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Reevaluate In Vitro CYP3A Index Reactions of Benzodiazepines and Steroids between Humans and Dogs

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Quantitative Determination of Metabolites in Incubations

Quantitative determinations were performed on ACQUITY UPLC coupled to Xevo TQ-S mass spectrometer (Waters, Milford, MA, USA). The detection was performed at the positive mode except that DCA, LCA and their metabolites were monitored at the negative mode. The capillary voltage, source temperature, desolvation temperature were set at 3.0 kV, 150°C and 550°C, respectively. The cone voltages and collision energies for analytes were set at 23 V and 24 eV (TRZ, m/z 343>308), 44 V and 26 eV (αh-TRZ, m/z 359>176), 26 V and 30 eV (4h-TRZ, m/z 359>273), 29 V and 25 eV (APZ, m/z 309>281), 40 V and 23 eV (αh-APZ, m/z 325>297), 20 V and 17 eV (4h-APZ, m/z 325>307), 31 V and 36 eV (MDZ, m/z 326>291), 35 V and 18 eV (1'h-MDZ, m/z 342>324), 20 V and 15 eV (4h-MDZ, m/z 342>325), 14 V and 44 eV (CLZ, m/z 327>192), 36 V and 20 eV (T, m/z 289>97), 27 V and 14 eV (6βh-T, 2αh-T, 2βh-T and 16αh-T, m/z 305>269), 49 V and 22 eV (PROG, m/z 315>109), 40 V and 27 eV (LCA, m/z 375>375), 80 V and 29 eV (DCA and HDCA, m/z 391>391), 80 V and 27 eV (1βh-DCA and 5βh-DCA, m/z 407>407), 80 V and 29 eV (DCA-2,2,4,4-D4, m/z 395>395), 80 V and 27 eV (CA-2,2,4,4-D4, m/z 411>411), respectively.

MDZ and its metabolites were separated on an ACQUITY BEH C18 column (1.7 μm, 50 mm × 2.1 mm) (Waters, Milford, MA) maintained at 40°C. The sample (1.0 μL) was eluted with the solvent conditions of 0.1% formic acid in water (mobile phase A) and acetonitrile (mobile phase B) delivered at a rate of 0.4 mL/min. A stepwise gradient profile was as follows: 0.0-1.1 min (70-5% A), 1.1-1.4 min (5% A), 1.4-1.6 min (5-70% A).

TRZ, APZ and their metabolites were separated on an ACQUITY HSS T3 column (1.8 μm, 50 mm × 2.1 mm) maintained at 40°C. The sample (1.0 μL) in the studies of TRZ and APZ was eluted at a flow rate of 0.45 mL/min with the mobile phase consisted of a mixture of 0.01% formic acid in water (mobile phase A) and acetonitrile (mobile phase B) by the following gradient: 0.0-0.2 min (95% A), 0.2-0.5 min (95-87% A), 0.5-0.8 min (87-82% A), 0.8-1.5 min (82-77% A), 1.5-2.0 min (77-72% A), 2.0-3.0 min (72-67% A), 3.0-3.7 min (67-63% A), 3.7-4.5 min (63-5% A), 4.5-4.8 min (5% A), 4.8-5.0 min (5-95% A).

T and 6βh-T were separated on an ACQUITY HSS T3 column (1.8 μm, 50 mm × 2.1 mm) maintained at 40°C. The sample (1.0 μL) in the studies of T was eluted with the mobile phase consisted of 0.01% formic acid in water (mobile phase A) and 0.01% formic acid in methanol (mobile phase B) delivered at a rate of 0.45 mL/min. A stepwise gradient profile was as follows:
0.0-0.3 min (70% A), 0.3-1.2 min (70-40% A), 1.2-3.2 min (40-5% A), 3.2-3.6 min (5% A), 3.6-4.0 min (5-70% A). The separation of 6βh-T from 2αh-T, 2βh-T and 16αh-T was achieved by matching the relative retention time of T and 6βh-T with reference to the ion chromatograms in previous report (Shou et al., 2003; Kandel et al., 2017).

Quantitative analysis of DCA, LCA and their metabolites was conducted as previously described (Yin et al., 2017; Zhu et al., 2018). The sample (5.0 μL) was injected into an ACQUITY BEH C18 Column (1.7 mm, 100 mm × 2.1mm) maintained at 45°C and eluted with the mobile phase consisted of 0.01% formic acid in water (mobile phase A) and acetonitrile (mobile phase B) at a flow rate of 0.45 mL/min with the following gradient: 0.0-0.5 min (95% A), 0.5-1.0 min (95-64% A), 1.0-2.0 min (64-74% A), 2.0-4.0 min (74-70% A), 4.0-6.0 min (70% A), 6.0-7.0 min (70-62% A), 7.0-9.0 min (62-55% A), 9.0-12.5 min (55-30% A), 12.5-13.0 min (30-0% A), 13.0-14.0 min (0% A), 14.0-14.1 (0-95% A) and 14.1-15.0 min (95% A).

The retention times of analytes were 4.12 (TRZ), 3.51 (αh-TRZ), 3.59 (4h-TRZ), 3.99 (APZ), 3.51 (αh-APZ), 3.37 (4h-APZ), 0.69 (MDZ), 0.67 (1'h-MDZ), 0.61 (4h-MDZ), 0.62 (CLZ), 2.33 (T), 1.69 (6βh-T), 1.80 (16αh-T), 1.90 (2αh-T), 1.99 (2βh-T), 2.74 (PROG), 11.02 (DCA), 6.30 (1βh-DCA), 4.83 (5βh-DCA), 12.76 (LCA), 9.00 (HDCA) min, respectively.
Figure S1. Kinetic plots of 1'-hydroxymidazolam (1'h-MDZ), 4-hydroxymidazolam (4h-MDZ), α-hydroxyalprazolam (αh-APZ), 4-hydroxyalprazolam (4h-APZ), α-hydroxytriazolam (αh-TRZ), 4-hydroxytriazolam (4h-TRZ), 1β-hydroxydeoxycholic acid (1βh-DCA), 5β-hydroxydeoxycholic acid (5βh-DCA), hyodeoxycholic acid (HDCA) and 6β-hydroxytestosterone (6βh-T) formation by human liver microsomes (HLM, squares) and dog liver microsomes (DLM, circles). Formation rate was plotted as a function of substrate concentration for 1-300 μM midazolam (MDZ, 10 min), 5-500 μM alprazolam (APZ, 60 min), 5-300 (HLM) or 500 μM (DLM) triazolam (TRZ, 10 min), 1-300 (HLM) or 400 μM (DLM) deoxycholate (DCA, 60 min), 1-150 μM lithocholate (LCA, 60 min), 1-300 μM testosterone (T, 15 min) with HLM and DLM (0.5 mg/mL), except for MDZ (0.1 mg/mL). Lines represented nonlinear regression curves. Data was shown as mean ± SD of triplicates.
Figure S2. Substrate degradation and metabolites formation of testosterone (T, A) and lithocholic acid (LCA, B) in 0.5 mg/mL of human liver microsomes (HLM, a) and dog liver microsomes (DLM, b). T (10 µM) and LCA (20 µM) were incubated for 15 and 60 min, respectively. T, LCA, 6β-hydroxytestosterone (6βh-T), and hyodeoxycholic acid (HDCA) were quantitated with standards, and androstenedione (AD), 2β-hydroxytestosterone (2βh-T), 16α-hydroxytestosterone (16αh-T), 3-dehydrolithocholic acid (3-dehydroLCA), and unknown were assessed by semiquantitative methods.

Note: identification of 2βh-T, 16αh-T, and AD was achieved by matching the relative retention times reported previously (Usmani et al., 2003).
Figure S3. Enzyme kinetic plots of 1'-hydroxymidazolam (1'h-MDZ), 4-hydroxymidazolam (4h-MDZ), α-hydroxyalprazolam (αh-APZ), 4-hydroxyalprazolam (4h-APZ), α-hydroxytriazolam (αh-TRZ), 4-hydroxytriazolam (4h-TRZ), 1β-hydroxydeoxycholic acid (1βh-DCA), 5β-hydroxydeoxycholic acid (5βh-DCA), 6β-hydroxytestosterone (6βh-T) formation by canine recombinant CYP2B11 (triangles), 3A12 (circles) and 3A26 (squares) (rCYP) over a large range of substrate levels. Formation rate was plotted as a function of substrate concentration for 1-300 μM midazolam (MDZ, 10 min), 5-500 μM alprazolam (APZ, 60 min), 5-500 μM triazolam (TRZ, 10 min), 1-400 μM deoxycholate (DCA, 60 min) and 1-300 μM testosterone (T, 15 min) with rCYP (50 pmole/mL). Lines represented nonlinear regression curves. Data was shown as mean ± SD of triplicate determinations.
Figure S4. Enzyme kinetic plots of 1'-hydroxymidazolam (1'h-MDZ), 4-hydroxymidazolam (4h-MDZ), α-hydroxyalprazolam (αh-APZ), 4-hydroxyalprazolam (4h-APZ), α-hydroxytriazolam (αh-TRZ), 4-hydroxytriazolam (4h-TRZ) formation by canine recombinant CYP2B11 (triangles), 3A12 (circles) and 3A26 (squares) (rCYP) within a lower substrate range. Formation rate was plotted as a function of substrate concentration for 1-6.25 μM midazolam (MDZ, 10 min), 1-12.5 μM alprazolam (APZ, 60 min), 1-6.25 μM triazolam (TRZ, 10 min) with rCYP (50 pmole/mL), respectively. Lines represented nonlinear regression curves. Data points were shown as mean ± SD of triplicate values.


