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Short Communication

UPLC-MS/MS analysis of dextromethorphan-O-demethylation kinetics in rat brain microsomes

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ABSTRACT

Formation of dextrorphan (DXT) from dextromethorphan (DXM) has been widely used to assess cytochrome P450 2D (CYP2D) activity. Additionally, the kinetics of CYP2D activity have been well characterized in the liver microsomes. However, studies in brain microsomes are limited due to the lower microsomal content and abundance of CYP2D in the brain relative to the liver. In the present study, we developed a micro-scale enzymatic incubation method, coupled with a sensitive UPLC-MS/MS assay for the quantitation of the rate of DXT formation from DXM in brain microsomes. Rat brain microsomes were incubated with different concentrations of DXM for various times. The reaction was stopped, and the proteins were precipitated by the addition of acetonitrile, containing internal standard (d_3-DXT). After centrifugation, supernatant (2 µL) was injected onto a UPLC, C18 column with gradient elution. Analytes were quantitated using triple-quadrupole MS/MS with electrospray ionization in positive ion mode. The assay, which was validated for accuracy and precision in the linear range of 0.25 nM to 100 nM DXT, has a lower limit of quantitation of 0.125 fmol on the column. Using our optimized incubation and quantitation methods, we were able to reduce the incubation volume (25 µL), microsomal protein amount (5 µg), and incubation time (20 min), compared with reported methods. The method was successfully applied to estimation of the Michaelis-Menten (MM) kinetic parameters of dextromethorphan-O-demethylase activity in the rat brain microsomes (mean ± SD, n = 4), which showed a maximum velocity of 2.24 ± 0.42 pmol/min/mg and a MM constant of 282 ± 62 µM. It is concluded that by requiring far less biological material and time, our method represents a significant improvement over the existing techniques for investigation of CYP2D activity in rat brain microsomes.

Keywords:

UPLC-MS/MS

CYP2D

Dextromethorphan-O-demethylase

Brain

Microsomes

Michaelis-Menten kinetics

1. Introduction

The dextromethorphan-O-demethylase (DOD) activity assay, where dextromethorphan (DXM) is biotransformed into the metabolite dextrorphan (DXT) (Fig. 1), is a commonly used tool to estimate cytochrome P450 (CYP) 2D6 (CYP2D6) function in humans [1, 2]. The DOD assay is also used in animal studies (especially in rats) to compare human CYP2D6 substrate specificities with those in other species [3-7]. In rodents, this activity is more generally assigned to the CYP2D subfamily, given there are multiple isoforms within this subfamily with similar homology and substrate affinities that overlap with the human CYP2D6 isoform [8-10].

In both humans and rats, CYP2D is expressed in both the liver and brain [11-13]. Although the liver is considered the main site of drug clearance and metabolism, studies have shown that the expression and activity of CYP2D in the brain can alter pharmacologic responses to centrally-acting drug [5, 6, 12, 14-17]. Functional characterization of CYP enzymes *in vitro* is typically achieved via subcellular fractionation and collection of the small vesicles formed by the endoplasmic reticulum, called microsomes. Hepatic microsomes are routinely used in drug metabolism assays given the high abundance of CYP protein located there [2]. The principal limitation for functional assays of CYP enzymes in the brain is their relatively low abundance, which is approximately 1-4% of that in the liver with the exception of some specific brain regions [11, 13, 18]. Indeed, the microsomal protein content in the brain (2-4 mg per gram of brain tissue) [19] is much lower relative to that in the liver (30-40 mg per gram of liver tissue) [20]. In rodent studies, this limitation is exacerbated by the small quantity of brain tissue in these species. Thus, there are few studies in the literature where the characterization of DOD kinetics in the brain microsomes has been attempted [3, 4, 7].

The reported *in vitro* studies of CYP2D activity in rat brain microsomes [3, 4, 7] tend to utilize much higher protein concentrations (50-1500 µg) and much longer incubation times (1 to 2 hours) than what are typically used with the hepatic microsomes. These conditions are apparently necessary so that the reaction can produce enough metabolite to be accurately quantified. However, this exacerbates the issue of overall lower amounts of microsomal protein available from a single brain, such that pooling of several brains to generate enough material for these assays is necessary [3, 4, 7]. Other studies [5, 6, 10] have utilized something called the "total membrane fraction," which is a pooling of both mitochondrial and microsomal membranes, to increase yield. However, these studies [5, 6, 10] still require long incubation times (2-3 hours) and high protein quantities (1.5-3 mg membrane protein/reaction).

To circumvent the barriers in the studies of DOD activity in rodent brain tissue, we present here an optimized micro-scale enzymatic incubation method, coupled with a validated and sensitive UPLC-MS/MS assay for quantification of DOD activity in rat brain microsomes. The relatively high analytical sensitivity of the assay, along with the use of a pure DXM substrate that is devoid of DXT impurity, allowed us to measure the lower concentrations of DXT produced at relatively low microsomal protein amounts and short incubation periods. The application of the method to determination of the Michaelis-Menten (MM) kinetic parameters of DXM demethylation in rat brain microsomes is also demonstrated. This method will be useful when applied to animal studies investigating the effect of drug-drug interactions or disease pathologies related to the CYP2D activity in the brain.

2. Materials and methods

2.1. Chemicals

Three different stock sources for dextromethorphan hydrobromide salt (DXM-HBr) were purchased from Sigma-Aldrich (St. Louis, MO, USA): DXM-HBr #1 (catalog #: D9684), DXM-HBr #2 (catalog #: PHR1018), and DXM-HBr #3 (catalog #: D2531). Dextromethorphan base reference standard (DXM-USP) was purchased from the United States Pharmacopeial Convention (Rockville, MD, USA). Analytical metabolite standard solutions for DXT tartrate and its stable isotope d_3-DXT were purchased from Cerilliant Corporation (Round Rock, TX, USA). UPLC/LC-MS grade solvents (acetonitrile, methanol, and water) were purchased from VWR (Radnor, PA, USA). All other reagents were analytical grade and were obtained from commercial sources.

2.2. Instrumentation

The UPLC-MS/MS system consisted of a Bruker EVOQ triple quadrupole mass spectrometer, attached to the Bruker Advance UPLC system with an integrated column oven, degasser, and a CTC PAL autosampler. The system was controlled and the data acquired and quantified by the Bruker MSWS 8 software. The chromatographic separation was achieved using a Phenomenex Kinetex 1.7 µm C18 (100 A, 50 x 2.1 mm) column, connected to a Phenomenex C18 SecurityGuard ULTRA (2.1 mm) pre-column and maintained at 40^0C. Samples (2 µL) were injected onto the column and eluted at a flow rate of 0.2 mL/min under gradient conditions consisting of solvent A (5 mM ammonium formate: formic acid, 100:0.05) and solvent B (acetonitrile: methanol: formic acid, 95:5:0.05). Gradient conditions were as follows: 0-0.5 minutes, 10% B; 0.5-4 min, linear gradient 10-90% B; 4-7 min, 90% B; 7 min, 10% B; 7-9 min,

10% B. However, MS data were collected from 1 to 4 min only, and the valve was diverted to waste before and after that time.

The mass spectrometer source utilized heated electrospray ionization (needle temperate 300^0C, flow 40 psi; cone temperature 300^0C, flow 20 psi; nebulizer gas flow 50 psi) in positive ion mode at 3000 V. Metabolite and internal standard (IS) were analyzed using selected reaction monitoring for the parent/fragment transitions for DXT (m/z 258 → 156.90) and d_3-DXT (m/z 261 → 156.90).

2.3. Standard curves

Stock solutions of DXT tartrate (equivalent to 1 mg/mL DXT base in methanol) and d_3-DXT (100 µg/mL in methanol) were used as supplied by Cerilliant. Dextrorphan tartrate was diluted to a concentration of 1 µg/mL (DXT base) in acetonitrile, and then further diluted to a working stock solution of 200 nM in 100 mM Tris buffer. For calibration curves, blank samples containing 0.2 mg/mL brain microsomal protein in Tris buffer were spiked with stock solutions in buffer to give concentrations of 0.25, 0.5, 1, 2, 5, 10, 20, 50, and 100 nM. The internal standard d_3-DXT was diluted to 10 nM in acetonitrile. Calibration curves were constructed by plotting the analyte: IS peak area ratios versus the added concentrations of DXT.

2.4. Sample preparation

Calibration standards or metabolic incubation samples (25 µL) in a microcentrifuge tube were mixed with 75 µL of an ice-cold solution of IS (10 nM) in acetonitrile. Subsequently, tubes were vortex-mixed for 10 sec and centrifuged at 12,000 g-force for 5 minutes to precipitate proteins. The supernatant was collected into HPLC vials for analysis.

2.5. Method validation

2.5.1. Accuracy and precision

The inter- and intra-run accuracies and precision of the method were evaluated based on the quantitation of quality controls at DXT concentrations of 0.25, 5, and 100 nM (n = 5 per each concentration) against calibration standards, which were prepared from different stock solutions. The accuracy was calculated by measured concentration x 100/nominal concentration. Precision was calculated as a percent of the relative standard deviation (R.S.D). The acceptable range for both inter- and intra-run accuracies was considered 85-115% for middle and high concentrations, and 80-120% for the low concentration [21]. The acceptable precision values were 15% (middle and high concentrations) and 20% (low concentration) [21].

2.5.2. Linearity

The linearity of the calibration curves was evaluated by the coefficient of determination (r^2) of the linear regression analysis of the analyte: IS peak area ratios versus spiked concentrations of DXT using a weight of 1/x, where x is the spiked concentration of DXT.

2.5.3. Overall and Matrix Recovery

The overall and matrix recoveries of DXT and IS from the samples containing brain microsomes (0.2 mg/mL protein) were determined in five replicates of three concentrations from the calibration curve (0.25, 5, and 50 nM). The overall recovery (recovery from protein precipitation plus matrix effect) was estimated by comparing samples and references prepared in the presence and absence of microsomes, respectively. The matrix recovery was estimated by preparing blank samples and references in the presence and absence of matrix, respectively, followed by protein precipitation and addition of DXT and IS directly to the resultant supernatants. In both cases, the absolute peak areas of DXT or IS in the samples were compared with those in their respective references, and recoveries were presented as percentages of the references.

2.6. DXT impurity in the DXM substrate from different sources

The extent of DXT impurities in DXM stock solutions (1000 μM), which were prepared from three different DXM-HBr powders and a DXM base reference standard (DXM-USP), were tested by quantitating DXT concentrations using the LC-MS/MS method. The extent of impurity was then calculated as the molar concentration of DXT impurity divided by the molar concentration of DXM, multiplied by 100.

2.7. Preparation of microsomes from whole rat brain

Frozen whole brains from adult (8 to 12-week old), male Sprague-Dawley rats were purchased from Innovative-Research (Novi, Michigan, USA). Whole brains were homogenized in ice-cold buffer (100 mM Tris, 0.2 mM EDTA, and 1.15% KCl; pH 7.4) at a 1:10 ratio using an electric motor-driven Potter-Elvehjem Teflon homogenizer. Homogenate was spun at 1,300 g for 5 minutes at 4^{o}C. The supernatant was collected, and the pellet washed with the homogenizing buffer and centrifuged again at 1,300 g for 5 minutes. The supernatants were combined and spun at 21,000 g for 12 minutes at 4^{o}C to pellet mitochondria. The supernatant was collected and spun at 110,000 g for 70 minutes to obtain microsomes. The supernatant was discarded, and the pellet was washed and centrifuged again using fresh homogenization buffer. The microsomal pellet was collected and re-suspended in a storage buffer (100 mM Tris, 0.2 mM EDTA, 1.15% KCl, 20% glycerol, 0.1 mM dithiothreitol, 22 μM butylated hydroxytoluene, and 0.1 mM phenylmethylsulfonyl fluoride; pH 7.4) and stored at -80^{o}C for later experiments. Total protein concentrations were estimated using the Bradford method.

2.8. Determination of the CYP2D-mediated O-demethylase activity

DXM stock solutions were prepared by dissolving the DXM-HBr and DXM-USP powders in water and 5 mM excess HCl solutions, respectively. After an initial study to compare the DOD

activities using DXM-HBr #3 and DXM-USP stock solutions at a DXM concentration of 1000 µM, all the MM experiments were conducted using DXM-USP at concentrations of 100, 200, 500, 1000, 1500, and 2000 µM. To determine the inter-animal variability, the MM curves were constructed using four different brain tissues . Microsomal protein (5 µg) was preincubated at 37^{o}C for 5 minutes in 100 mM Tris-HCl buffer pH 7.4 with DXM substrate in a final volume of 25 µL. The reaction was initiated by the addition of 1 mM NADPH. After 20 minutes of incubation at 37^{o}C, reactions were terminated by the addition of ice-cold acetonitrile (75 µL), containing 10 nM IS (d_3-DXT). Each reaction mixture was paired with its own control sample, containing all the elements of the reaction mixture with the exception of NADPH. The reactions in the control samples were immediately (time zero) terminated by the addition of ice-cold acetonitrile containing internal standard. Samples were centrifuged, and the supernatants were loaded into HPLC vials for LC-MS/MS analysis. The nonlinear regression analysis of the metabolism rate-substrate concentration data was fitted to a one-enzyme MM model to estimate the maximum velocity (V_{max}) and MM constant (K_m) using GraphPad Prism software (La Jolla, CA, USA).

2.9. Stability of DXT in brain microsomal incubations and during autosampler storage

To confirm stability of the generated DXT in the brain microsomal incubations, DXT (5 nM) was added to the microsomal incubation samples, described above, in the presence of NADPH but without the addition of substrate. The concentration of DXT (5 nM) was selected to be close to the concentration generated near the K_m of our reactions. The samples were incubated at 37^{o}C and subjected to LC-MS/MS analysis at 0, 5, 10, 15, and 20 min after incubation (*n* = 3/each time point). Additionally, the samples, after protein precipitation, were kept in the autosampler

(10^{o}C) and re-injected 24 hours later to determine the autosampler stability of the processed samples.

3. Results and discussion

3.1. UPLC-MS/MS assay characteristics

Figure 2 depicts the chromatograms of brain microsomal matrix for a blank brain microsomal sample, the lowest concentration of DXT (0.25 nM) in the calibration curve, and the IS (d_3-DXT). Under the chromatographic conditions described in the methods section, DXT and IS eluted at 2.75 min as sharp peaks with low baseline noises.

The overall and matrix recoveries of DXT from the brain microsomal samples at low, middle, and high concentrations in the calibration curve are presented in Table 1, along with their respective internal standard (single concentration of 10 nM). Whereas the matrix recovery is influenced by the matrix only, the overall recovery reflects both matrix recovery and recovery from the protein precipitation procedure. The recovery of DXT and IS from the matrix, based on the absolute peak areas, ranged from 91% – 95% and 96% to 102%, respectively, indicating a lack of matrix effect. Similarly, the overall recoveries of DXT (91% – 106%) and IS (89% – 108%) were relatively high, suggesting an almost complete recovery from the protein precipitation method and confirming the lack of matrix effect on the samples (Table 1). For both cases, the recoveries were independent of the concentration of DXT (one-way ANOVA). Most of the current LC-MS/MS assays that quantitate DXT have been developed for measurement of DXM and its metabolites in plasma [22] or blood [23] and require extraction procedures to remove substantial matrix effect of the plasma or blood. The lack of matrix effect in our studies, which uses protein precipitation, is most likely due to the fact that our micro-scale microsomal assay contains only 5 µg of proteins, as opposed to substantial proteins in the plasma and blood.

However, our protein precipitation method may not be suitable for plasma or blood samples because it may cause significant matrix effect.

Calibration curves constructed in the range of 0.25-100 nM (prepared in brain microsomal matrix) were linear with r^2 values of ≥ 0.99 for the inter- and intra-run experiments. The representative equation for the standard curves (calculated from the averages of the inter-day calibration curves) was $y = 0.0337\,x - 0.00126$, where y and x refer to the DXT: IS peak ratio and the concentration of DXT, respectively. The results of intra- and inter-day accuracy and precision experiments are reported in Table 2. The values fell within the limits of the FDA guidelines; the accuracy was within 94.1-111%, and the precision values (R.S.D.) were ≤ 14.9%. Based on the accuracy and precision values (Table 2), the lower limit of quantitation (LLOQ) for DXT was 0.25 nM, which translates to an equivalent of 0.125 fmol DXT on the column.

Previous studies of the CYP2D-mediated DOD activity in the brain have relied on GC-MS [7] or HPLC with UV [4], fluorescence [5, 6], electrochemical [10], or MS/MS [3] detectors for quantification of DXT. These assays reported LLOQs or detection limits between 2 fmol to 2 pmol, with the HPLC-MS/MS method [3] reporting the highest sensitivity. Others have also reported HPLC or LC methods coupled with tandem mass spectrometry for the DOD assay, mostly for microsomal samples from the liver [2, 24] or intestinal [20] tissues. However, these assays may not be directly transferrable to the brain microsomes because of low abundance of CYP450 in the brain tissue. The only reported LC-MS/MS assay for DOD activity in the brain [3] is based on an assay originally developed for the liver microsomes [24]. Although reporting a detection limit of 2 fmol, the assay [3] requires 50 µg of microsomal protein and uses bucetin as an IS, which shows a different retention time than DXT [24]. The use of deuterated DXT as IS in our assay is more advantageous compared with an IS with a different structure, which may show

a different response to matrix effect or inter- and intra-run changes to the sensitivity of the MS detector. Additionally, our LLOQ for DXT (0.125 fmol) is a considerable improvement to the LLOQ reported in the literature for the DOD assay. The higher sensitivity of our assay, compared with the other MS/MS assays [2, 3, 20, 24] may be partly due to our use of UPLC instead of HPLC. Additionally, our method has a simpler sample preparation (protein precipitation with acetonitrile) compared to those methods that use a more complicated liquid-liquid extraction method for determination of DOD activity in the brain [5-7, 10, 20].

3.2. DXT impurity in the DXM substrates from different sources

Almost all of the studies of DOD activity use DXM hydrobromide (DXM-HBr), which is significantly more water soluble than DXM base. However, when we tested three commercially available powders of DXM-HBr, we noticed measurable concentrations of DXT impurity in the substrate. When quantified, the concentrations of DXT in 1000 µM stock solutions of the powders ranged from 4.17 to 9.72 nM, which translates to a very low impurity of 0.000417% to 0.000972%, based on the molar concentrations of DXT and DXM (Table 3). We also purchased and tested an analytical reference standard of DXM base powder (DXM-USP). The DXM-USP was dissolved in dilute HCl solutions for solubility purposes. The concentration and extent of impurity of DXT in the DXM-USP were >10 fold lower than the corresponding values for the DXM-HBr powders (Table 3).

The relatively low level of impurity of DXT in the substrate powders (Table 3) may be insignificant for DOD activity assays in the liver microsomes, which have high DOD activity. However, the impurity could potentially lead to significant errors when the production rate of DXT is relatively low, as is the case with the brain microsomes. Therefore, we tested the effects of DXT impurities on the formation rate of DXT in brain microsomes using 1000 µM

concentrations of DXM-HBr #3, which had the lowest degree of impurity among the three tested HBr powders (Table 3), and DXM-USP with a 10-fold lower impurity. Figures 3A and 3B demonstrate the chromatograms of DXT present in the DXM-HBr #3 and DXM-USP powders, respectively, immediately (time zero) and 20 min after the incubation of the brain microsomal samples with 1000 μM of the substrates. As expected from the data in Table 3, the DXT impurity peak in the DXM-HBr #3 sample (4.36 ± 0.22 nM, Fig. 3A) was ~ 10-fold larger ($p < 0.0001$, $n = 5$) than that in the DXM-USP sample (0.453 ± 0.044 nM, Fig. 3B). Additionally, for the DXM-HBr #3 substrate, the DXT impurity peak appeared to substantially contribute to the DXT peak after 20 min of incubation with brain microsomes (Fig. 3A). However, the contribution of small impurity in the DXM-USP to the 20-min incubation sample seemed to be minimal (Fig. 3B).

The higher impurity in the DXM-HBr #3 powder resulted in a significantly ($p < 0.01$, two-tailed unpaired t-test, $n = 5$) higher (40%) DOD activity with this substrate as compared with the activity after the incubation with DXM-USP (Fig. 3C). However, when the production of the metabolite by brain microsomes was normalized to the background peak at time zero, the DOD activities for the two substrates became similar (Fig. 3D). Therefore, for our incubation studies with brain microsomes, we used DXM-USP in addition to measurement of the DXT at both time zero and 20 min to account for the presence of any impurities.

3.3. Optimization of DOD incubation conditions

Figures 4A and 4B depict the brain microsomal activity of DOD as a function of time and microsomal protein concentrations, respectively. As demonstrated in Fig. 4A, the amount of metabolite formed was linear up to 40 min of incubation time in the presence of 0.2 mg/mL protein and 200 μM DXM. Additionally, the formation rate was linear across the tested protein concentrations (0.1, 0.2, 0.3, and 0.4 mg/mL) after 20 min incubation of the samples with 200

μM DXM (Fig. 4B). From these experiments, we determined that the optimum incubation conditions for our kinetic experiments were 0.2 mg/mL brain microsomal protein (5 μg protein for an incubation volume of 25 μL) and 20 min of incubation time. Compared with our incubation method, which uses 5 μg microsomal protein, previous studies estimating DOD activity in rat brain microsomes have used much higher brain microsomal proteins of 50 μg [3], 250 μg [4], or 1500 μg [7]. This decrease in protein amount per reaction allows for the use of single rat brain microsomal preparation as opposed to the pooling of multiple brains to yield enough microsomal material for MM studies of multiple enzymes. Additionally, a reaction time of 20 minutes was sufficient to produce enough metabolite for the analysis, compared to the 1-2 hours' time typically needed [3, 4, 7].

Figure 4C shows the stability of the metabolite under the optimized reaction conditions as a function of incubation time. As demonstrated (Fig. 4C), there were no significant changes in the DXT concentrations with time, which indicates that the metabolite is stable for up to 20 minutes of incubation with NADPH and 5 μg of brain microsomal protein. Additionally, the re-injection of the same samples stored for 24 hours in the autosampler (10^{o}C) did not reveal any significant changes in the concentrations of DXT (Figure 4C) or the absolute peak values of the DXT and IS (data not shown) as a result of autosampler storage.

3.4. Estimation of DOD kinetics in rat brain microsomes

The kinetics of DOD activity in brain microsomes collected from four different animals are shown in Figure 4D. We observed an expected MM curve that plateaued between substrate concentrations of 1000 and 2000 μM. The estimated MM kinetic parameters (mean ± SD, n = 4 animals) were a V_{max} of 2.24 ± 0.42 pmol/min/mg and a K_m of 282 ± 62 μM. Only two other studies, Jolivalt et al. [4] and Voirol et al. [7], have reported DOD kinetics in rat brain

microsomes. These studies used far higher protein amounts (250 and 1500 µg) and much longer incubation times (120 and 90 minutes), compared with our study (5 µg protein and 20 min incubation time). Whereas Jolivalt et al. [4] reported V_{max} and K_m values of 1.40 pmol/min/mg and 400 µM, respectively, a V_{max} of 0.65 pmol/min/mg and a K_m of 120 µM were reported by Voirol et al. [7]. Our estimated K_m value (282 µM) lies in between the values reported by these two studies (120 and 400 µM). However, the V_{max} values reported in these studies are somewhat lower than our observations (2.24 pmol/min/mg). In addition to these two studies, a study by Asai et al. [3] measured the DOD activity in the microsomes from the brain cerebellum, cortex, and hippocampus only at a single substrate concentration of 50 µM. In their study, the highest DOD activity was observed in the cerebellum microsomes, which was 0.03 pmol/min/mg. However, the DOD activity in the cortical and hippocampal microsomes was half of that in the cerebellum. This activity is far lower than what we would expect from our whole brain microsomal samples (~0.5 pmol/min/mg at 100 µM dextromethorphan, Fig. 4D). The lower DOD activities or V_{max} reported in the literature for brain microsomes may be due to a variety of factors. First, all three studies [3, 4, 7] used an NADPH-regenerating system, which contained $MgCl_2$ that can reportedly [7] inhibit CYP450 metabolism. In our preliminary studies, we also observed inhibition of DOD activity when using $MgCl_2$ in our reaction buffer (data not shown). Additionally, these studies used longer incubation times of 60 min [3], 90 min [7], or 120 min [4]. Thus, the longer incubation times may be responsible for the lower metabolic capacity. This could be due to the inherent instability that has been reported for CYP450 in brain samples [10, 13].

In studies described here, we followed the rate of formation of DXT as opposed to the rate of decline in the concentrations of the substrate. This is because MM studies are normally carried

out at substrate concentrations that are expected to remain almost constant during the incubation period. Indeed, the concentrations of DXT formed during our 20-min microsomal incubation time from substrate concentrations of 100 – 2000 μM were on average equal to 2.32 – 8.39 nM, which are equal to 2.32% and 0.420% of the initial substrate concentrations, respectively. Therefore, the concentrations of the substrate remains virtually constant during the incubation period.

4. Conclusions

In this communication, we have described a micro-scale incubation method and a sensitive and validated LC-MS/MS assay for the estimation of DEX demethylation activity in the rat brain microsomes. Collectively, these methods allowed us to substantially reduce the incubation volume, microsomal protein amount, and the length of incubation. The method was successfully applied to the estimation of CYP2D MM kinetics of the DOD activity in the brain microsomes. By requiring far less biological material and time, our method represents a significant improvement over the existing techniques for investigating CYP2D activity in rat brain microsomes.

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Table 1

Overall and matrix recoveries (mean ± SD) of dextrorphan and internal standard (IS) from the brain microsomal samples at low (0.25 nM), medium (5 nM), and high (50 nM) concentrations of DXT in the calibration curves (n = 5). The IS concentration was the same (10 nM) in all the samples.

Concentration (nM)	Overall Recovery (%)		Matrix Recovery (%)	
	Dextrorphan	IS	Dextrorphan	IS
0.25	106 ± 18	108 ± 8	91.0 ± 25.5	95.9 ± 3.76
5	94.1 ± 11.5	96.2 ± 11.5	94.6 ± 8.24	96.2 ± 5.94
50	91.0 ± 16.1	89.4 ± 15.3	95.3 ± 4.83	102 ± 4.64

Table 2

Intra- and inter-run accuracy and precision values of the lowest (0.25 nM), middle (5 nM), and highest (100 nM) concentrations of dextrorphan in calibration curves for brain microsomes (n = 5).

Concentration (nM)	Intra-run		Inter-run	
	Accuracy	R.S.D. (%)	Accuracy	R.S.D. (%)
0.25	102	14.9	111	14.5
5	98.2	6.5	94.1	7.8
100	100	9.4	101	1.6

Table 3

Extent of dextrorphan (DXT) impurity (mean ± SD) in 4 different commercially-available powders of dextromethorphan (n = 5).

DXM Stock (1000 μM)	DXT impurity	
	Conc. (nM)	%
DXM-HBr #1	9.72 ± 0.68	0.000972 ± 0.000068
DXM-HBr #2	9.42 ± 0.23	0.000942 ± 0.000023
DXM-HBr #3	4.17 ± 0.11	0.000417 ± 0.000011
DXM-USP	0.401 ± 0.044	0.0000401 ± 0.0000044

Figure legends

Figure 1. Schematic of the dextromethorphan-O-demethylase (DOD) reaction by CYP2D enzyme,

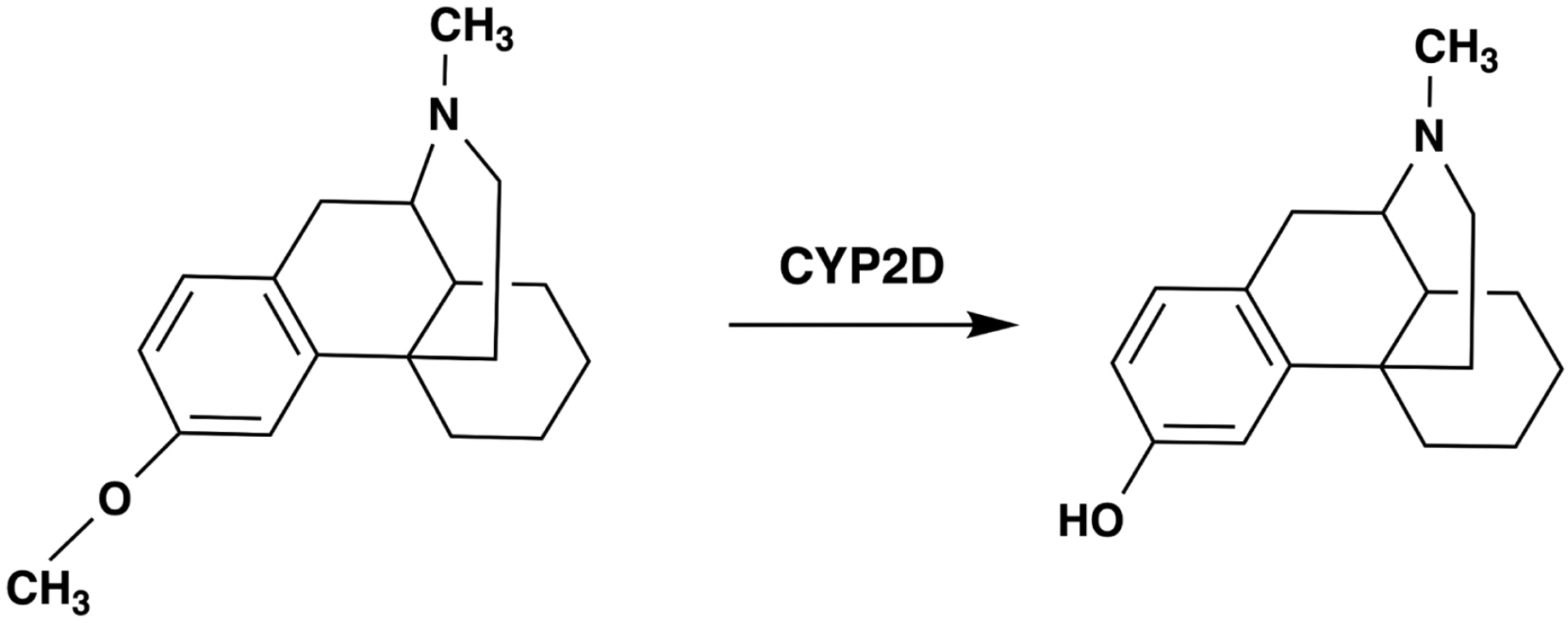
where dextromethorphan (DXM) is converted to dextrorphan (DXT).

Figure 2. Representative chromatograms of a blank brain microsomal sample (A), the lowest dextrorphan concentration (0.25 nM) in the calibration curve (B), and the internal standard (C).

Figure 3. Effects of DXT impurity in the substrate on the DOD assay. (A) and (B): Representative chromatograms of dextrorphan in the brain microsomes immediately and 20 min following microsomal incubation with 100 µM DXM-HBr #3 (A) or DXM-USP (B); (C) and (D): The estimated rates of metabolite (DXT) formation after 20 min of brain microsomal incubation at 37^{o}C without (C) or with (D) correction for the impurity. ** denotes $p < 0.01$, based on unpaired, two-tailed t test.

Figure 4. DOD assay characteristics (A-C) and Michaelis-Menten (MM) kinetics (D) in rat brain microsomal incubations. For time linearity (A), rat brain microsomes (n = 4) were incubated at 0.2 mg/mL protein with 200 µM DXM. For protein linearity (B), rat brain microsomes (n = 4) were incubated at varying protein concentrations with 200 µM DXM for 20 minutes. For metabolite stability (C), dextrorphan (5 nM) was incubated (37^{o}C) in brain microsomes (0.2 mg/mL) in the presence of NADPH (1 mM), and samples (n = 3) were analyzed immediately (First Injection) and after 24 hours of storage (10^{o}C) in the autosampler (Second Injection). For MM kinetics (D), incubations were carried out at 37^{o}C at 0.2 mg/mL protein in a final volume of 25 µL with 1 mM NADPH for 20 minutes (n = 4 different brains). Symbols and bars represent mean and SD of experimental data, respectively, and the lines are based on linear regression

analysis (A, B, and C) or a one-enzyme, Michaelis-Menten fit to the data (D). The slopes of the linear regression lines in C were not significantly different from zero.



Dextromethorphan (DXM) **Dextrorphan (DXT)**

Figure 1

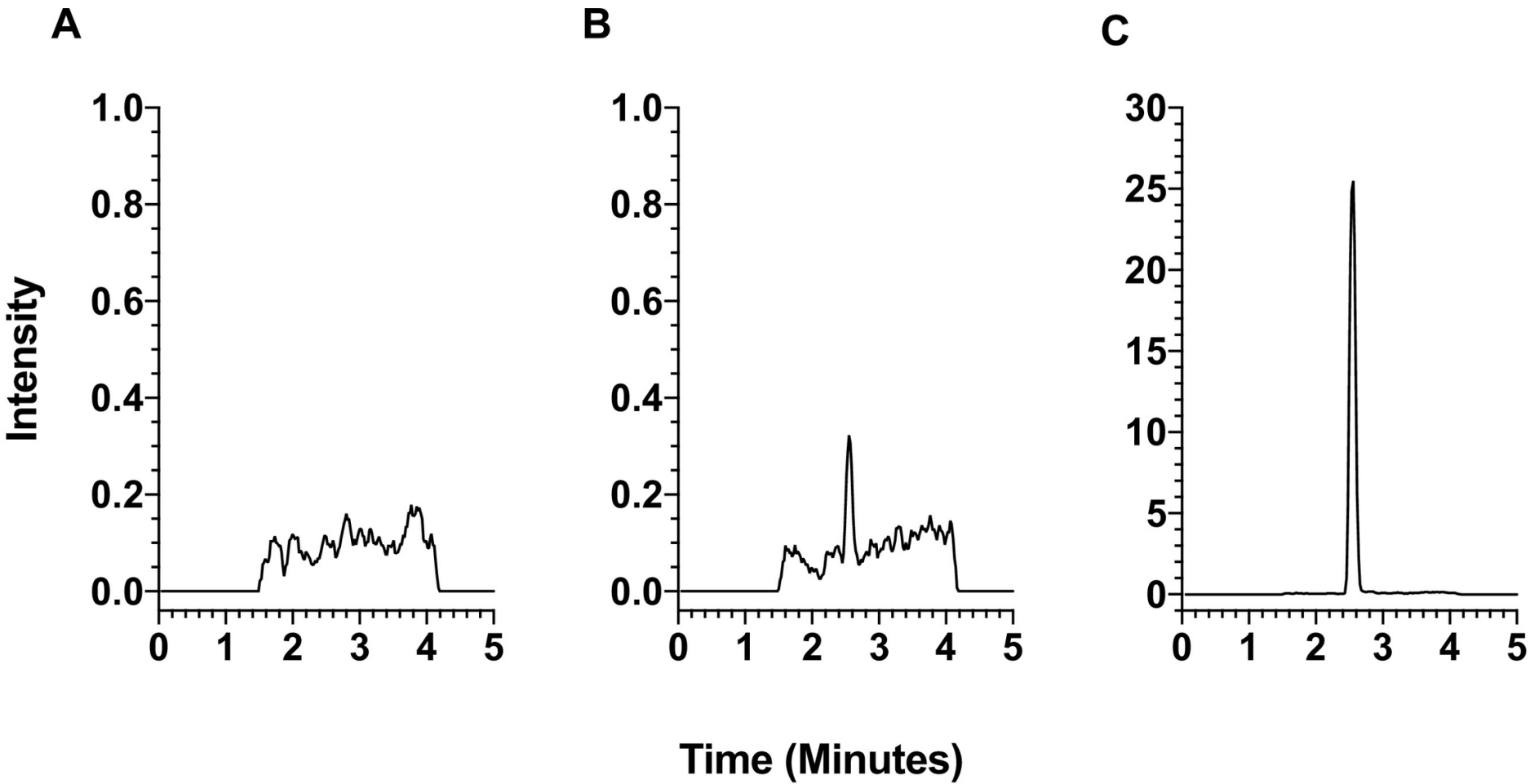

A
B
C
Intensity
1.0
0.8
0.6
0.4
0.2
0.0
30
25
20
15
10
5
0
0 1 2 3 4 5
Time (Minutes)

Figure 2

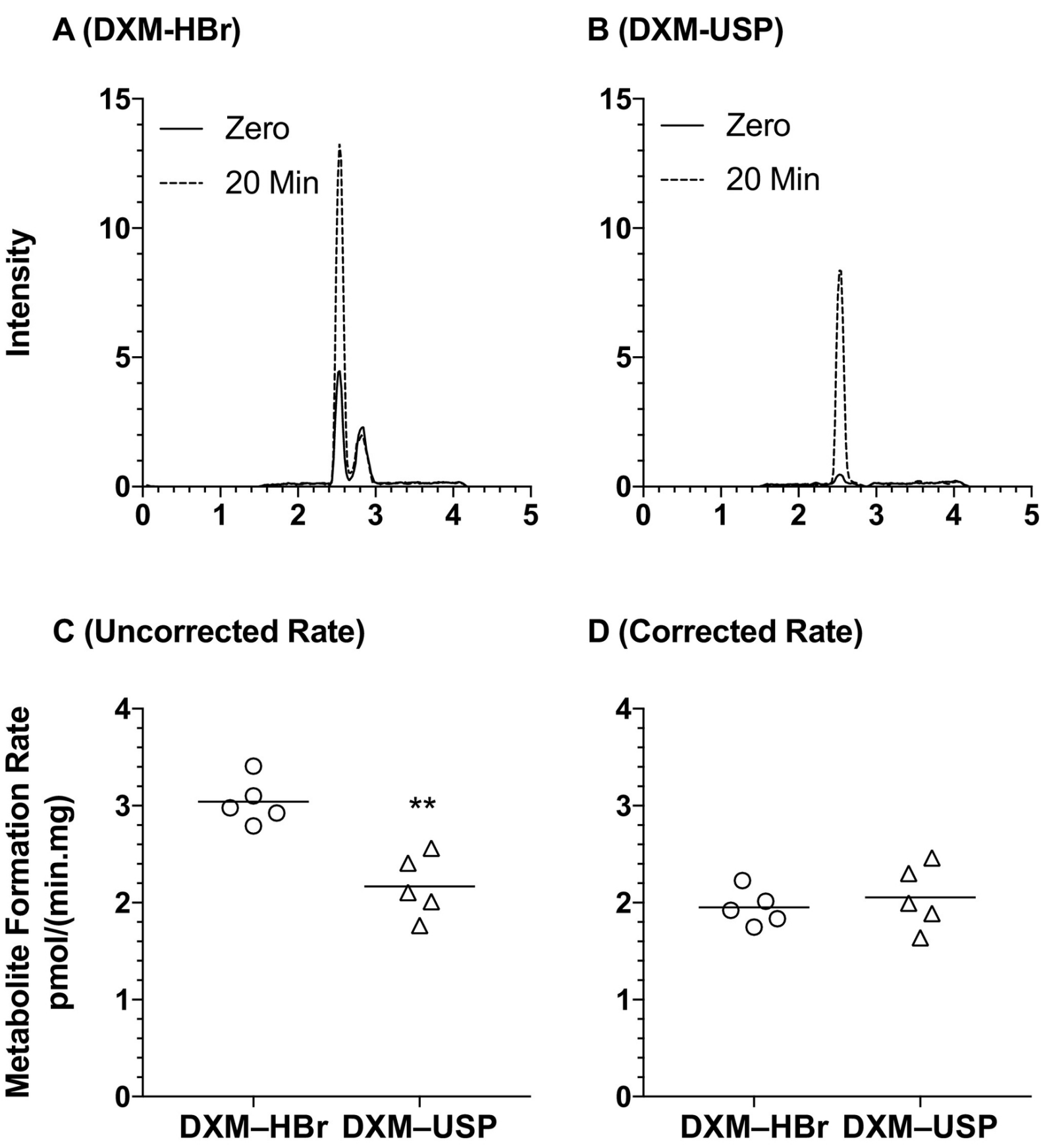
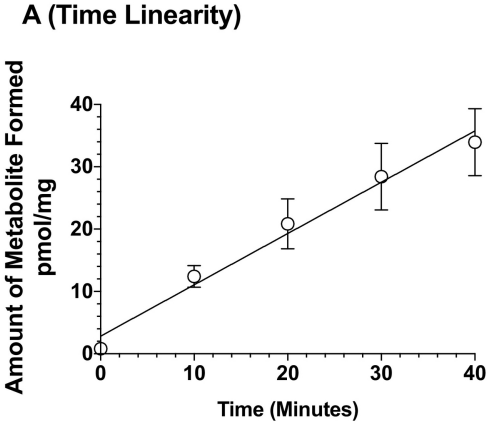
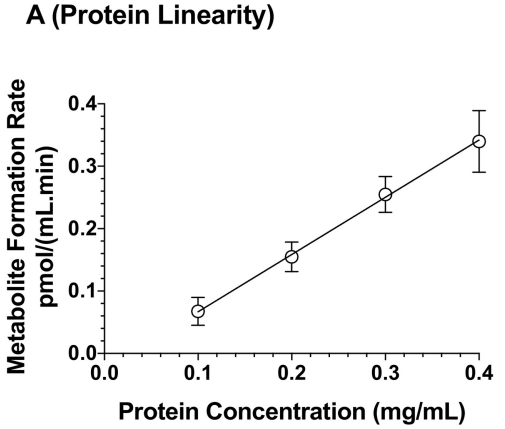
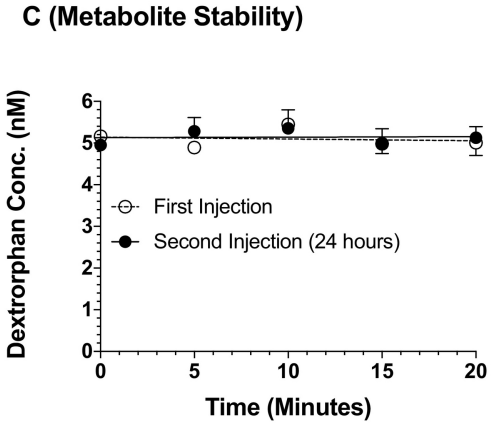

A (DXM-HBr)
B (DXM-USP)
Zero
20 Min
Intensity
15
10
5
0
0 1 2 3 4 5
C (Uncorrected Rate)
D (Corrected Rate)
Metabolite Formation Rate
pmol/(min.mg)
4
3
2
1
0
**
DXM–HBr DXM–USP

Figure 3


A (Time Linearity)
Amount of Metabolite Formed
pmol/mg
0
10
20
30
40
Time (Minutes)


A (Protein Linearity)
Metabolite Formation Rate
pmol/(mL.min)
0.0
0.1
0.2
0.3
0.4
Protein Concentration (mg/mL)


C (Metabolite Stability)
Dextrorphan Conc. (nM)
0
1
2
3
4
5
6
First Injection
Second Injection (24 hours)
0
5
10
15
20
Time (Minutes)

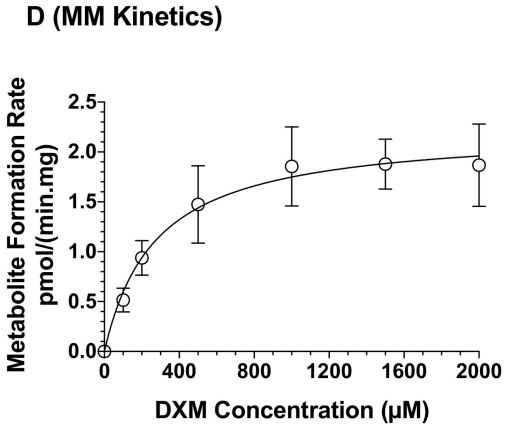

D (MM Kinetics)
Metabolite Formation Rate
pmol/(min.mg)
0.0
0.5
1.0
1.5
2.0
2.5
0
400
800
1200
1600
2000
DXM Concentration (µM)

Figure 4