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# IN VITRO PREDICTION AND IN VIVO VERIFICATION OF ENANTIOSELECTIVE HUMAN TOFISOPAM METABOLITE PROFILES

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d) Non-standard abbreviations: BID, twice a day; TID, three times a day

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## ABSTRACT

*In vitro* studies were conducted to elucidate the metabolic profiles of and the responsible enzymes for the metabolism of (*R*)- and (*S*)-tofisopam (1-(3,4-dimethoxyphenyl)-5-ethyl-7,8-dimethoxy-4-methyl-5H-2,3-benzodiazepine). Large differences were observed between the two enantiomers. The major metabolite in incubations of 500 ng/ml (≈1.3 μM) (*R*)-tofisopam in human liver microsomes corresponded to demethylation of the methoxy group at the 4 position of the phenyl ring (M3). Incubating (*R*)-tofisopam with recombinant P450 or with human liver microsomes and isoform selective P450 chemical inhibitors indicated that M3 was primarily catalyzed by CYP2C9. Similar incubations with S-tofisopam indicated that the primary metabolite was due to demethylation of the methoxy group at the 7 position of the benzodiazepine ring (M1), and this reaction was catalyzed primarily by CYP3A4. The primary metabolites of both enantiomers were further demethylated to form a common di-demethylated metabolite (M5), where the methoxy groups at positions 4 and 7 are demethylated. Analysis of plasma and urine samples from human clinical trials confirmed the *in vitro* observations. Subjects orally treated with 200 mg BID (*R*)-tofisopam had a 2 hr M1:M3 plasma ratio of 1:29 and a ratio of 1:123 in urine; whereas, patients orally administered (*S*)-tofisopam at 150 mg TID mg/kg had opposite M1 to M3 ratios of 8:1 in plasma and 6:1 in urine.

## INTRODUCTION

The use of pure enantiomers as pharmaceuticals has greatly increased the awareness of the effect of chirality on the interactions of small molecules with proteins. In addition to the development of new drugs, several older racemic drugs such as nefopam and thioridazine have been re-introduced as pure enantiomers (Brian, 2001). In cases where one of the enantiomers shows greater activity, the use of pure enantiomers can have key advantages such as improved pharmacokinetics or decreasing non-specific toxicity problems. There are instances in which toxicity has been linked to one member of a pair of stereoisomers, not necessarily the active isomer (FDA, 1992).

Racemic tofisopam (1-(3,4-dimethoxyphenyl)-5-ethyl-7,8-dimethoxy-4-methyl-5H-2,3-benzodiazepine) is approved in 18 countries including Japan, South Korea, Russia, and Hungary and has been used for many years to treat disorders of the autonomic nervous system (Pakkanen et al., 1980; Hovi-Viander et al., 1985; Andrasi et al., 1987; Nedogoda and Parshev, 2000). Recently the individual enantiomers have been evaluated for their pharmacological activity. Animal models on both (*R*)- and (*S*)-tofisopam suggest that they decreased visceral hypersensitivity, have no effect on normal GI function, and normalize GI motility in stressed conditions. (*R*)-Tofisopam has an improved activity for irritable bowl syndrome and is currently in Phase IIb clinical trials. Metabolism of racemic tofisopam in microsomal incubations from multiple species was previously reported (Elekes et al., 1981; Tomori et al., 1982; Tomori et al., 1984) and the primary metabolites were identified. These metabolites corresponded to demethylation of one or more of the four tofisopam methoxy groups by cytochrome P450. All previous studies detailing tofisopam metabolism were conducted on racemic tofisopam instead of

individual enantiomers and did not evaluate the specific enzymes responsible for tofisopam metabolism.

In the present study, we present data on the stereoselective metabolism of the individual enantiomers of tofisopam. The concentration of tofisopam 50 and 500 ng/ml (≈0.13 and 1.3 µM) was chosen to represent clinical concentrations. *In vitro* studies with recombinant P450 enzymes and with human liver microsomes, (*R*)- and (*S*)-tofisopam showed different metabolite profiles for the two enantiomers. The primary enzymes responsible for generation of the individual metabolites were identified. These *in vitro* results were confirmed by analyses of samples from human clinical trials.

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## MATERIALS AND METHODS

*Chemicals used*. Pure enantiomers of tofisopam, racemic metabolites, and $d_6$-tofisopam were synthesized by Cerilliant (Round Rock, TX). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) in the highest purity available. Human liver microsomes (Lot No: 821-1) pooled from 15 individuals (male and female) were obtained from CellzDirect (Austin, TX). Recombinant P450 were *E. coli* expressed and purchased from CYPex (purchased through Xenotech, Lenexa, KS). When multiple reductase levels were available for individual P450 isoforms, the higher level was purchased.

*Analytical method*. The method was validated for a range of 1.00 to 500 ng/mL based on analysis of 0.500 mL sample (recombinant P450 incubates, microsomal incubates or clinical plasma or urine samples). Tofisopam and its six major reported metabolites, M1-M6 (Klebovich and Abermann, 1993) were analyzed with a single injection. Metabolite naming convention is shown in Table 1. Samples were extracted into 2 ml of ethyl acetate, dried, reconstituted in 100 µl of mobile phase and 5 µl was injected onto a Micromass Quattro II LC-MS/MS operating in positive ion mode with a heated nebulizer equipped with a Luna Silica HPLC column from Phenomenex Inc. (3 µm 4.6x250 mm) using a mobile phase of hexane:acetone:THF:TFA (112.5:137.5:12.5:1). The column was held in a column heater maintained at 70$^{o}$C. Over the 15-minute run time, analytes were resolved based on isocratic chromatographic separation and mass differences. Analytes were quantitated based upon the peak areas of product ions of tofisopam, *m/z* 383.4→298.4; M1-M4, *m/z* 369.4→284.3; and M5-M6,

*m/z* 355.4→242.2 measured against the peak areas of product ions of the internal standard $d_6$-tofisopam, m/z 389.4→304.4. Quantitation was performed using weighted ($1/x^2$) linear least squares regression analyses generated from calibration standards extracted immediately prior to each run.

*Incubations with a recombinant P450 enzyme*. Incubations consisted of 5 pmoles of recombinant P450, 500 ng/ml, ≈1.3 μM, tofisopam (or metabolite), and 100 mM potassium phosphate buffer, pH 7.4, in a final incubation volume of 0.5 ml. Incubations were initiated by the addition of NADPH (1 mM final concentration) and stopped after 15 minutes by the addition of 0.5 mL ethyl acetate and vortexing. An additional 1.5 ml of ethyl acetate was added to the samples just prior to the liquid:liquid extraction. Incubations to determine $K_m$ and $V_{max}$ were conducted similarly but the enzyme level was decreased to 2 pmoles of recombinant P450 and the incubation time was shortened to 5 minutes to make sure tofisopam did not become limiting in the reactions. Tofisopam concentration of 0.033, 0.1, 0.33, 1, 3.3, 10, 33, and 100 μM were examined. Tofisopam stock solutions were prepared in methanol and the final concentration of methanol in the reactions was 0.4% methanol.

*Incubations with human liver microsomes*. All microsomal incubations were conducted in 100 mM phosphate buffer, pH 7.4, initiated by the addition of NADPH (1 mM final concentration), and stopped by the addition of 0.5 mL ethyl acetate and vortexing after a predetermined incubation time period. Pilot microsomal incubations were conducted to determine appropriate concentrations of tofisopam and human liver microsomal protein, as well as incubation time. On the basis of these pilot incubations, the following incubation conditions were used for the remainder of the microsomal work:

500 ng/mL tofisopam, 0.2 mg/mL human liver microsomes and a 10-minute incubation time.

The selective inhibition of human liver microsomal enzymes during incubations with (*R*)- and (*S*)-tofisopam were monitored for the conversion of these tofisopam enantiomers to their primary metabolites M1 and M3 and the secondary metabolite M5 (Table 1). Additionally, in order to evaluate the enzymes involved in the formation of M5, racemic M1 and M3 were used as starting substrates. Incubations were conducted in duplicate and samples were analyzed as previously described. The following P450 isoform selective inhibitors were utilized (Masimirembwa et al., 1999; Sai et al., 2000; Bjornsson et al., 2003): CYP1A2, furafylline, 30 µM; CYP2C9, sulfaphenazole, 10 µM; CYP2C19, (*S*)-mephenytoin, 250 µM; CYP2D6, quinidine, 10 µM; and CYP3A4, ketoconazole, 10 µM. Inhibitor stock solutions were prepared in appropriate solvents (Hickman et al., 1998), either methanol (sulfaphenazole, quinidine, and ketoconazole) or acetonitrile (furafylline and (*S*)-mephenytoin) and contributed 0.4% organic solvent to the final incubation. The formation of M1, M3, and M5 were quantitated and compared against appropriate solvent controls (no inhibitor). Furafylline, the selective inhibitor used for CYP1A2, inhibits through direct competitive inhibition for the binding pocket and through the formation of a reactive imidazomethide intermediate (Racha et al., 1998) which leads to CYP1A2 inactivation. Because the inactivation of furafylline is time dependent, furafylline is a more potent inhibitor if the microsomes are preincubated. We did not preincubate furafylline containing samples because we did not want the rate of tofisopam metabolism to be artificially decreased due to thermal inactivation of microsomal proteins and because 30 µM furafylline sufficiently inhibits CYP1A2

without pre-incubation. Another common CYP1A2 inhibitor α-naphthoflavone was not used because it has been shown to alter the binding geometry of benzodiazepines within CYP3A4 (Cameron et al., 2005).

*Extraction procedure for recombinant P450 or microsomal incubates.* 0.5 mL incubates were treated with the addition of 2.0 mL ethyl acetate (0.5 mL was used to stop the reaction and an additional 1.5 ml was added just prior to the extraction) and 25 µL internal standard ($d_6$-tofisopam at 2.0 µg/mL in methanol). The organic layer was transferred to a new tube, evaporated to dryness, reconstituted in mobile phase and injected.

*Extraction procedure for clinical plasma or urine samples.* Clinical samples were obtained from trials performed in the Netherlands designed to evaluate repeated escalating doses for safety and pharmacokinetics. All patients were fully consented and appropriate regulations were followed. (*R*)-tofisopam patients were doses 100 mg and 200 mg BID for one week, oral capsules. The Day 7 200 mg BID samples are presented in the manuscript. Plasma (two and four hours post-dose) and urine (pooled 0 to 12 hours post-dose) samples were evaluated for tofisopam and its major metabolites. The data presented represents 13 healthy caucasian female volunteers, 56 +/- 5 years of age. (*S*)-tofisopam patients were dosed 50 mg and 150 mg TID for a week, oral capsules. The Day 7 150 mg TID samples were evaluated for metabolite profiles and are presented in the manuscript. The data represents 14 healthy volunteers (12 caucasian, 1 oriental, 1 black), 3 male (ages 23, 26, 42) and 11 female (age 55 +/- 3).

Plasma and urine samples were stored at -80$^{o}$C and thawed immediately prior to analysis. Samples were analyzed with and without Glusulase® (PerkinElmer Life

Sciences, cat# NEE-154) treatment to remove glucuronide or sulfate conjugates resulting from phase II metabolism. For Glusulase® treated samples, 2.0 µL of Glusulase® solution was added to 0.500 mL of sample. Samples were then incubated at room temperature for 1 hour prior to analysis. Samples were treated by the addition of 25 µL internal standard ($d_6$-tofisopam at 2.0 µg/mL in methanol), then extracted using solid phase extraction cartridges (Bond Elut C18 200mg, 3mL, Cat# 1210-2025, Varian) eluted with methanol, evaporated to dryness, reconstituted in mobile phase and injected. Samples that were above the standard curve range were diluted with blank plasma or urine and re-evaluated. Appropriate dilution QCs were included to verify the appropriateness of the dilution scheme.

## RESULTS

*Analytical Method.* To evaluate the metabolites of tofisopam, an analytical method was developed. The similar polarity of the possible monodemethylated metabolites (M1 – M4) yielded poor results with reverse phase chromatography columns. Chromatography was further complicated by shoulders caused by the conformational changes of the diazepine rings. With column heating to 70$^{o}$C, the peaks were successfully resolved using normal phase chromatography. A sample chromatogram is shown in Figure 1. It was not necessary to develop an enantiomeric method since incubations were performed with pure enantiomers. In addition, there is no evidence of bioconversion between the two enantiomers of the compound. The method was further refined to achieve satisfactory results in recombinant P450 and microsomal incubates, human plasma, and urine. The final conditions are reported in the Materials and Methods section. Specific fragment ions for the individual monodemethylated metabolites could be obtained; however, under the conditions required to generate unique fragments the molecule was extensively fragmented and there was minimal signal intensity. All four of the potential monodemethylated metabolites efficiently fragmented at the diazepine ring giving acceptable signal intensity with parent and daughter ions of the same masses. Therefore, a decision was made to choose the transition with the best signal intensity and to concentrate efforts on obtaining baseline resolution of the monodemethylated metabolite peaks. Synthetic standards were included in runs to verify the identity of individual peaks.

*Recombinant P450 Incubations*. Incubations contained 5 pmoles of recombinant cytochrome P450 and were conducted with 500 ng/ml (≈1.3 μM) of tofisopam to

evaluate the potential pathways for tofisopam metabolism. Separate incubations containing either (*R*)- or (*S*)-tofisopam were conducted and the generation of monodemethylated metabolites was monitored. Additional incubations with racemic M1 or M3 were set up to evaluate the conversion of M1 or M3 to the didemethylated metabolite M5. The rate of demethylation at the 7 position of the benzodiazepine ring to form M1 was highest for CYP3A4 and second highest for CYP3A5 with both enantiomers, Figure 2. Similarly, the rates for conversion of M3 to M5, which also involves demethylation at the 7 position of the benzodiazepine ring, was highest for the same enzymes. The rate of demethylation at the 4 position of the phenyl ring to form M3 was greatest for CYP2C9 followed by CYP2C19 for both enantiomers and CYP1A2 for the S enantiomer. Conversion of M1 to M5, which also involves demethylation at the 4 position of the phenyl ring, was catalyzed at the highest rate by CYP2C9 and CYP2C19.

Kinetic constants were determined for all of the enzymes that demonstrated the potential to metabolize tofisopam, CYP1A2, CYP2C9, CYP2C19, CYP3A4, and CYP3A, plus CYP2C8 which was indicated in a previous study *(Niwa et al., 2005)* as being involved in the metabolism of racemic tofisopam. To assure the experimentally determined rates were conducted under initial rate conditions, the amount of recombinant P450 was reduced from 5 pmol to 2 pmol and the incubation time was decreased from 15 minutes to 5 minutes. The Tofisopam concentrations between 0.033 to 100 µM were evaluated. The intrinsic clearance of M1 formation, as determined by $V_{max}/K_m$, was over thirty times higher for CYP3A4 than for any of the other enzymes tested, Table 2. The higher intrinsic clearance observed for CYP3A4 was primarily due to its low $K_m$ of 0.8 µM. The enzyme with the highest observed intrinsic clearance for M3 formation is

CYP2C9. This is in agreement with the data in figure 2. It should be pointed out that at high tofisopam concentrations multiple P450 could metabolize tofisopam. Indeed, all six of the P450 tested were able to generate both M1 and M3 in incubations of (*S*)-tofisopam. These are represented in Table 2 with an asterisk as their $K_m$ was above 100 μM.

In the recombinant incubations, all isoforms were evaluated at the same enzyme concentration. Physiologically there are large differences in the expression levels of the different isoforms. Additionally, recombinant P450 from different commercial vendors have reported $V_{max}$ values that differ by several folds. To get a better idea of the most likely physiological routes of tofisopam metabolism, further evaluation was conducted using pooled human liver microsomes.

*Pilot Human Liver Microsomal Incubations.* Pilot incubations were set up to determine the conditions to conduct the phenotyping study. Before setting up the phenotyping study, several factors needed to be determined. First, the effects of incubation time and microsomal protein concentrations on the formation rates of all metabolites of interest needed to be determined for the optimal incubation conditions for (*R*)- and (*S*)-tofisopam metabolism in human liver microsomes. Second, the major metabolites of both (*R*)- and (*S*)-tofisopam needed to be confirmed in human liver microsomes. Finally, conditions where the tofisopam concentration would not become limiting during the experiments needed to be found. In order to evaluate all these parameters, pilot incubations were carried out with two substrate concentrations (50 or 500 ng/mL for (*R*)- or (*S*)-tofisopam), three concentrations of human liver microsomes (0.05, 0.2, or 1.0 mg/mL), and five incubation time intervals (0, 10, 20, 40, or 60 minutes). Results of the 500 ng/mL pilot incubations are shown in Figure 3 for (*R*)-

tofisopam and in Figure 4 for (*S*)-tofisopam. Similar trends were observed for the 50 ng/mL incubations (data not shown). The major metabolites for all incubations were found to be M1, M3, and M5. Levels of M2, M4, and M6 were insignificant. On the basis of these pilot incubations, phenotyping experiments were conducted using 500 ng/mL substrate, 0.2 mg/mL human liver microsomes and a 10-minute incubation time. These conditions were chosen because sufficient levels of the metabolites were generated, and could be quantitated even if 90% inhibition were observed during the chemical inhibitor experiments. Additionally, tofisopam levels were decreased by less than 30% during the course of the reactions.

Evaporation of methanol in the tofisopam spiking solutions was observed during the pilot incubation experiments and accounts for the sum of metabolites exceeding 500 ng/ml. This does not affect the conclusions on the optimal incubation conditions. All study spiking solutions were freshly prepared and stored on ice.

*Chemical inhibition of individual P450 in human liver microsomes.* The metabolism of (*R*)- and (*S*)-tofisopam and racemic M1 and M3 were investigated by setting up incubations containing selective chemical inhibitors of cytochromes P450. Chemical inhibitors were evaluated for all of the isozymes that were shown to have the potential to metabolize tofisopam or its primary metabolites in the recombinant P450 incubation experiments. Quinidine, a selective inhibitor of CYP2D6, was also evaluated and as expected did not significantly inhibit tofisopam metabolism. The incubation of (*R*)-tofisopam with human liver microsomes primarily generated the monodemethylated metabolite M3 (demethylation at the 4 position of the phenyl ring), with a calculated rate of formation of 57.0 ng/min/mg when uninhibited. The rate of M3 formation was

observed to decrease by 93% when the CYP2C9 inhibitor sulfaphenazole was added to the incubation, and by 64% when the CYP3A4 inhibitor ketoconazole was added to the incubation (Figure 5). It should be pointed out that although ketoconazole is referred to as a selective CYP3A4 inhibitor in this manuscript and elsewhere in the literature; it is known to inhibit other isozymes as well. For example, inhibition constants ($K_i$ values) for ketoconazole have been determined to be 8, 7, and 0.03-0.4 for CYP2C9, CYP2C19, and CYP3A4, respectively (Emoto et al., 2003). In the current study, ketoconazole was used at 10 μM**,** to completely inhibit CYP3A4; however, some inhibition of CYP2C9 and CYP2C19 can be expected. Chemical inhibitors of the remaining P450 isozymes did not appear to inhibit M3 formation by more than 40%. Demethylation at the 7 position of the benzodiazepine ring to form M1 was a minor metabolite of (*R*)-tofisopam metabolism in human liver microsomes and M1 had a calculated rate of formation of 4.0 ng/min/mg, significantly lower than the rate of M3 formation. The rate of M1 formation was decreased by over 95% when the CYP3A4 inhibitor ketoconazole was added to the incubation. Other chemical inhibitors did not appear to inhibit M1 formation. Curiously, addition of the CYP2C9 inhibitor, sulfaphenazole, resulted in a 62% increase in the rate of M1 formation. It is likely that the increased M1 concentration was due to decreasing the further metabolism of M1 to form M5 by blocking CYP2C9. This conclusion is supported by the results from incubations of racemic M1 to form M5. In those experiments, addition of sulfaphenazole resulted in a 92% inhibition of the rate of M5 formation (see below).

The calculated rate of M5 formation from (*R*)-tofisopam in human liver microsomes was 3.04 ng/min/mg; however, this rate is the result of two demethylation

steps. The formation of M5 is dependent upon the presence of either M1 or M3, and to further complicate the determination of the rate of M5 formation in human liver microsomal incubations of (*R*)-tofisopam, the concentrations of M1 and M3 change significantly over the course of the incubation. Even with these limitations, the observed effects of the addition of selective inhibitors on M5 formation can still provide valuable information. Starting with (*R*)-tofisopam, the rate of M5 formation decreased by 90% when the CYP2C9 inhibitor sulfaphenazole was added to the incubation and 96% when the CYP3A4 inhibitor, ketoconazole was added to the incubation (Figure 5). These findings are supported by the data presented above for M1 and M3 formations, since the formation of M5 is dependent upon the formation of either M1 or M3 from tofisopam. Specifically, as mentioned above, M3 formation from (*R*)-tofisopam, which requires demethylation at the 4 position of the phenyl ring, was observed to be primarily catalyzed by CYP2C9. The formation of M1 from (*R*)-tofisopam, which requires demethylation at the 7 position of the benzodiazepine ring, was observed to be catalyzed by CYP3A4. Because the generation of the secondary metabolite M5 requires demethylations at both of these positions, it is not surprising that inhibition of either the CYP2C9 or the CYP3A4 pathways inhibited M5 formation.

In contrast to (*R*)-tofisopam, the major metabolite of (*S*)-tofisopam metabolism by human liver microsomes was M1, with a calculated rate of formation of 57 ng/min/mg when uninhibited. The rate of M1 formation was observed to decrease by over 99% when the CYP3A4 inhibitor ketoconazole was added to the incubation (Figure 6). Selective chemical inhibitors of the remaining P450 isozymes did not appear to inhibit M1 formation by more than 40%. The rate of M3 formation from (*S*)-tofisopam, which was

observed to be 7.4 ng/min/mg, was almost 8 times lower than the rate of M1 formation. The rate of M3 formation decreased by 51% upon addition of the CYP2C9 inhibitor sulfaphenazole and 76% upon addition of the CYP3A4 inhibitor ketoconazole (Figure 6). Similar to the incubations using (*R*)-tofisopam, the formation of M1 appeared to be catalyzed by CYP3A4 and formation of M3 appeared to be generated by both CYP2C9 and CYP3A4. CYP3A4 appears to play a more significant role in M3 formation for the *S* enantiomer than for the *R* enantiomer. This observation was reinforced by the recombinant P450 results in Figure 2.

In order to further clarify the P450 isozymes responsible for tofisopam metabolism, synthetic racemic M1 and M3 were incubated with human liver microsomes in the presence of the same selective chemical inhibitors. Results from these incubations agreed and reinforced the results of the previous enantiomeric tofisopam incubations. When the racemic tofisopam metabolite, M1, was used as the substrate, it was possible to follow the conversion of M1 to M5 under conditions that better approximate first order enzyme kinetics. The calculated rate of M5 formation was 71.2 ng/min/mg. The rate of M5 formation decreased by 92% when the CYP2C9 inhibitor sulfaphenazole was added to the incubation and 54% when the CYP3A4 inhibitor ketoconazole was added to the incubation (Figure 7). This result was expected from observations of earlier incubations where the demethylation at the 4 position of the phenyl ring was catalyzed by both enzymes. When the racemic tofisopam metabolite M3 was used as the substrate, the calculated rate of M5 formation was 21.9 ng/min/mg, less than 1/3 of the rate of M5 formation when M1 was the substrate. The rate of M5 formation did not appear to be affected by the CYP2C9 inhibitor sulfaphenazole, but was decreased by >99% when the

CYP3A4 inhibitor ketoconazole was added to the incubation. This was also found to be in agreement with the enantiomeric tofisopam incubations. The proposed reaction schematic for (*R*)- and (*S*)-tofisopam are shown in Figures 8 and 9, respectively.

*Evaluation of Clinical Samples.* Based upon the *in vitro* experiments, it was expected that clinical samples from an (*R*)-tofisopam study would have M3 as the primary metabolite whereas samples from an (*S*)-tofisopam study would have M1 as the primary metabolite. Plasma and urine samples were from a previously conducted study evaluating safety and pharmacokinetics in a 7 day repeated dose study. Plasma sample were 2 and 4 hr following the final oral dose. Tofisopam metabolites were found to be extensively glucuronidated, similar to reports by previous researchers (Klebovich and Abermann, 1993). As predicted from our *in vitro* experiments, the (*R*)-tofisopam plasma samples had a significantly higher concentration of M3 than M1 with a M1 to M3 ratio of 1:29 at the two-hour time point, Table 3. Conversely, in (*S*)-tofisopam clinical samples, the M1 to M3 ratio was 8:1, Table 4. Similarly, results from the analysis of human urine samples was also in agreement to the *in vitro* data. Urine was pooled over a 12 hour period post drug administration. Similar to human plasma samples, human urine samples were highly glucuronidated. The ratio of M1 to M3 for (*R*)-tofisopam clinical samples was 1:123 and for (*S*)-tofisopam was 6:1, Table 5.

## DISCUSSION

The first major hurdle in the current study was the development of an acceptable analytical method. Due to the similar polarity of the metabolites, normal phase chromatography was employed. Earlier studies on metabolites of tofisopam were unable to obtain clean chromatographic separation of the four potential monodemethylated products (Tomori et al., 1982). Aided by improvement in the commercially available chromatography columns, we were able to develop an LC-MS/MS method for this study to resolve tofisopam and its metabolites in a single injection.

In this study, tofisopam metabolites M1, M3 and M5 were observed at significant concentrations in human recombinant P450, human liver microsomal incubates, and in human plasma and urine. Although additional tofisopam metabolites (M2, M4 and M6) in species other than human were reported in the literature (Klebovich and Abermann, 1993), they were found in inconsequential levels in the present study. The ions monitored by the LC-MS/MS assay would have detected all of the mono- and di-demethylated tofisopam metabolites. Further demethylation to form tri- and tetra-demethylated products have also been reported (Klebovich and Abermann, 1993). We did not focus on these metabolites as they appear to be minor.

It is clear that the metabolism of tofisopam is affected by stereospecificity. To our knowledge, this is the first report of stereoselective metabolism of tofisopam. A recent study using recombinant P450 evaluated the ability of individual isozymes to metabolize a number of psychotropic drugs, including racemic tofisopam (Niwa et al., 2005). The study followed tofisopam depletion so no information was given about the metabolites generated. CYP3A4 was reported to have the highest rate of tofisopam

depletion and CYP2C9 was reported to have approximately 1/5 of the CYP3A4 activity and CYP2C8 was 1/10. In our study, we observed that CYP2C8 did not metabolize tofisopam or its metabolites at significant rates at the clinically significant tofisopam concentrations, but CYP2C19 and CYP3A5 could contribute to tofisopam metabolism. Rates calculated by recombinant enzymes need to be placed in physiological context as the concentration of individual P450 isoforms is highly variable. Average hepatic CYP2C9 levels have been reported to be 5-fold higher than CYP2C19 levels in human (Lasker et al., 1998). Given the difference in expression levels and that the specific activity of CYP2C9 was significantly higher than CYP2C19, it is not surprising that the human liver microsomal studies indicated that CYP2C9 was more important than CYP2C19 to tofisopam metabolism.

Although the incubation of tofisopam with human liver microsomes generates three major metabolites, there are only two sites of metabolism. The sites of tofisopam metabolism are at the 7 position of the benzodiazepine ring and at the 4 position of the phenyl ring. Both methoxy groups at these positions are demethylated. Data collected in this study showed that demethylation at the 7 position of the benzodiazepine ring is catalyzed by CYP3A4. This is supported by the strong inhibition of M1 formation from both (*R*)- and (*S*)-tofisopam and by the inhibition of M5 formation from M3 upon the addition of the CYP3A4 inhibitor ketoconazole. Demethylation at the 4 position of the phenyl ring appears to proceed primarily through CYP2C9, with some contributions from CYP2C19 for both enantiomers and CYP3A4 for (*S*)-tofisopam. While CYP2C19 may be contributing to the overall generation of M3, it is not surprising that the CYP2C19 inhibitor, (S)-mephenytoin, did not significantly inhibit this reaction as the majority of

the M3 produced appears to be via CYP2C9. CYP3A appears to have a larger role in the demethylation at the 4 position of the phenyl ring with (*S*)-Tofisopam than with (*R*)-Tofisopam, as suggested by the larger effect of ketoconazole and the results from recombinant P450.

The metabolites detailed in the current phenotyping study are the major metabolites in man and should give a good prediction of the metabolism of tofisopam in man. Analysis of clinical urine and plasma samples from both tofisopam enantiomers were in good agreement with the *in vitro* results. Literature reports describing the metabolites and routes of excretion after a radioactive dose of racemic tofisopam (Klebovich and Abermann, 1993) indicate that M1, M3 and M5 account for 86% of the total metabolites in human. Additional metabolites primarily corresponded to the further oxidation of tofisopam to tri- and quatro-demethylated metabolites. Tofisopam was eliminated in both urine and feces with a 2:1 ratio. While we do not have fecal samples, the clinical plasma and urine samples should give a good approximation of the M1:M3 ratio in man.

In conclusion, *in vitro* experiments with recombinant P450 enzymes and with human liver microsomes have shown that the chiral center of tofisopam is significant to the metabolites generated by hepatic metabolism. The primary enzymes responsible for the generation of the individual tofisopam metabolites have also been identified and the metabolic profiles of (*R*)-Tofisopam and (*S*)-Tofisopam clinical samples support the *in vitro* findings.

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## FIGURE LEGENDS

Figure 1: LC-MS/MS Chromatograms of Reference Standards of Tofisopam, M1, M2, M3, M4, M5, and M6. Tofisopam and its six reported metabolites were resolved by time and mass in a single injection. The following mass transitions were used for the analysis: tofisopam, *m/z* 383.4→298.4; M1-M4, *m/z* 369.4→284.3; and M5-M6, *m/z* 355.4→242.2.

Figure 2: Incubations with Recombinant P450 Isozymes. Incubations containing 5 pmoles of recombinant P450 and 500 ng/ml (≈1.3 µM) tofisopam (or metabolite) were initiated by the addition of NADPH stopped after 15 minutes.

Figure 3: Pilot Incubation Plots for (*R*)-Tofisopam. Incubations containing 500 ng/mL (≈1.3 µM) (*R*)-Tofisopam were carried out with 0.05 (diamonds), 0.2 (squares) and 1.0 (triangles) mg/mL pooled human liver microsomes. Aliquots were taken at 0, 10, 20, 40 and 60 minutes.

Figure 4: Pilot Incubation Plots for (*S*)-Tofisopam. Incubations containing 500 ng/mL (≈1.3 µM) (*S*)-Tofisopam were carried out with 0.05 (diamonds), 0.2 (squares) and 1.0 (triangles) mg/mL pooled human liver microsomes. Aliquots were taken at 0, 10, 20, 40 and 60 minutes.

Figure 5: Effects of Selective Inhibitors on the Metabolism of (*R*)-Tofisopam. Incubations containing 500 ng/mL (≈1.3 µM) (*R*)-Tofisopam and 0.2 mg/mL pooled human liver microsomes were incubated for ten minutes in the presence of the following P450 isozyme specific chemical inhibitors: furafylline (CYP1A2), 30 µM; sulfaphenazole (CYP2C9), 10 µM; S-mephenytoin (CYP2C19), 250 µM; quinidine (CYP2D6), 10 µM; and ketoconazole (CYP3A4), 10 µM. Rates are displayed in panel A and percent inhibition is displayed in panel B.

Figure 6: Effect of Selective Inhibitors on the Metabolism of (*S*)-Tofisopam. Incubations containing 500 ng/mL (≈1.3 µM) (*S*)-Tofisopam and 0.2 mg/mL pooled human liver microsomes were incubated for ten minutes in the presence of the following P450 isozyme selective chemical inhibitors: furafylline (CYP1A2), 30 µM; sulfaphenazole (CYP2C9), 10 µM; S-mephenytoin (CYP2C19), 250 µM; quinidine (CYP2D6), 10 µM; and ketoconazole (CYP3A4), 10 µM. Rates are displayed in panel A and percent inhibition is displayed in panel B.

Figure 7: Effect of Selective Inhibitors on the formation of M5 from Racemic M1 and Racemic M3. Racemic M1 or M3, 500 ng/mL(≈1.4 µM), was incubated with 0.2 mg/mL pooled human liver microsomes for ten minutes in the presence of the following P450 isozyme selective chemical inhibitors: furafylline (CYP1A2), 30 µM; sulfaphenazole (CYP2C9), 10 µM; S-mephenytoin (CYP2C19), 250 µM; quinidine (CYP2D6), 10 µM; and ketoconazole (CYP3A4), 10 µM. Rates are displayed in panel A and percent inhibition is displayed in panel B.

Figure 8: (*R*)-Tofisopam Metabolism Schematic.

Figure 9: (*S*)-Tofisopam Metabolism Schematic.

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| Compound | Chemistry | Chemical Name |
|---|---|---|
| Tofisopam | Parent | 1-(3,4-dimethoxyphenyl)-5-ethyl-7,8-dimethoxy-4-methyl-5H-2,3-benzodiazepine |
| M1 | Demethylated at the 7 Position of the Benzodiazepine Ring | 1-(3,4-dimethoxyphenyl)-5-ethyl-7-hydroxy-8-methoxy-4-methyl-5H-2,3-benzodiazepine |
| M2 | Demethylated at the 8 Position of the Benzodiazepine Ring | 1-(3,4-dimethoxyphenyl)-5-ethyl-8-hydroxy-7-methoxy-4-methyl-5H-2,3-benzodiazepine |
| M3 | Demethylated at the 4 Position of the Phenyl Ring | 1-(4-hydroxy-3-methoxyphenyl)-5-ethyl-7,8-dimethoxy-4-methyl-5H-2,3-benzodiazepine |
| M4 | Demethylated at 3 Position of the Phenyl Ring | 1-(3-hydroxy-4-methoxyphenyl)-5-ethyl-7,8-dimethoxy-4-methyl-5H-2,3-benzodiazepine |
| M5 | Demethylated at the 4 Position of the Phenyl Ring and the 7 Position of the Benzodiazepine Ring | 1-(4-hydroxy-3-methoxyphenyl)-5-ethyl-7-hydroxy-8-methoxy-4-methyl-5H-2,3-benzodiazepine |
| M6 | Demethylated at the 3 Position of the Phenyl Ring and the 7 Position of the Benzodiazepine Ring | 1-(3-hydroxy-4-methoxyphenyl)-5-ethyl-7-hydroxy-8-methoxy-4-methyl-5H-2,3-benzodiazepine |

Table 1. Chemical Names for Tofisopam and its Primary Metabolites

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(*R*)-Tofisopam

| | M1 Formation | | | M3 Formation | | |
|---|---|---|---|---|---|---|
| | $K_m$ (μM) | $V_{max}$ (pmol/min/pmol) | $V_{max}/K_m$ | $K_m$ (μM) | $V_{max}$ (pmol/min/pmol) | $V_{max}/K_m$ |
| 1A2 | - | | | - | | |
| 2C8 | - | | | 52 | 0.43 | 0.0083 |
| 2C9 | - | | | 7.6 | 17 | 2.2 |
| 2C19 | 24 | 0.34 | 0.014 | 20 | 1.8 | 0.090 |
| 3A4 | 0.9 | 1.8 | 2.0 | * | | |
| 3A5 | 17 | 0.82 | 0.048 | * | | |

(*S*)-Tofisopam

| | M1 Formation | | | M3 Formation | | |
|---|---|---|---|---|---|---|
| | $K_m$ (μM) | $V_{max}$ (pmol/min/pmol) | $V_{max}/K_m$ | $K_m$ (μM) | $V_{max}$ (pmol/min/pmol) | $V_{max}/K_m$ |
| 1A2 | * | | | * | | |
| 2C8 | * | | | * | | |
| 2C9 | * | | | 0.9 | 1.9 | 2.1 |
| 2C19 | 49 | 0.47 | 0.0096 | 9.1 | 1.8 | 0.20 |
| 3A4 | 0.8 | 3.3 | 4.1 | 81 | 0.92 | 0.011 |
| 3A5 | 10 | 1.4 | 0.14 | * | | |

Table 2: Kinetic constants for tofisopam using recombinant P450s. An asterisk indicates that the respective metabolite was generated, but the $K_m$ was above 100 μM. A dash indicates that the respective metabolite was not observed even at 100 μM tofisopam.

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**R Plasma**

| Metabolite | 2 hr (ng/mL) | 2 hr* (ng/mL) | 4 hr (ng/mL) | 4 hr* (ng/mL) |
|---|---|---|---|---|
| Tofisopam | 94.0 +/- 80.9 | 93.3 +/- 80.0 | 41.1 +/- 32.4 | 39.6 +/- 32.6 |
| M1 | 2.02 +/- 0.48 | 26.0 +/- 4.6 | 0.90 +/- 0.29 | 15.5 +/- 4.0 |
| M2 | 1.19 +/- 0.69 | 1.71 +/- 0.86 | 0.73 +/- 0.36 | 1.11 +/- 0.46 |
| M3 | 74.8 +/- 37.9 | 757 +/- 167 | 47.7 +/- 19.8 | 549 +/- 119 |
| M4 | 0.00 +/- 0.00 | 0.03 +/- 0.11 | 0.04 +/- 0.13 | 0.00 +/- 0.00 |
| M5 | 35.6 +/- 12.3 | 95.6 +/- 37.3 | 24.4 +/- 8.3 | 70.5 +/- 32.6 |
| M6 | 0.00 +/- 0.00 | 0.00 +/- 0.00 | 0.00 +/- 0.00 | 0.00 +/- 0.00 |

* Samples were incubated with glucylase for 1 hour to remove gulcuronide and sulfate conjugates.

Table 3: Analysis results of (*R*)-Tofisopam clinical plasma samples. Plasma samples were obtained 2 and 4 hours after oral administration of (*R*)-tofisopam, 200 mg capsule. Data are the averages of 13 healthy female volenteers and represent the mean +/- the standard deviation.

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**S Plasma**

| Metabolite | 2 hr (ng/mL) | 2 hr* (ng/mL) | 4 hr (ng/mL) | 4 hr* (ng/mL) |
|---|---|---|---|---|
| Tofisopam | 188 +/- 102 | 186 +/- 103 | 69.2 +/- 47.4 | 68.5 +/- 46.2 |
| M1 | 145 +/- 34.4 | 726 +/- 126 | 52.7 +/- 11.5 | 433 +/- 95 |
| M2 | 0.40 +/- 0.32 | 0.74 +/- 0.46 | 0.17 +/- 0.13 | 0.37 +/- 0.31 |
| M3 | 21.0 +/- 7.8 | 88.2 +/- 31.1 | 7.83 +/- 3.70 | 45.9 +/- 18.6 |
| M4 | 0.20 +/- 0.15 | 1.28 +/- 0.44 | 0.05 +/- 0.07 | 0.77 +/- 0.35 |
| M5 | 78.2 +/- 17.1 | 210 +/- 48.5 | 37.2 +/- 10.7 | 136 +/- 39 |
| M6 | 3.33 +/- 1.02 | 4.70 +/- 1.05 | 1.14 +/- 0.41 | 2.34 +/- 0.60 |

* Samples were incubated with glucylase for 1 hour to remove gulcuronide and sulfate conjugates.

Table 4: Analysis results of (S)-Tofisopam clinical plasma samples. Plasma samples were obtained 2 and 4 hours after oral administration of (*S*)-tofisopam, 150 mg capsule. Data are the averages of 14 healthy male and female volenteers and represent the mean +/- the standard deviation.

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**Urine Data**

| Metabolite | (*R*)-Tofisopam 12 hr (ng/mL) | (*R*)-Tofisopam 12 hr* (ng/mL) | (*S*)-Tofisopam 12 hr (ng/mL) | (*S*)-Tofisopam 12 hr* (ng/mL) |
|---|---|---|---|---|
| Tofisopam | 46.9 +/- 26.4 | 45.7 +/- 24.7 | 8.43 +/- 8.50 | 9.93 +/- 8.08 |
| M1 | 20.0 +/- 4.52 | 208 +/- 70 | 1370 +/- 470 | 13800 +/- 3800 |
| M2 | 0.42 +/- 0.27 | 1.16 +/- 4.19 | 1.73 +/- 5.60 | 7.74 +/- 8.24 |
| M3 | 418 +/- 232 | 25600 +/- 13500 | 286 +/- 166 | 2280 +/- 1140 |
| M4 | 0.00 +/- 0.00 | 0.00 +/- 0.00 | 0.59 +/- 2.19 | 38.4 +/- 20.4 |
| M5 | 472 +/- 169 | 2160 +/- 1310 | 5760 +/- 1900 | 12100 +/- 3400 |
| M6 | 0.82 +/- 0.62 | 0.00 +/- 0.00 | 152 +/- 65.1 | 150 +/- 64.7 |

* Samples were incubated with glucylase for 1 hour to remove gulcuronide and sulfate conjugates.

Table 5: Analysis results of (*R*)- and (*S*)-Tofisopam clinical urine samples. Urine was pooled for the 12 hours after oral administration of either 200 mg (*R*)-Tofisopam or 150 mg (*S*)-Tofisopam. (*R*)-Tofisopam data are the averages of 13 healthy female volenteers and (*S*)-Tofisopam data are the averages of 14 healthy male and female volenteers.

Figure 1

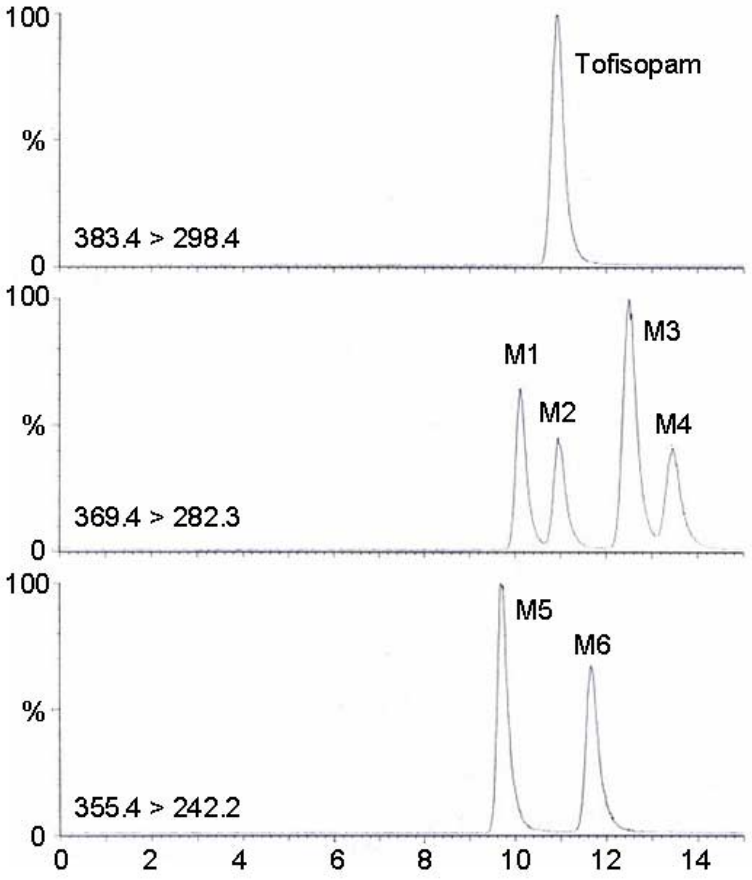

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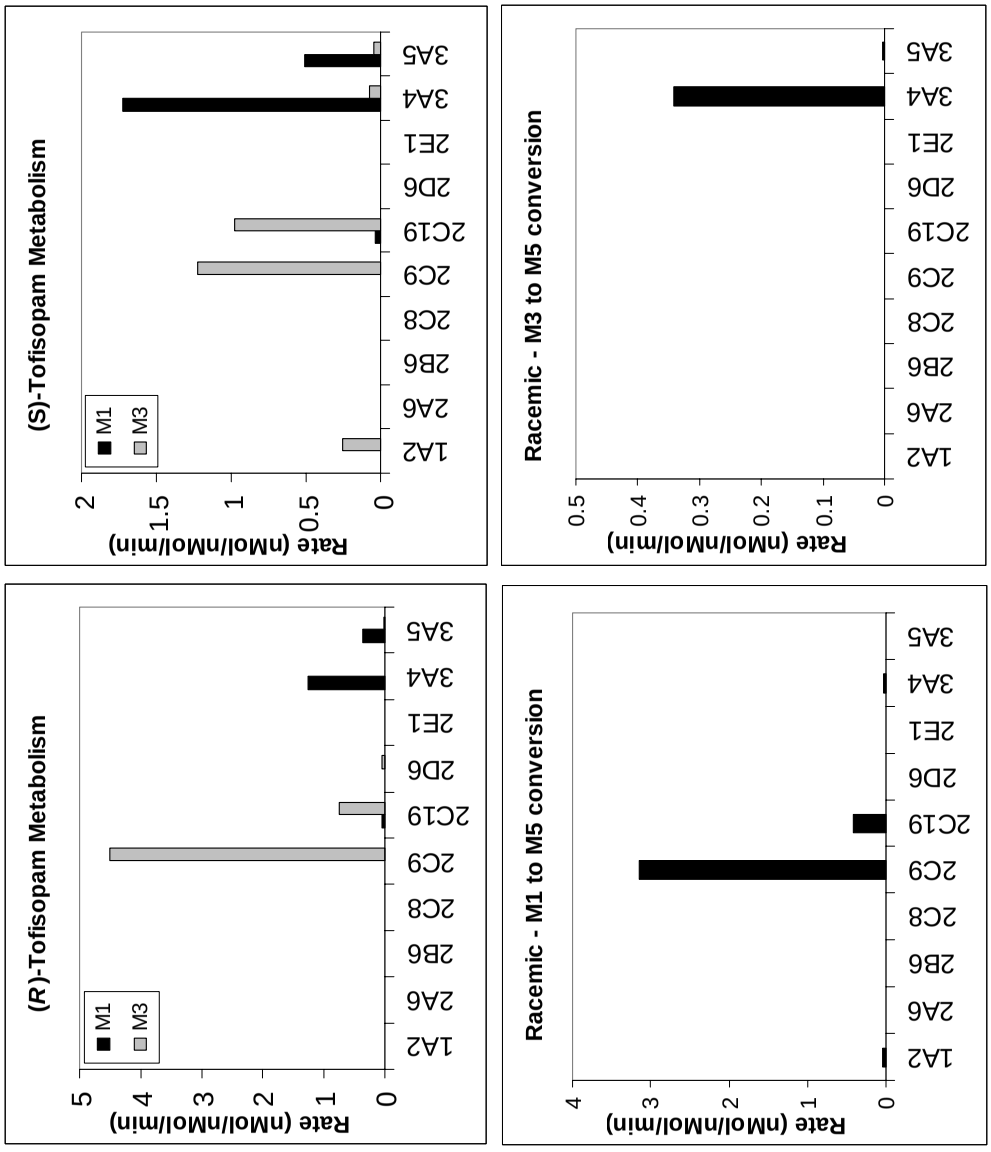
Figure 2



# Figure 3

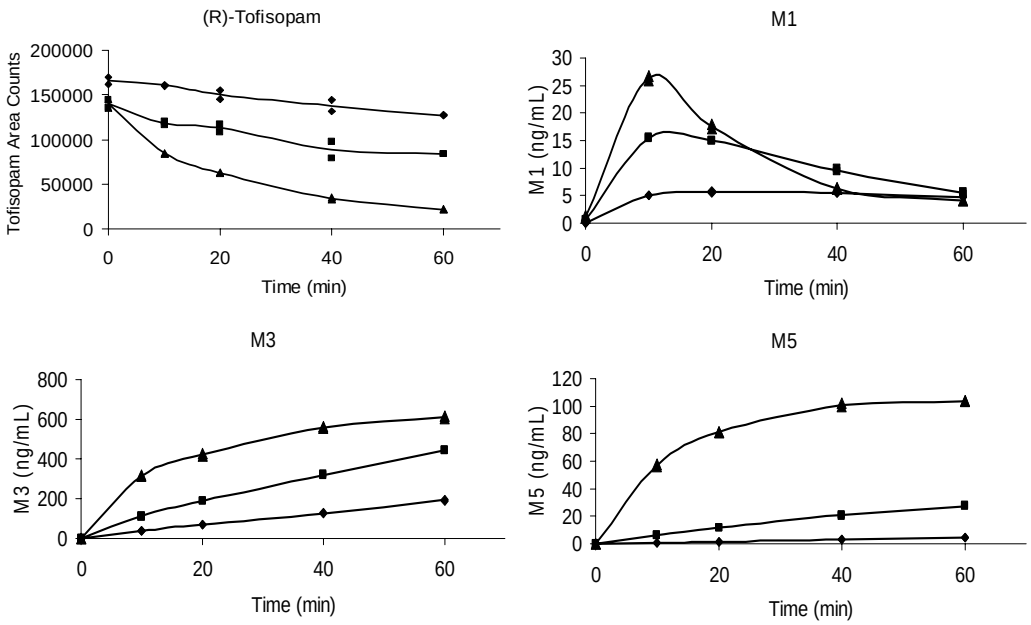


Figure 4

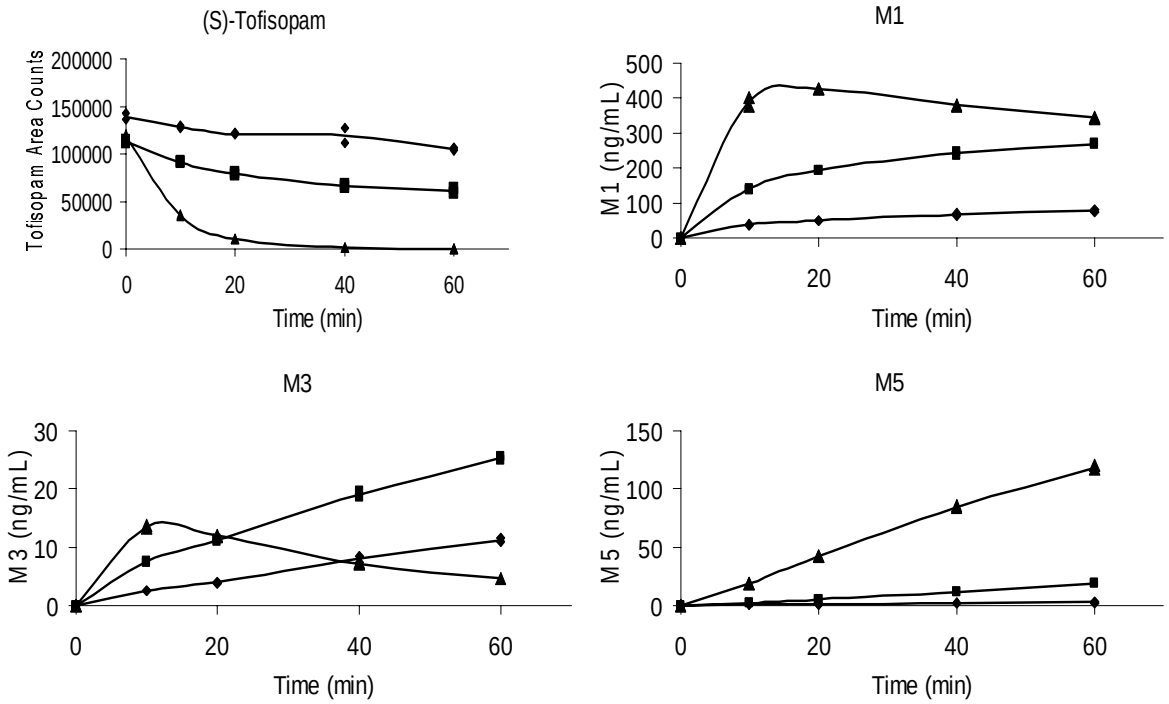


Figure 5

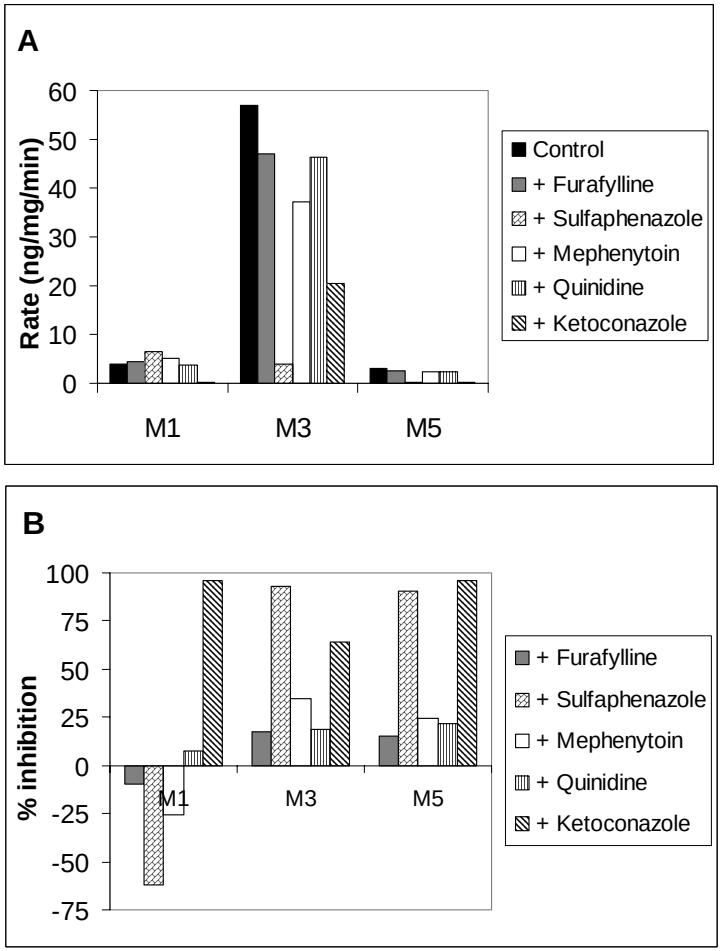


Figure 6

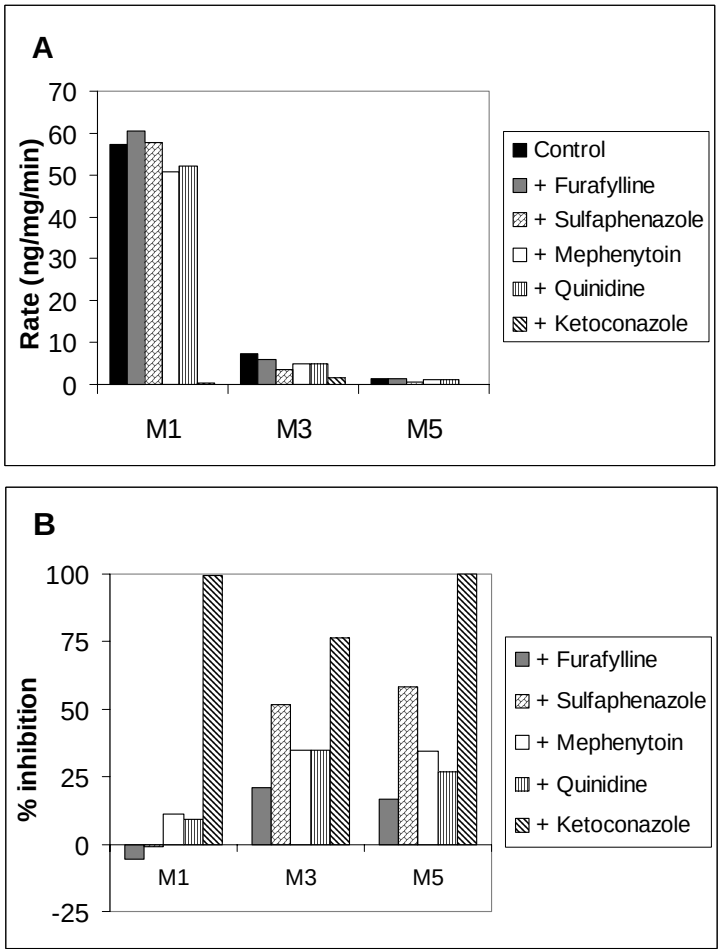


Figure 7

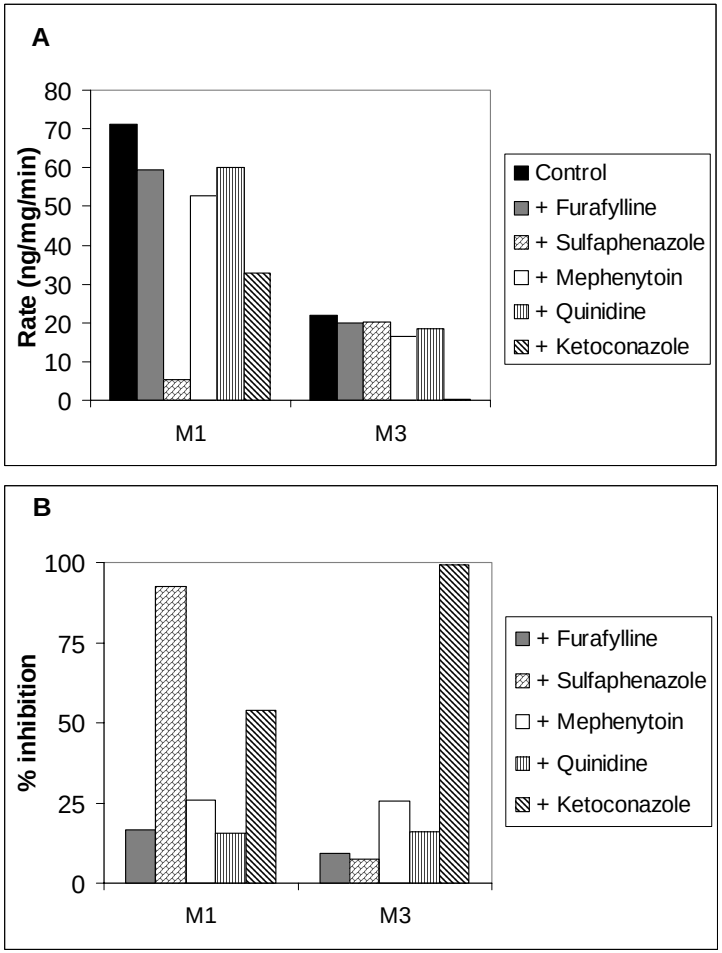


Figure 8

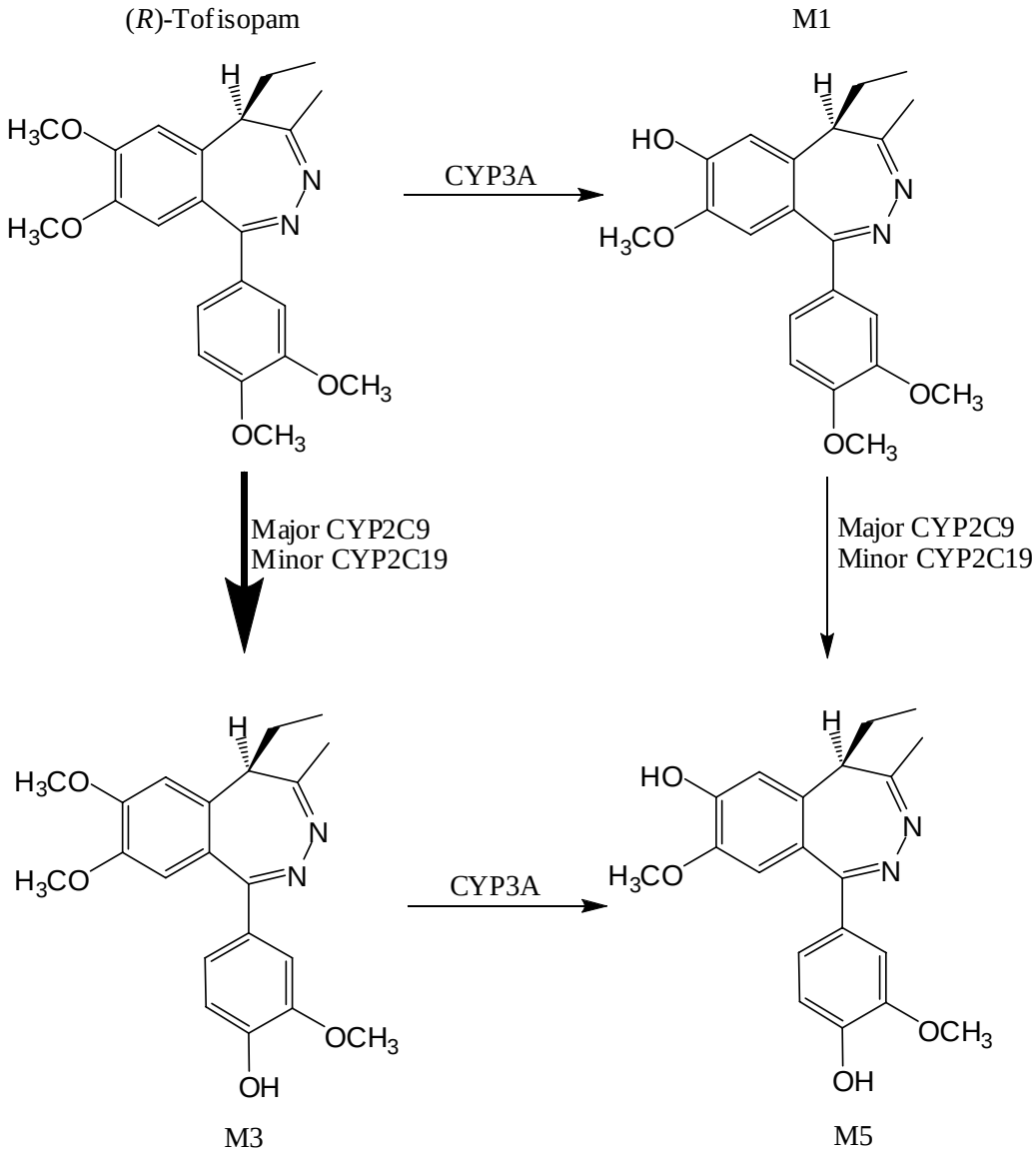


Figure 9