CYP3A, 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 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, in press): only midazolam was found to cause a moderate inhibition of BUP elimination and 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-buprenorphine (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; Pontani et al., 1985), which have remained unidentified. Human cytochrome P450 (CYP) 3A4 was demonstrated to be the major isoform involved in Nor-BUP formation in two reports (Iribarne 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 of Moody et al.
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 CYPs 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 who received a single dose of 4 mg sublingually. In our experience, the steady-state 24h 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 CYP 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, USA). Trimethoprim was obtained from Glaxo-Welcome (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 were purchased from BD GENTEST (Woburn, MA, USA).

CYP 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, 2E1, 3A4, 3A5 or 3A7 as previously described (Lacroix et al., 1997; Sonnier and Cresteil, 1998; Treluyer et al., 1997). The level of expression of CYP proteins was roughly equivalent, as demonstrated by immunoblots of cellular microsomes with appropriate antibodies and calibration with baculovirus-insect-cell expressed CYP (BD GENTEST, Woburn, MA, USA) and measurement of relevant monooxygenase activities using fluorescent substrates (methoxy-, ethoxy, or benzyloxy-trifluoromethylcoumarin or cyanoethoxycoumarin). 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 CYP3A4 and 3A5, but has no effect on the catalytic activity of other CYP proteins. Cells were cultured at 37°C under 5% CO₂ in D-MEM containing 10% fetal calf serum. 75cm² flasks containing cells at near confluence were incubated with 20µM 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 CYP
In preliminary experiments, linearity of Nor-BUP formation was checked at 0.2 µM BUP using a range of CYP contents (10-100 pmol/ml), microsomal protein concentrations (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 to 50 µM) or Nor-BUP (0.2 µM). Substrates and microsomes were pre-incubated 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 CYP isoforms to BUP metabolism**

The results obtained with recombinant CYPs 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 (Woburn, MA, USA). The scaled individual CYP reaction velocity (Vᵢ) was calculated by multiplying the experimental reaction velocity by the RAFᵢ value. This scaling process integrates the hepatic abundance of each CYP isoform and the differences in activity between cDNA-expressed enzymes and human liver microsomal CYP.

Results were model-fitted using the Michaelis-Menten equation and the kinetic parameters calculated by non-linear regression analysis using Winreg 3.1 (available online: http://www.unilim.fr/pages_perso/jean.debord/winreg/winreg1.htm, accessed December 1st, 2004). The intrinsic clearance (Clᵢᵣ) was estimated by the Vᵢᵣ/Kᵢᵣ ratio.

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

$$ RCᵢ (%) = \frac{Vᵢ(S)}{\sum Vᵢ(S)} (Eq.1) $$
Chemical inhibition experiments

Co-incubation 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 procedure, except that 50 µl of the inhibitor working solution were pre-incubated 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 percent 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 UI/ml) in 1 M acetate buffer (pH 4.8) during 2 hours 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 USA). Samples diluted ½ with water were loaded on the cartridge, which had been previously activated with 1 ml methanol followed by 1 ml H₂O. The cartridge was rinsed with 1 ml of 0.1 N HCl. BUP and metabolites were then eluted with 1 ml methanol containing 2 % ammonium hydroxide. The extracts were evaporated to dryness and reconstituted in 50 µl 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. 5 µl of extract were injected on an X-Terra MS C18, 3.5 µm reversed-phase column (Waters, Milford, Mass, USA). 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 / acetonitrile, 10/90; v/v) in solution B (2 mM ammonium formate pH 3.0), as follows: 0-2 min, 5% A; 20 min, 90% A; 21 min, 90% A; 21-23 min, 5% A; 25 min, 5% A. Detection was performed using a QTRAP tandem mass spectrometer with linear ion-trapping capabilities in the third quadrupole (Applied-Biosystems/Sciex, Foster city, CA). A Turbo-Ion spray 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 Q1 Selective Ion Monitoring (SIM) mode using the hypothetical m/z ratios of the most probable metabolites. Secondly, the nature of the metabolites detected was confirmed using the enhanced product ion scan mode (EPI) where 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, 7 calibrators (from 0.5 to 100 ng/ml BUP or Nor-BUP) were prepared in water, and incubation samples were also diluted (1/2 to 1/40) with water. The determination procedure involved solid phase extraction with Extrelut-3 cartridges, 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 analysed 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 analysed with each run.
RESULTS

Characterization of BUP metabolic pathways using CYP 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 minutes 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 Cl_int (Table 1).

Individual contribution of CYP450 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 (0.25-1.5µM) decreased Nor-BUP formation in a concentration-dependent manner up to 1.5 µM (corresponding to approx. 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 100µM (maximum inhibition: 30.8 %) (Table 2).

The effect of ketoconazole at each tested concentrations 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, approx. 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 CYP 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 0.2-50 µM.

**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 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. 5 A; RT=9.1 and 9.6), 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, 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 CYP3A4, CYP3A5 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 ½ in acetonitrile, in order to avoid excessive extraction selectivity), and in vivo using LC-MS/MS in the single-quadrupole full scan or Single Ion Monitoring (SIM) modes, 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 long 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 inter-individual 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 (K_m=15µM) with those reported by Iribarne et al. (1997) (K_m=89 ± 45 µM) or Kobayashi et al. (1999) (K_m=39 ± 9 µM). Part of this difference might also be 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 of Moody et al. (2002), conducted with BUP concentrations closer to this clinical range, suggested the involvement of other CYPs (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. CYP 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 approx. ¾ of Nor-BUP production. According to chemical inhibition experiments, CYP
3A4 would account for approx. 65% and CYP 2C8 for approx. 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 be a source of interindividual exposure variability (Huang et al., 2004). Further studies, comparing the activity of CYP 3A5 -genotyped or -immuno-quantified 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 that they produced Nor-BUP; ketoconazole inhibited BUP utilization more efficiently than Nor-BUP production by HLM; and BUP in vitro metabolism using Ad 293 CYP 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 hypothetic residual effect of UDP-glucuronosyl transferase 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 CYP 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 CYP3A4 by ketoconazole or of CYP2C8 by trimethoprim showed that approx. 90% of BUP was metabolized by CYP 3A and approx. 5% by CYP2C8. Thus, other CYPs (i.e. CYP 2C18, 2C19 and 2C9) apparently play a minor role in BUP metabolism (approx. 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, as 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, so 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 under-estimate 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 understood, the results of this study might help understand their interaction mechanism.
References


Footnotes

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Figure 1. Comparison between the amount of Nor-BUP produced and the amount of BUP consumed (nmol) during 24-hour incubation of BUP (100 nmol) with CYP Ad 293 transfected cells and native Ad293 cells. Results are presented as the mean of duplicate experiments ± standard deviation.

Figure 2. Enzymatic kinetic modeling of Nor-BUP formation in pooled human liver microsomes (crosses), CYP 3A4 (solid circles) and CYP 2C8 (empty circles) using the Michaelis-Menten function. Results are presented as the mean of duplicates obtained with recombinant CYPs and scaled to HLM following the RAF method.

Figure 3. Chromatograms of BUP and Nor-BUP hydroxylated metabolites in the Q1 Single Ion Monitoring (Q1 SIM) mode corresponding to incubation of 0.2 µM BUP (A) or Nor-BUP (B) with human liver microsomes.

Figure 4. Enhanced product ion scan (EPI) mass spectra of OH-BUP (A) and OH-Nor-BUP (B) produced by human liver microsomes in vitro, as well as OH-BUP glucuronide (C) and OH-Nor-BUP glucuronide (D) detected in a patient’s urine sample.

Figure 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.

Figure 6. Buprenorphine metabolic pathways in humans.
TABLE 1: Nor-BUP production kinetic parameters (mean estimate ± standard error) computed from BUP incubation with CYP 3A4 and CYP 2C8 Supersomes and scaled to HLM using the RAF approach.

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<th>$K_m$ (µM)</th>
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<td>CYP 3A4</td>
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<td>CYP 2C8</td>
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TABLE 2: Inhibition of BUP metabolism by ketoconazole and trimethoprim: results are expressed as percent inhibition of either BUP consumption or Nor-BUP production by pooled human liver microsomes *in vitro* (mean of duplicate).

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<th>Inhibition of Nor-BUP production (%)</th>
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<td><strong>Ketoconazole (µM)</strong></td>
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FIGURE 1

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FIGURE 2
FIGURE 3
FIGURE 5
FIGURE 6