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# Novel metabolites of buprenorphine detected in human liver microsomes and human urine

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Abbreviations used are: HLM, human liver microsomes; P450, cytochrome P450; MAb, monoclonal antibody; NADPH GS, NADPH generating system; LC-ESI-MS/MS, liquid chromatography-electrospray ionization-tandem mass spectrometry; SIM, selected ion monitoring; CID, collision-induced dissociation; RAF, relative activity factor.

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## Abstract

The in vitro metabolism of buprenorphine was investigated to explore new metabolic pathways and identify the cytochrome P450s (P450s) responsible for the formation of these metabolites. The resulting metabolites were identified by liquid chromatography-electrospray ionization-tandem mass spectrometry. In addition to norbuprenorphine, two hydroxylated buprenorphine (M1 and M2) and three hydroxylated norbuprenorphine (M3, M4 and M5) metabolites were produced by human liver microsomes (HLMs), with hydroxylation occurring at the tert-butyl group (M1 and M3) and at unspecified site(s) on the ring moieties (M2, M4 and M5). Time-course and other data suggest: buprenorphine is N-dealkylated to form norbuprenorphine, followed by hydroxylation to form M3; buprenorphine is hydroxylated to form M1 and M2, followed by N-dealkylation to form M3 and M4 or M5. The involvement of selected P450s was investigated using cDNA-expressed P450s coupled with scaling models, chemical inhibition, monoclonal antibody (MAb) analysis and correlation studies. The major enzymes involved in buprenorphine elimination and norbuprenorphine and M1 formation were P450s 3A4, 3A5, 3A7, and 2C8; while 3A4, 3A5 and 3A7 produced M3 and M5. Based on MAb analysis and chemical inhibition, the contribution of 2C8 was higher in HLM with higher 2C8 activity, while 3A4/5 played a more important role in HLM with higher 3A4/5 activity. Examination of human urine from subjects taking buprenorphine showed the presence of M1 and M3; most of M1 was conjugated while 60-70% of M3 was unconjugated.

## Introduction

Buprenorphine, a semi-synthetic derivative of the alkaloid thebaine (Lewis, 1973), is a partial μ-opioid agonist and κ-opioid antagonist (Cowan et al., 1977). It was first developed as an analgesic for moderate to severe pain in the early 1970s, but is currently more widely used as a replacement therapy for opiate-dependence. Buprenorphine has comparable effects to methadone in regard to treatment of opiate-dependent patients (Strain et al., 1996; Johnson et al, 2000), but has reduced risk due to the "ceiling effect" associated with its partial μ-opioid agonist properties (Walsh et al., 1994; Walsh, et al., 1995).

Absorption, distribution, metabolism and excretion studies of buprenorphine have been carried out in humans using gas chromatography-mass spectrometry (Cone et al., 1984), and in animals using thin-layer chromatography of tritiated-buprenorphine (Brewster et al., 1981; Pontani et al., 1985). These studies suggested that buprenorphine was mainly metabolized by N-dealkylation and glucuronidation of both buprenorphine and norbuprenorphine. A tentative 6-O-demethyl norbuprenorphine in free and conjugated form was observed in rat urine (Pontani et al., 1985) and some unknown polar metabolites were found in rat bile samples (Brewster et al., 1981). No evidence was given for additional metabolites in humans (Cone et al., 1984). A recent study by Picard et al. (2005) using liquid chromatography-tandem mass spectrometry, however, identified the presence of two hydroxylated metabolites, one of buprenorphine and one of norbuprenorphine, in human liver microsomes (HLM) and urine samples from patients treated with buprenorphine. Buprenorphine N-dealkylation is mainly catalyzed by cytochrome P450 (P450) 3A4 (Iribarne et al., 1997; Kobayashi et al., 1998), with involvement of P450 3A5 and 2C8 (Moody et al., 2002; Picard et al., 2005). The involvement of specific P450s in production of the hydroxylated metabolites was limited to a finding that trace amounts of hydroxy-

buprenorphine were produced by P450 3A4, 3A5 and 3A7 transfected cell lines (Picard, et al., 2005).

In our previous study we observed a higher rate of buprenorphine elimination than norbuprenorphine formation in HLM, suggesting there might be some other routes for metabolism of buprenorphine or its metabolites (Chang and Moody, 2005). In this paper we report a study of the metabolism of buprenorphine in HLM and analysis of human urine from subjects treated with buprenorphine. The identification of new metabolites was achieved by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) and the involvement of P450s in the formation of new metabolites was clarified using cDNA-expressed human P450s and correlations with a panel of HLMs. The contribution of each enzyme was estimated by inhibitory analysis using monoclonal antibodies (MAbs) and chemical inhibitors in phenotyped HLMs, and also predicted by relative activity factor (RAF) and immunoquantification scaling approaches. Based on our results an extended biotransformation profile is proposed for buprenorphine.

## Experiment

**Materials.** Buprenorphine (for incubation), D-glucose 6-phosphate monosodium salt, glucose 6-phosphate dehydrogenase, β-NADP sodium salt, EDTA disodium salt, $MgCl_2$, β-glucuronidase (from Helix pomatia which also has sulfatase activity), trimethoprim, 8-methoxypsoralen, sulphaphenazole and quinidine were obtained from Sigma/Aldrich Chemical Corp. (St. Louis, MO). Furafylline was obtained from Synergy House (Manchester, UK). Buprenorphine (for analysis), $d_4$-buprenorphine, norbuprenorphine and $d_9$-norbuprenorphine were purchased from Cerilliant (Round Rock, TX). Ketoconazole was obtained from ICN Biomedicals Inc. (Aurora, OH). 10-Hydroxybuprenorphine, buprenorphine N-oxide and 10-oxobuprenorphine were provided by Reckitt Benckiser Healthcare Limited (Dansom Lane, UK). The liver samples were obtained from Tissue Transformation Technologies (Edison, NJ). Insect cell cDNA-expressed human P450s (Supersomes) and fifteen phenotyped HLM were purchased from BD-Gentest (Woburn, MA). Inhibitory MAbs to human P450 3A4/5 and 2C8 were provided by the National Cancer Institute of the National Institutes of Health (Bethesda, MA). All aqueous reagents were prepared in purified water (specific resistance >18.2 mΩ/cm) obtained by a Milli-Q Plus water purification system.

**In vitro incubations of buprenorphine with HLM.** Microsomes were prepared from human liver by differential centrifugation as described by Nelson et al. (2001). The first centrifugation was at 9,000 g, the homogenization buffer contained 0.25 M sucrose, and 10 strokes of homogenization were used. HLM prepared in our lab are not thoroughly phenotyped; to enhance the probability of having a representative amount of different P450 enzymes, pooled HLM (n=5) were used for initial metabolites identification studies. The incubation mixture (final volume

500 µl) contained incubation buffer (0.1 M phosphate buffer pH 7.4 with 1.0 mM EDTA and 5.0 mM $MgCl_2$), a NADPH generating system (NADPH GS) composed of 10 mM glucose-6-phosphate, 1.2 mM NADP, and 1.2 units of gluscose-6-phosphate, 0.5 mg/ml microsomal protein, and 10 µM buprenorphine or norbuprenorphine. The reaction was initiated by adding the NADPH GS, and incubated at 37°C in a shaking water bath for the specified times. For qualitative studies, following a 30-min incubation the mixture was adjusted to pH>10 with 50 µl 1N NaOH followed by extraction with a mixture of n-butyl chloride and acetonitrile (4:1, v/v). For quantitative studies, the reaction was terminated by the addition of 200 µl ice-cold methanol and the samples were stored at -75°C until analysis.

**In vitro incubations of buprenorphine with recombinant human P450s.** The metabolism of buprenorphine and norbuprenorphine was evaluated in microsomes prepared from insect cells transfected with cDNAs encoding for human P450 1A2, 2A6, 2B6, 2C8, 2C9*1, 2C18, 2C19, 2D6*1, 2E1, 3A4, 3A5 and 3A7. Supersomes that co-expressed cytochrome $b_5$ were used where available, this was not the case for 1A2, 2C18 and 3A5. Buprenorphine or norbuprenorphine (10 µM) was incubated at 37°C for 20 min with 25 pmol P450 in incubation buffer described above. Control insect cell microsomes were used at the mean protein concentration averaged over all of the supersomes. All reactions were initiated by addition of the NADPH GS and stopped by the addition of 200 µl ice-cold methanol after which the samples were stored at -75°C until analysis.

**Inhibition of buprenorphine metabolism using MAbs.** The role of P450 3A4/5 and 2C8 was measured by the addition of the P450 target specific MAb, either alone or in combination, to the reaction mixture using the procedure proposed by Yang et al. (1999). The recommended

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volumes (10 µl) of MAbs specific for P450 3A4/5 or 2C8 were mixed with phenotyped HLMs in 0.5 ml of incubation buffer and preincubated for 5 min at 37°C. Tubes were then placed on ice, buprenorphine was added (final concentration 10 µM) and the reaction was initiated by addition of the NADPH GS. The reaction continued for specified times at 37°C and terminated with 200 µl ice-cold methanol. Ten microliters of egg lysozyme was used as a control.

**Chemical inhibition studies.** The effect of the selective P450 inhibitors on buprenorphine metabolism was first studied in pooled HLM. Subsequently more extensive studies were performed in phenotyped HLMs using the selective P450 3A4/5 inhibitor ketoconazole 2 µM (Newton et al., 1995; Sai et al., 2000) and the selective P450 2C8 inhibitor trimethoprim 100 µM (Wen et al., 2002). The inhibitor and buprenorphine (final concentration 10 µM) were added to the reaction mixture and the reaction was initiated by the addition of the NADPH GS in a 37°C shaking water bath. The reaction continued for specified times and was terminated by the addition of 200 µl methanol. The incubation sample with no inhibitor served as control.

**Correlation studies.** HLM from 15 individual donors along with data for P450 specific enzyme activities, provided by BD-Gentest, were used to study the relationship between the metabolism of buprenorphine and the metabolism of selective P450 substrates. The ability of HLM from each donor to metabolize buprenorphine was correlated with the P450-specific enzyme activities for each sample. The assay was performed with 10 µM buprenorphine and incubated for the specified times.

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**In vivo metabolism of buprenorphine.** Twenty four-hour postdose urine samples were collected from seven subjects who had been maintained on a daily sublingual dose of 16 mg buprenorphine for at least 21 days. A 1-ml aliquot of each urine sample was adjusted to pH 5 with sodium acetate buffer (0.1 M) and treated with 5,000 units of β-glucuronidase (containing sulfatase). The mixture was incubated at 50°C for 16 hours. Another aliquot of the urines was analyzed without hydrolysis. Blank urine samples also underwent hydrolysis to control for interference arising from endogenous materials.

**LC-ESI-MS/MS analysis.** The quantification of buprenorphine and norbuprenorphine (or semi-quantification of hydroxylated metabolites) in incubation samples and urine samples was performed using a modification of our previously described LC-ESI-MS/MS method (Moody et al., 2002). The incubation samples were made basic (pH>10) by the addition of 50 µl of 1 N NaOH and extracted with a 4-ml mixture of n-butyl chloride and acetonitrile (4:1, v/v); the organic layer was dried under $N_2$. The final residue was reconstituted to a volume of 75 µl using initial mobile phase and 20 µl was injected into the LC.

Mass spectrometric analysis was performed on a TSQ 7000 or TSQ Quantum (Thermo Finnigan, San Jose, CA) equipped with a triple-quadrupole MS and an ESI source operated at 4.5 kV. The MS was set to scan for positive ions. Quantification was performed by selected reaction monitoring (SRM) transitions *m/z* 468 to *m/z* 396 (buprenorphine), *m/z* 414 to *m/z* 101 (for TSQ 7000) (norbuprenorphine), *m/z* 472 to *m/z* 400 ($d_4$-buprenorphine) and *m/z* 423 to *m/z* 110 ($d_9$-norbuprenorphine). The semi-quantification of hydroxylated metabolites by SRM is described in detail in the results section. MS/MS conditions used were 3.0 mTorr argon collision gas and 45 eV collision potential. When the Quantum was used, we found that

norbuprenorphine had better sensitivity when the survivor molecular ion was monitored (i.e. 22 eV collision potential with *m/z* 414 to *m/z* 414) (Huang et al., in press). The liquid chromatograph was a Hewlett-Packard Series 1100 HPLC (Agilent Technologies, Palo Alto, CA). The chromatographic separations were conducted on a 3 μM YMC ODS-AQ™ column (2.0 × 50 mm cartridge) (Waters, Milford, MA). The mobile phase was MilliQ $H_2O$ (A) and $CH_3CN$ (B), both containing 0.1% formic acid. The gradient elution went from 97% A at 1 min to 80% A at 3 min, holding for 5 min, then decreased to 20% A at 10 min, holding for 2 min.

Qualitative studies were performed on an Inertsil C18 column (250 × 2.1 mm i.d.), packed with 3-μM particles (Bellefonte, PA). Isocratic elution was performed at 81% A with a flow-rate of 0.25 ml/min. The screening of metabolites by mass spectrometry was based on full scan, selected ion monitoring (SIM), constant neutral loss scan, precursor ion scan and product ion scan. The constant neutral loss scan of 54 u was used to detect the metabolites that undergo a loss of cyclopropylmethyl group. The precursor ion scans of *m/z* 396 and *m/z* 101 were used to detect the metabolites that can produce typical fragment ions at *m/z* 396 and *m/z* 101 under the collision-induced dissociation (CID) conditions. The product ion scan was used to identify the metabolites.

## Results

In our previous studies on in vitro metabolism of buprenorphine, we focused on use of substrate concentrations that approached therapeutic plasma concentrations (Moody et al., 2002; Chang and Moody, 2005). As the purpose of this study was to identify new metabolites, we have used a higher concentration, 10 μM, for in vitro experiments to enhance our ability to detect what might be minor metabolites. This concentration, which is less than reported $K_m$ for buprenorphine metabolism (Kobayashi et al., 1998), still meets criterion suggested by Bjornsson et al., (2003) for P450 phenotyping studies. The in vivo relevance will be shown from studies in human urine.

**Buprenorphine elimination and norbuprenorphine formation in HLM.** When buprenorphine (10 μM) was incubated with pooled HLM (n=5), norbuprenorphine formation only accounts for 46% and 37% of buprenorphine elimination at 20 min and 60 min incubation time, respectively (data not shown). Higher buprenorphine elimination compared to norbuprenorphine formation suggested that other biotransformation pathways for buprenorphine or its metabolites exist in HLM.

**Mass spectrometric analysis of buprenorphine.** Under the CID-MS/MS conditions, the characterized product ions generated from protonated molecular ions of buprenorphine (*m/z* 468) were at *m/z* 414, *m/z* 396 and *m/z* 101 (Moody et al., 2002). A $[M-54]^+$ peak at *m/z* 414 (referred to as the a-moiety) showed the removal of a cyclopropylmethyl group. The peak at *m/z* 396 (referred to as the b-moiety) was formed by combination of the loss of a methyl group and

cleavage of a tert-butyl group instead of loss of cyclopropylmethyl group and a water molecule, which was confirmed by the presence of a high abundant product ion at *m/z* 400 produced from $d_4$-buprenorphine (*m/z* 472) (data not shown). This assignment was consistent with previous work reported by Polettini and Huestis (2001). At the low mass range, a fragment ion at *m/z* 101 (referred to as the c-moiety) was assigned to the alkyl side-chain $HOC(CH_3)C(CH_3)_3^+$ at C-7 and it can lose a water molecule to form the fragment ion at *m/z* 83. Another fragment ion at *m/z* 55 corresponds to the cyclopropylmethyl group.

**Identification of in vitro phase I metabolites of buprenorphine.** In HLM, the major metabolite, norbuprenorphine, formed by N-dealkylation of buprenorphine has been studied in great detail. In the current study, different scan modes of the triple quadrupole MS were used to screen for unknown metabolites. Constant neutral loss scan of 54 u and precursor ion scan of *m/z* 396 and *m/z* 101 showed the presence of hydroxylated buprenorphine and norbuprenorphine. In initial experiments norbuprenorphine was found to readily form an adduct ion with acetonitrile (plus 41 u) which shows better response on the mass spectrometer used than the protonated molecular ion. As such, the acetonitrile adduct ion was used to determine norbuprenorphine structurally related metabolites. The *m/z* 484 and *m/z* 471 ions correspond to the hydroxylated buprenorphine protonated molecular ion and hydroxylated norbuprenorphine adduct ion with acetonitrile. There are four peaks in the SIM chromatogram at *m/z* 484 and three peaks at *m/z* 471 (Fig. 1). At retention times 9.83 min (M1) and 12.13 min (M2) (Fig. 1A), and retention times 6.34 min (M3), 7.87 min (M4) and 9.96 min (M5) (Fig. 1B), the peaks are absent in the chromatograms of the corresponding blank control samples. Peaks at retention times 14.90 min (I1) and 16.62 min (I2) in the SIM chromatogram of *m/z* 484 were also present in the

control samples incubated with heat-inactivated microsomes, and their amounts did not change with changes in incubation time suggesting these two peaks are probably inert impurities.

When HLM were incubated with buprenorphine, the microsomal protein precipitated with methanol and the supernatant directly injected into the LC-MS/MS, the same, and no additional metabolites were observed. Selected ion monitoring of other possible metabolites, such as O-demethyl, N-oxide, and di-hydroxyl metabolites, showed negative results. The oxidative degradation compounds of buprenorphine found in sublingual tablets, i.e., 10-hydroxybuprenorphine, buprenorphine N-oxide and 10-oxobuprenorphine were not detected in microsomal samples using comparisons with the reference compounds. 6-O-Demethyl norbuprenorphine, which was tentatively identified in rat bile (Pontani et al., 1985), was not identified in HLM.

The structure of the metabolites has been proposed by interpreting their product ion mass spectra and comparison with that of parent drug. The CID product ion mass spectrum of M1 (Fig. 2A) presented the strongest peak at *m/z* 396 suggesting that the b-moiety is intact. The *m/z* 414 ion in the CID product ion scan of buprenorphine shifted by 16 for M1 and meanwhile the *m/z* 55 ion was present indicating that the cyclopropylmethyl group was intact and hydroxylation had occurred at the a-moiety. The absence of *m/z* 101 ion confirmed that the addition of a hydroxyl group was on the c-moiety. In consideration of the spatial hindrance and molecular stability, the hydroxylation occurred at the tert-butyl group.

CID-MS/MS spectrum of M2 presents characteristic product ions at *m/z* 430 and *m/z* 412, and a strong fragment ion at *m/z* 101 (Fig. 2B). The presence of *m/z* 430 and *m/z* 101 ions suggests that cyclopropylmethyl group and alkyl side-chain at C-7 position are intact, the addition of a hydroxyl group might occur at one of the ring moieties.

Three peaks were observed in the SIM chromatogram of *m/z* 471 (Fig. 1B). The characteristic fragment ion *m/z* 101 corresponding to the alkyl side-chain at C-7 was absent in the product ion scan of M3 (Fig. 3A), while present in the product ion scan of M4 (Fig. 3B) and M5 (Fig. 3C). It suggests that the hydroxylation of M3 is similar to M1 and the addition of oxygen is on the tert-butyl group. The hydroxylation of M4 and M5 is similar to M2 and the hydroxyl group is on one of the ring moieties, but the exact hydroxyl position could not be determined.

**The time-course of hydroxylated metabolites formation in HLM.** After incubation of 10 μM buprenorphine with HLM, the amount of M1, M2, M3, M4 and M5 was determined by SRM of *m/z* 484 to 396 (M1), *m/z* 484 to *m/z* 101 (M2), *m/z* 471 to 202 (M3), and *m/z* 471 to 101 (M4 and M5) transitions, respectively. The amount was expressed as peak area ratio in comparison with internal standard $d_4$-buprenorphine since no standard compound was available. The rate of formation of M1 was greater than M3 and M5, as indicated by the slope of the curves at earlier incubation times. The amount of M1 decreased after 10 min, suggesting that it might undergo further metabolism (Fig. 4A). Only M3 was detected in HLM incubated with 10 μM norbuprenorphine and it increased linearly up to 60 min (Fig. 4B). The metabolites M2 and M4 were not detected by SRM.

**Screening of 12 cDNA-expressed human P450s in the metabolism of buprenorphine.** Consistent with our previous study using 21 nM buprenorphine (Moody et al, 2002), incubation of 10 μM buprenorphine with 12 human baculovirus insect cell-expressed P450 isoenzymes (25 pmol) showed that the 3A family and 2C8 were the major enzymes involved in buprenorphine elimination and norbuprenorphine formation (data not shown). The most efficient enzyme for

M1 formation was P450 3A5, followed by 2C8, 3A4 and 3A7. The formation of M3 and M5 was mediated by P450 3A4, with a smaller contribution of 3A7 and 3A5. No metabolism was observed with other P450s and control insect microsomes (Fig. 5A). Incubation of 10 μM norbuprenorphine with P450s only produced M3, which was mainly mediated by 3A4 and to a much lesser extent by 3A5 (Fig. 5B).

**The contribution of individual P450s to buprenorphine metabolism in HLM.** *MAbs analysis and chemical inhibition*. Based on our P450s screening data together with previously reported results (Moody et al., 2002; Picards et al., 2005), P450 3A4, 3A5, 3A7 and 2C8 are the major enzymes involved in the elimination of buprenorphine. In addition, a preliminary experiment in pooled HLM using other selective P450 inhibitors: 5 μM furafylline (1A2), 5 μM 8-methoxypsoralen (2A6), 20 μM sulphaphenazole (2C9) and 10 μM quinidine (2D6) did not show any significant inhibition on buprenorphine metabolism. Therefore, the study on the contribution of individual P450 focused on 3A4/5 and 2C8. The individual contribution of 3A4/5 and 2C8 was determined by measuring metabolite(s) formation and buprenorphine elimination in phenotyped HLMs following the addition of MAbs (see Yang et al., 1999; Krausz et al., 2001 for specificity of MAbs) or chemical inhibitors. Based on time-course results, norbuprenorphine and M1 formation were evaluated at 10 min; all others at 30 min. The percentage of inhibition observed with the addition of a MAb or chemical inhibitor determined its contribution to the total metabolism (Table 1). In the current study two phenotyped HLMs with different relative activities of 3A4/5 and 2C8 were used. HLM 452013 had higher 2C8 and lower 3A4/5 activity while HLM 452164 had higher 3A4/5 and lower 2C8 activity. In HLM 452013, the contributions of 2C8 to the elimination of buprenorphine, and the formation of norbuprenorphine

and M1 were 4.8-11.9 times higher than 3A4/5 according to MAbs analysis, and 1.6-3.5 times higher based on chemical inhibition. In HLM 452164, the data are the same for MAb analysis and chemical inhibition, and the contribution of 3A4/5 was higher than that of 2C8. In both HLMs, the contribution of 3A4/5 was higher than 2C8 for the formation of M3 and M5. No significant difference was observed by increasing the amount of MAbs from 10 µl to 20 µl.

*Scaling of cDNA-expressed P450 activities.* RAFs were determined using the average of the enzyme activities for the 15 phenotyped HLM used in this study divided by the enzyme activities provided by BD Gentest for the cDNA-expressed P450s (Crespi, 1995; Venkatakrishnan, 2000). The immunoquantification abundances were from another previously described (Neff and Moody, 2001) BD Gentest data-bank of 7 HLM; the abundance of 2C8, which was not provided, was estimated from 2C9 abundance and the finding of Lapple et al. (2003) that the average content of 2C8 is 64.2% of 2C9. The predicted contributions of individual P450s are shown in Table 2. Using RAFs P450 3A contributed the most to buprenorphine elimination (78.1%) and norbuprenorphine formation (48.4%) followed by 2C8, with contribution of 14.5% and 36.4%, respectively. For the formation of M1, 2C8 was predicted to contribute most (70.2%) followed by 3A (29.2%). The estimated contribution of 3A increased and 2C8 decreased when the immunoquantitative data were used (Table 2).

**Correlation study.** The rates of formation of metabolites and buprenorphine elimination were determined in 15 individual HLMs and the data were correlated with the P450 phenotyped activities provided by the vendor. The results for correlations with 3A and 2C8 activities are shown in Table 3. Significant correlations ($p<0.05$) between testosterone 6β-hydroxylation

catalyzed by P450 3A were observed with buprenorphine elimination and the formation of each metabolite. For the formation of M1 and M5, the significant correlations were only observed by excluding three HLMs with highest 3A activities. The only significant correlation with paclitaxel 6α-hydroxylation, catalyzed by 2C8, was with norbuprenorphine formation. The power of these correlation experiments depends, in part, on the extent of the inter-HLM variation in activity; the greater the range within a liver bank the more power it has to establish a significant correlation. It should be noted that the range of 3A activity (highest activity HLM/lowest activity HLM) in the 15 HLM was 15.8; that of 2C8 activity was only 5.4. Correlations with other P450 activities (activity range in parentheses) 1A2 (15.3), 2A6 (14.3), 2B6 (20), 2C9 (4.9), 2C19 (171), 2D6 (7.9), 2E1 (3.2) and 4A11(37.7) were also investigated (data not shown). The only other significant correlations observed were M1 formation with 2E1 activity, chlorzoxazone 6-hydroxylation (R=0.550).

**In-vivo studies.** Human urine samples with or without hydrolysis by β-glucuronidase (containing sulfatase) were analyzed by LC-ESI-MS/MS. The product ion mass spectrum and the retention time on the LC when compared to that in HLM incubations demonstrated that buprenorphine can be metabolized to form M1 and M3 in vivo (data not shown). Semi-quantitation of M1 and M3 was determined from peak area ratios of metabolite to $d_4$-buprenorphine, and those determined in hydrolyzed urine compared to those determined in non-hydrolyzed urine (Table 4). The negligible amount of M1 in non-hydrolyzed urine sample in comparison to hydrolyzed samples suggests that M1 is significantly conjugated with glucuronide in vivo. The smaller difference between hydrolyzed M3 and non-hydrolyzed M3 (mean=68.5%, range 53-100% of unconjugated) suggests that it is excreted, for the most part, as

the unconjugated form (Table 4). Non-hydrolyzed urine was also extracted by solid-phase (Huang et al., in press) in order to directly examine the conjugated buprenorphine and metabolites. Neutral loss scans of 176 (glucuronide conjugates) and 80 (sulfonate conjugates) and SRM (transition of molecular ion of interest to –176 and –80) were performed. Glucuronide conjugates were identified for buprenorphine, norbuprenorphine, M1 and M3. Only norbuprenorphine showed evidence for a sulfonate conjugate at approximately 1% of its glucuronide conjugate (data not shown).

## Discussion

Here, for the first time, we demonstrated the presence of five new phase I metabolites of buprenorphine using HLM, as identified by LC-ESI-MS/MS. No other metabolites were found, even with different extraction and LC separation methods. The results are different from the study by Picard et al. (2005), in which a single hydroxylated buprenorphine and a single hydroxylated norbuprenorphine were found in HLM and the hydroxylation was proposed to occur on the phenyl-ring. As the mass fragmentations they presented did not extend below *m/z* 300, it is difficult to assign the two metabolites they identified to ones we identified in this study.

The time-course of M1 formation decreased after 10 min of incubation, suggesting that M1 was a transient metabolite that underwent further metabolism. This was further confirmed from the inhibition experiments. When P450 3A activity was blocked by anti-3A4/5 or ketoconazole, more M1 was present at 30 minutes than in the control samples. M3 was formed by HLM incubated with buprenorphine as well as norbuprenorphine suggesting that M3 was formed from buprenorphine N-dealkylation followed by hydroxylation; buprenorphine hydroxylation followed by N-dealkylation is also possible, but can only be established by addition of M1 to HLM, which is currently not possible. M5 was produced in incubations with buprenorphine but not with norbuprenorphine suggesting that M5 was formed by buprenorphine hydroxylation followed by N-dealkylation. Due to lack of metabolite reference standards the quantity of metabolites could not be determined. Neither M2 nor M4 were identified in HLM or urine examined by SRM, suggesting they are minor metabolites. M5 seen in HLM was not determined in urine, which suggests it is either a minor metabolite or eliminated by other routes, such as biliary excretion. The extended biotransformation profile of buprenorphine in HLM is summarized in Scheme 1.

In the current work, we used recombinant P450s and correlation studies to study the involvement of P450s in buprenorphine metabolism, the individual contribution of each P450 was estimated using MAb-based inhibitory analysis, chemical inhibition, RAF and immunoquantification scaling approaches. In previous studies, MAbs have not been used, and scaling and correlation studies have been limited to the involvement of P450 3A (Kobayashi et al., 1998; Picard et al., 2005).

Based on the MAb inhibition analyses, the contribution of 3A4/5 and 2C8 to the buprenorphine N-dealkylation was significantly different in HLMs from different donors. In HLM 452013 with higher 2C8 activity, the 2C8 enzyme played an important role for the elimination of buprenorphine (77%) and the formation of norbuprenorphine (83%). Chemical inhibition confirmed the minor role of 3A4/5 in this source of HLM, but could only ascribe ≈30% of activity to 2C8. In HLM 452164 with higher 3A4/5 activity, the results obtained from MAbs analyses and chemical inhibition were the same and they were consistent with the previous finding that the most active enzyme was 3A4/5 for buprenorphine N-dealkylation. The significant correlations between 3A activities and buprenorphine elimination (R=0.896) and norbuprenorphine formation (R=0.776) in 15 HLMs, together with the predicted contribution using RAF and immunoquantification approaches supported this finding in the HLM with higher 3A4/5 activity.

Other oxidative metabolites of buprenorpine or of norbuprenorphine have only recently been described; first by Picard et al. (2005) and now in this study. The involvement of specific P450 enzymes in the formation of these new metabolites was previously limited; Picard et al. (2005) only mentioned the formation of trace amounts of hydroxy-buprenorphine by expressed P450s 3A4, 3A5 and 3A7. In the current study, the formation of the 5 new metabolites was examined

using 12 cDNA-expressed human P450s. The activities of P450s involved in M1 formation are in the order of 3A5>2C8>3A4>3A7. Based on the MAbs analysis, in HLM with higher 2C8 activity, the contribution of 2C8 (67%) was higher than 3A4/5 (14%); in HLM with higher 3A4/5 activity, 3A4/5 (48%) showed higher contribution than 2C8 (18%). Significant correlation (R=0.683) between M1 and testosterone 6β-hydroxylation catalyzed by 3A suggested that generally 3A played an important role in the formation of M1. The relative activities of P450s involved in M3 and M5 formation were ranked as 3A4>3A7>3A5 (M3) and 3A4>3A5 (M5), respectively. (Note: the contribution of P450 3A5 may be an underestimate, as it was not available with co-expressed cytochrome $b_5$. Cytochrome $b_5$ enhances some of 3A5 activities, much as it does 3A4 (Yamaori et al., 2003)). The strongest correlation between metabolite formation and testosterone 6β-hydroxylation was noted for M3 (R=0.963) and M5 (R=0.921), which confirmed that 3A was the most important enzyme responsible for their formation in HLM. In conclusion, the 3A family has been shown to be the most important enzyme(s) involved in the metabolism of buprenorphine. P450 2C8, relative to its liver content was involved in the formation of norbuprenorphine and M1, as well as buprenorphine elimination.

In correlation studies, significant correlations for the formation of M1 and M5 with 3A were only observed after excluding three HLMs with highest activity. Possible explanations include: at higher 3A activity the formation of M1 and M5 will not increase linearly with the increase of 3A content; another consideration may be that high 3A activity could result from higher 3A5 content. A significant correlation for M1 formation with 2E1 activity was inconsistent with the cDNA-expressed P450 studies; 2E1 activity only varied 3.2-fold in our 15 HLMs, which demonstrated the limitation of correlation studies for this P450 using our current phenotyped liver bank.

In some individuals, P450 3A5 can make up to 50% of total 3A in human liver (Kuehl et al., 2001). Determination of the contribution of 3A5 versus 3A4 in metabolism of a drug is complicated by the lack of specificity of antibodies and inhibitors for these two members of the 3A family. The cDNA-expressed P450s offer one opportunity to discriminate between the two, but scaling requires some idea of relative abundance. As the immunoquantitation of 3A5 varies extensively, it would not be prudent to just insert a mean content into the immunquantitation approach used in Table 2. To provide some estimate of the contribution of 3A5 to buprenorphine elimination and norbuprenorphine and M1 formation, we have used RAF analysis. This, however, also requires some assumptions. While the activity of the probe substrate in cDNA-expressed P450s is known, the amount of the activity due to 3A4 and 3A5 in HLM cannot yet be ascertained. We have therefore made estimations with the assumptions that either 3A4 and 3A5 have equal activity for testosterone 6-hydroxylation in HLM (Fig.6A- C), or that 3A5 in HLM, as was found in cDNA-expressed P450s, has one-tenth of the testosterone 6-hydroxylation in HLM (Fig. 6D-F). Using these assumptions one can then make calculations with increasing amounts of 3A5. When this is done with 10% increments, the results seen in Fig. 6 were achieved. The greater the activity of P450 3A5 for a substrate (M1 > norbuprenrophine > buprenorphine elimination), the less it needs to be present to exert a major influence, and diminish the contribution of other P450 enzymes. This is less so when 3A5 activity towards the probe was assumed to be one-tenth of 3A4. Activity probably lies somewhere in between.

The results of this current study provide more information on the in vitro and in vivo metabolism of buprenorphine and ensuing metabolites. Identification of new metabolic pathways of a drug is of importance for its overall ADME, potential for drug-drug interactions and safety. The clinical relevance of the current study in this regard is hampered by two main

limitations. First, it can be argued that the use of concentrations well above therapeutic (10 μM) as we used in the in vitro studies may compromise conclusions in regard to involvement of the P450s. A recent pharmaceutical perspective on the conduct of in vitro and in vivo drug interactions studies (Bjornsson et al., 2003), however, suggested that as long as the concentration used is less than the $K_m$, useful phenotyping information can be obtained. Reported $K_m$s for buprenorphine metabolism range from 30.1 to 48.5 μM (Kobayashi et al., 1998). Secondly, due to lack of reference material, neither Picard et al., (2005) nor we have been able to quantitate the abundance of the newly identified metabolites. At this time, we can only point to the discrepancy in buprenorphine elimination versus norbuprenorphine formation to suggest that other metabolic pathways have some importance in the intrinsic clearance of buprenorphine. This may be through routes of direct metabolism of buprenorphine (M1 and M2 formation), which could be of importance in drug-drug interactions and safety issues, or through further metabolism of norbuprenorphine (M3, and M4 and M5 formation), which could also impact safety issues, but would only have drug-drug interaction importance if norbuprenorphine is an active metabolite (a contested issue, see Huang, et al. 2001; Ohtani, et al. 1995). In short, additional pathways of buprenorphine metabolism have been identified. The role of P450 2C8, as well as 3A4 and 3A5, in buprenorphine metabolism has been extended; the importance of their individual contributions depend in part on individual contents of each P450.

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Legends for figures:

**Figure 1.** The SIM chromatograms of the hydroxylated buprenorphine metabolites M1 and M2 (A) and the hydroxylated norbuprenorphine metabolites M3, M4 and M5 (B) following incubation of 10 μM buprenorphine for 30 min with HLM at 1.0 mg protein/ml. The dashed line is the control sample incubated with heat-inactivated HLM.

**Figure 2.** CID product ion mass spectra of M1 (A) and M2 (B) and deduced structures.

**Figure 3.** CID product ion mass spectra of M3 (A), M4 (B) and M5 (C), and deduced structures.

**Figure 4.** The time-course of M1 (◆ ), M3 (■ ) and M5 (● ) formation in HLM incubated with 10 μM of buprenorphine (A), and time-course of M3 formation in HLM incubated with 10 μM norbuprenorphine (B). The microsomal protein content is 0.5 mg/ml. The amount of metabolites was expressed as peak area ratio of metabolites to internal standard $d_4$-buprenorphine. Each point is the mean of duplicate experiments.

**Figure 5.** Metabolism in cDNA-expressed human P450s. The formation of M1, M3, and M5 incubated with 10 μM buprenorphine (A); and the formation of M3 incubated with 10 μM norbuprenorphine (B). The incubation time was 20 min and cDNA-expressed P450 content was 25 pmol. Data represent the mean of duplicate incubations. Since nmol P450 added and incubation times were constant, results for new metabolite formation are just given as peak area ratios. Control insect cell microsomes were used at the mean protein concentration averaged over all of the supersomes.

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**Figure 6.** An estimate of the impact of percent P450 3A that is 3A5 has on contribution of P450s to the elimination of buprenorphine (A and D) and the formation of norbuprenorphine (B and E) and M1 (C and F). RAF scaling was performed with the percentage of total 3A being 3A5 increased and 3A4 decreased in 10%-increments. For Figures A-C it was assumed that 3A5 activity towards the probe substrate was equal to that of 3A4. For Figures D-E it was assumed that 3A5 activity is one-tenth that of 3A4 as is found in cDNA-expressed P450s. Note: 3A5 participation is adjacent to 3A4, the area between 3A5 and the top of the graph represents participation of other P450s.

**Scheme 1**. The proposed phase I metabolic pathways of buprenorphine in HLM.

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Table 1. The effect of immuno- and chemical inhibition on the metabolism of buprenorphine

| | Elimination of | Formation of | | | |
|---|---|---|---|---|---|
| Inhibitor | Bup | Nor | M1 | M3 | M5 |
| | | (% Inhibition) | | | |
| A) HLM 452013: higher 2C8, lower 3A4/5 | | | | | |
| Anti-3A4/5 | 12 | 7 | 14 | 74 | 87 |
| Anti-2C8 | 77 | 83 | 67 | 14 | 12 |
| Ketoconazole | 18 | 14 | 4 | 89 | 100 |
| Trimethoprim | 28 | 32 | 14 | 38 | 0 |
| B) HLM 452164: lower 2C8, higher 3A4/5 | | | | | |
| Anti-3A4/5 | 76 | 60 | 48 | 95 | 94 |
| Anti-2C8 | 3 | 18 | 18 | 0 | 0 |
| Ketoconazole | 77 | 59 | 48 | 95 | 90 |
| Trimethoprim | 7 | 13 | 16 | 38 | 7 |

Note: The testosterone 6 hydroxylase activities (3A4/5) of HLMs 452013 and 452164 are 890 and 4100, respectively; the paclitaxel 6 -hydroxylase activities (2C8) are 380 and 78, respectively. The activities expressed as pmol product per (mg protein×minute).

The recommended volumes (10 l) of MAbs specific for P450 3A4/5 or 2C8 was used. The concentrations of ketoconazole and trimethoprim were 2 μM and 100 μM, respectively.

Results are the mean of duplicate incubations. Formation of norbuprenorphine and M1 were determined after 10 minute incubations; all others after 30 minute incubations.

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**Table 2.** Relative activity factor (RAF) versus immunoquantitation scaling of cDNA-expressed P450 activity for buprenorphine (Bup) utilization and formation of norbuprenorphine (Nor) and M1

| | RAF | | | Immunoquantitation | | |
|---|---|---|---|---|---|---|
| P450 | Bup | Nor | M1 | Bup | Nor | M1 |
| | (Percent contribution) | | | | | |
| 1A2 | 2.1 | 0.3 | 0.4 | 1.1 | 0.2 | 0.3 |
| 2A6 | 4.8 | 0.0 | 0.0 | 1.3 | 0.0 | 0.0 |
| 2B6 | 0.0 | 0.0 | 0.1 | 0.0 | 0.0 | 0.3 |
| 2C8 | 14.5 | 36.4 | 70.2 | 6.4 | 21.9 | 47.5 |
| 2C9 | 0.0 | 5.1 | 0.0 | 0.0 | 1.0 | 0.0 |
| 2C19 | 0.1 | 0.1 | 0.1 | 0.3 | 0.4 | 0.5 |
| 2D6 | 0.4 | 0.0 | 0.0 | 0.3 | 0.0 | 0.0 |
| 2E1 | 0.0 | 9.7 | 0.0 | 0.0 | 0.8 | 0.0 |
| 3A | 78.1 | 48.4 | 29.2 | 90.5 | 75.6 | 51.4 |

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**Table 3** Correlations between buprenorphine metabolism and P450 3A and 2C8 activities

| Reaction | Correlation Coefficient (R) | |
|---|---|---|
| | Testosterone 6ß-hydroxylation (3A) | Paclitaxel 6α-hydroxylation (2C8) |
| Buprenorphine elimination | 0.896* | 0.381 |
| Norbuprenorphine formation | 0.776* | 0.598* |
| M1 formation | 0.683*† | - 0.261 |
| M3 formation | 0.963* | 0.316 |
| M5 formation | 0.921*† | - 0.212 |

The experiment was performed with 10 μM of buprenorphine in a panel of 15 human liver microsomes. The incubation time was 10 min (30 min for M5). Data are the means of duplicate experiments.

* Significant correlation, $p<0.05$.

† Data were obtained by excluding three HLMs with highest 3A activity.

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**Table 4** The relative amount of M1 and M3 in hydrolyzed (*) and non-hydrolyzed human urine samples from seven subjects.

| Metabolites | Subjects | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| | Peak area ratio | | | | | | |
| M1 | 0 | 0.025 | 0.005 | 0.014 | 0 | 0.007 | 0.008 |
| M1* | 0.192 | 3.997 | 0.247 | 0.402 | 0.084 | 0.838 | 1.469 |
| M3 | 0.335 | 0.531 | 0.714 | 0.419 | 0.185 | 0.517 | 0.145 |
| M3* | 0.563 | 0.921 | 0.928 | 0.788 | 0.178 | 0.870 | 0.211 |

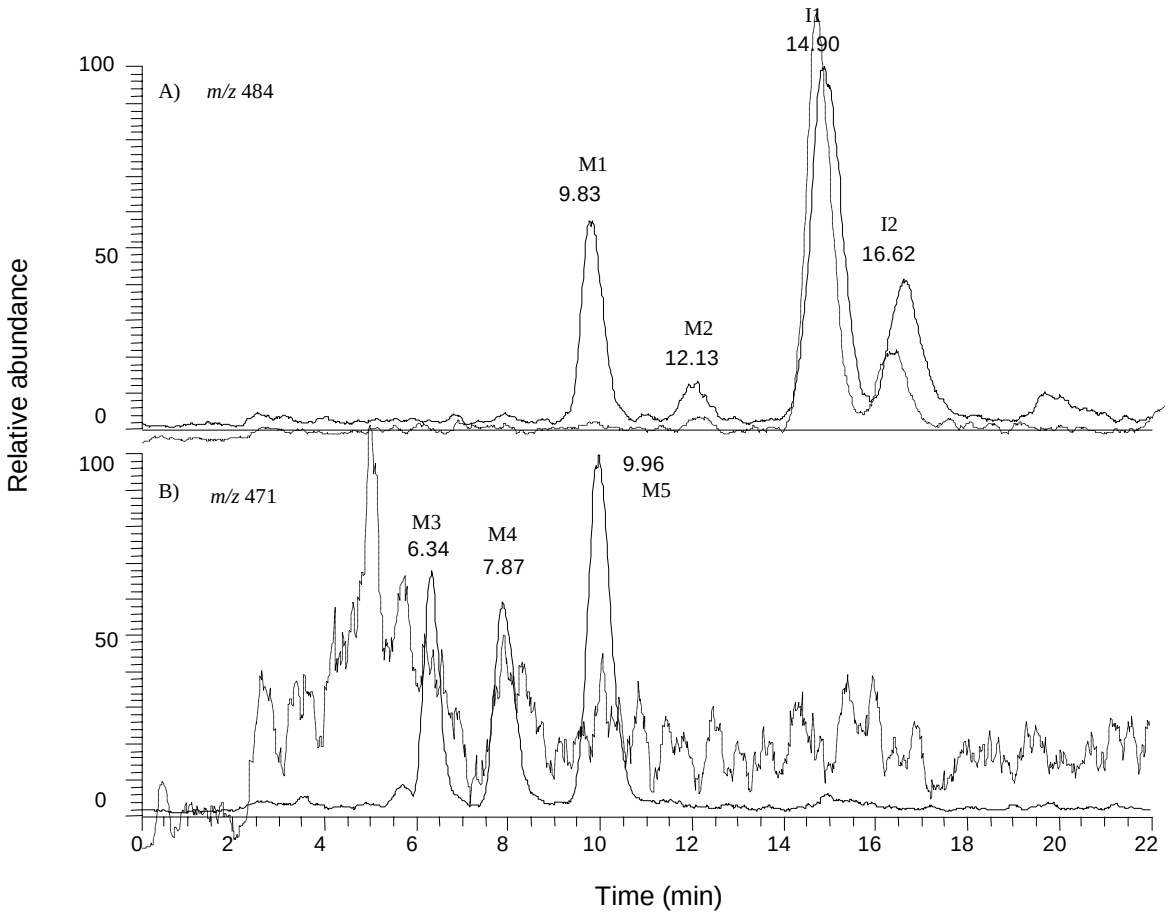


Figure 1

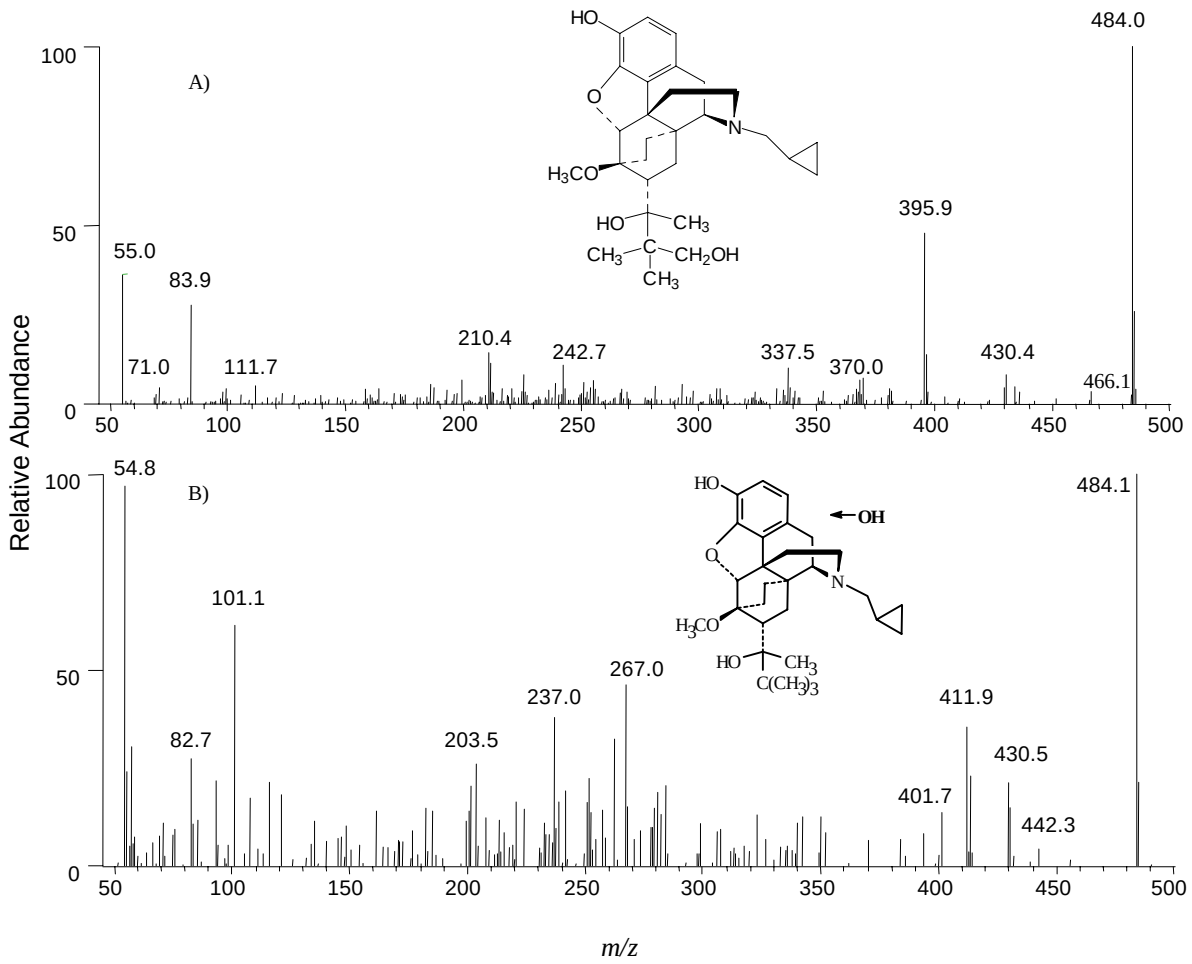


Figure 2

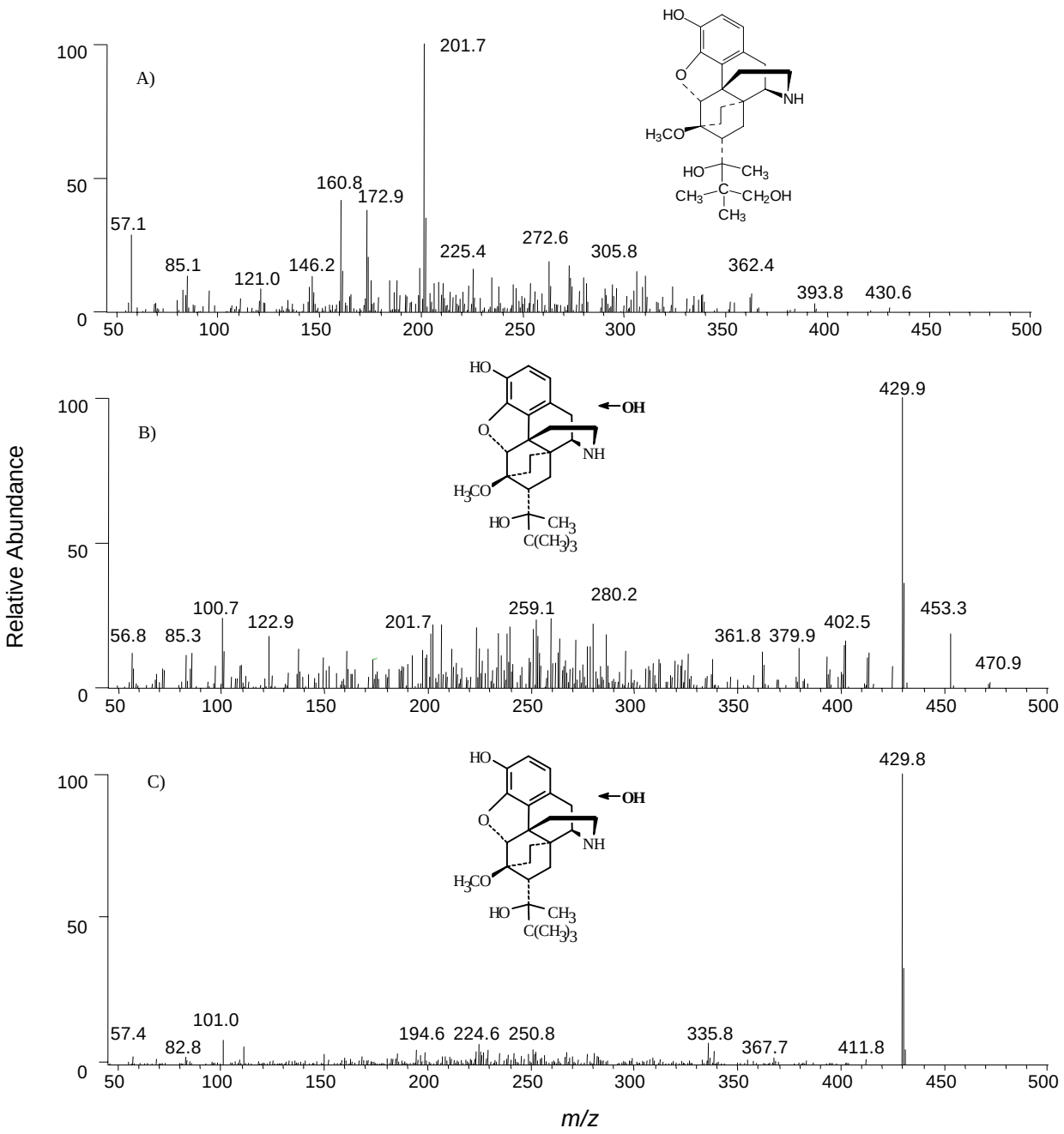


Figure 3

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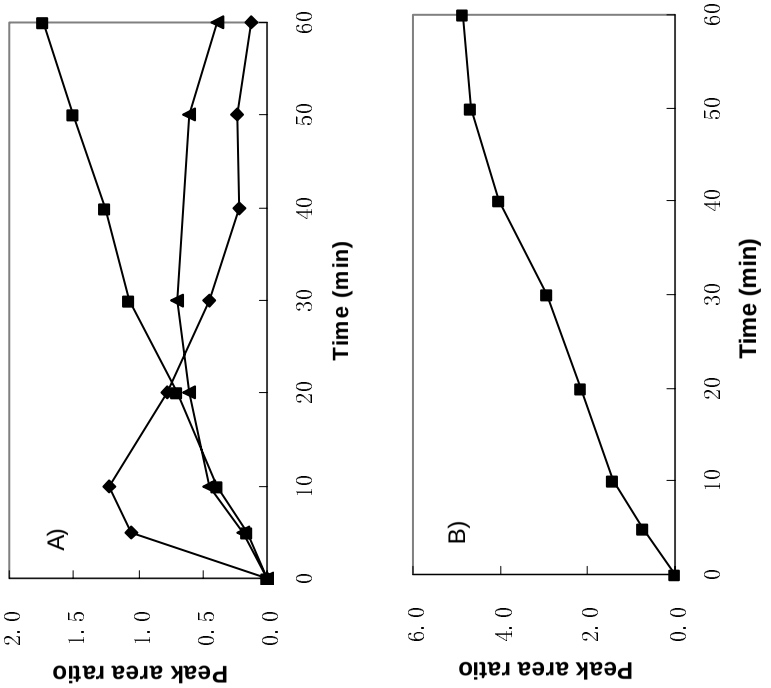


Figure 4

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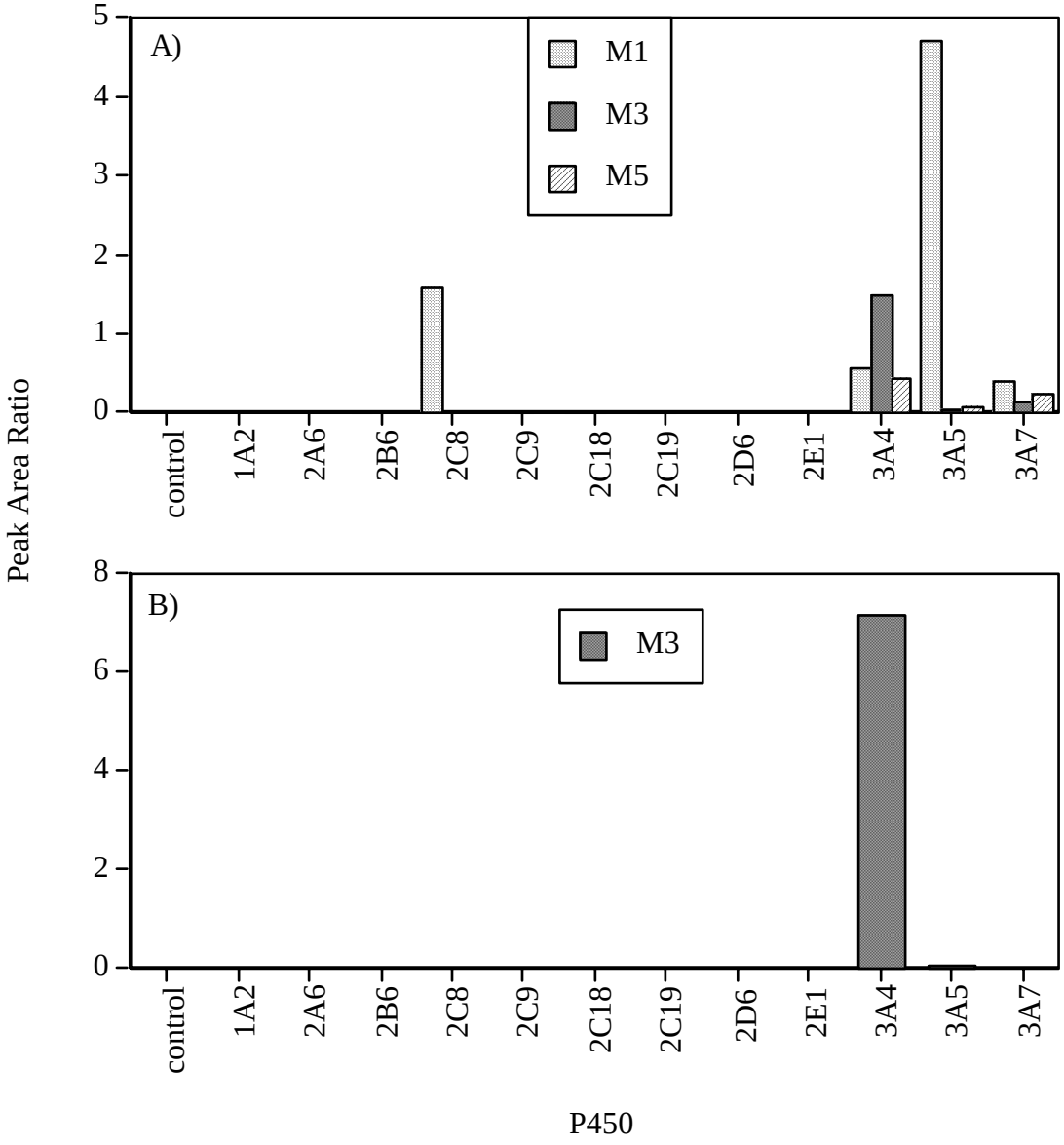


Figure 5

Scheme 1

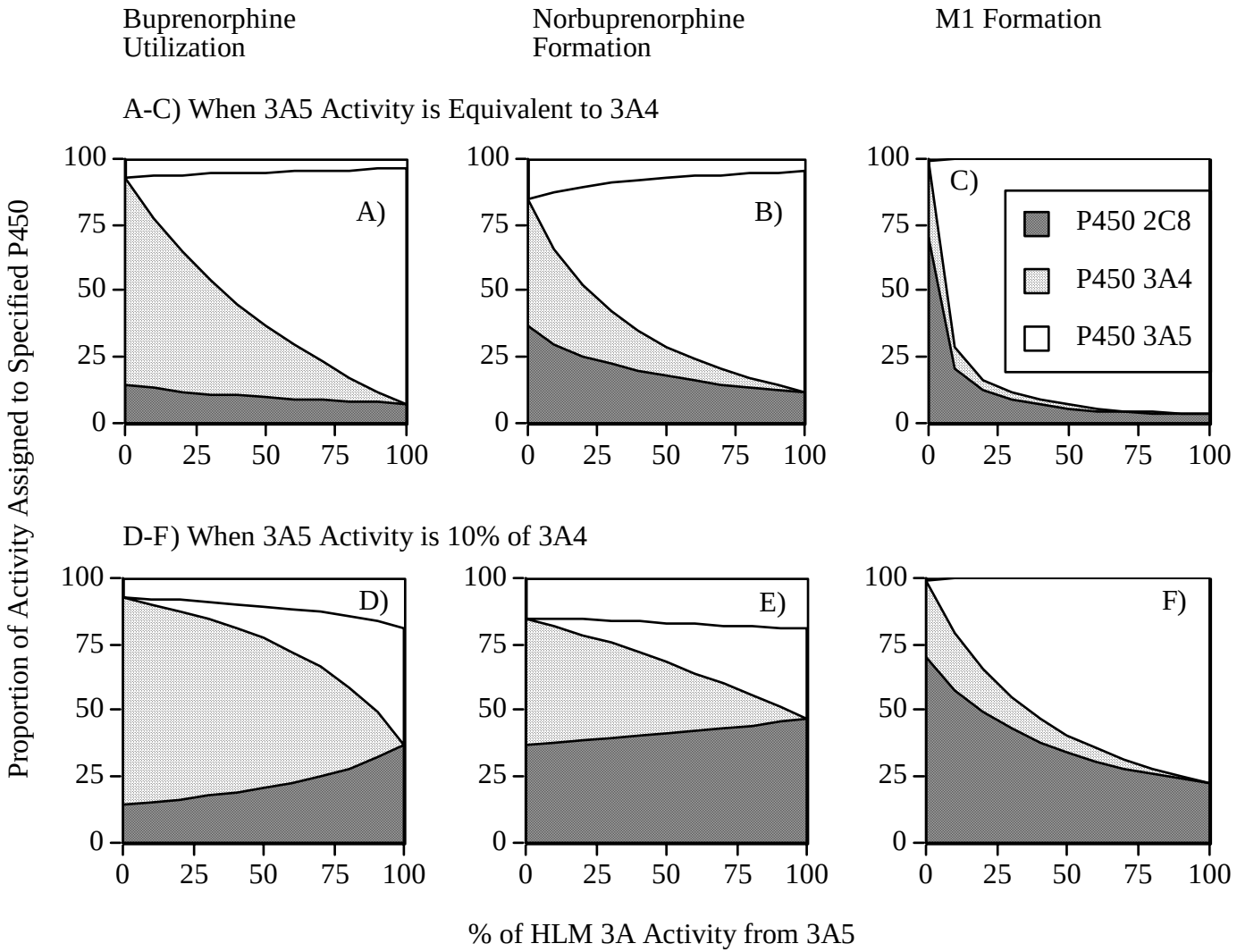


Figure 6