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Development of an *in vitro* incubation procedure for screening of CYP2D6 intrinsic clearance

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Abstract

The *in vitro* intrinsic clearances (CL_{int}) for the metabolism of *p*-methoxymethamphetamine (PMMA) and fluoxetine by the CYP2D6 enzyme were calculated using a steady-state (SS) approach and a new general enzyme (GE) method, which measures the formation of product and the depletion of substrate as a function of time. For PMMA, the SS experiment resulted in a CL_{int} of 2.7 ± 0.2 μL pmol $2D6^{-1}$ min^{-1} and the GE experiment resulted in a CL_{int} of 3.0 ± 0.6 μL pmol $2D6^{-1}$ min^{-1}. For fluoxetine, the SS experiment resulted in a CL_{int} of 0.33 ± 0.17 μL pmol $2D6^{-1}$ min^{-1} and the GE experiment resulted in a CL_{int} of 0.188 ± 0.013 μL pmol $2D6^{-1}$ min^{-1}. We used two kinetic modeling techniques that can accommodate atypical kinetic models. We also show that the addition of fluoxetine results in a 10-fold decrease in the observed intrinsic clearance of PMMA, confirming that fluoxetine is a potent inhibitor of the liver enzyme CYP2D6.

Keywords: Drug metabolism; *p*-Methoxymethamphetamine; Fluoxetine; CYP2D6; Inhibition; Enzyme kinetics

1. Introduction

1.1. Pharmacokinetics and drugs of abuse

Pharmacokinetics is the field of study that encompasses the disposition of drugs in the body, specifically absorption, distribution, metabolism, and excretion [1]. In particular, metabolism studies include the determination of the specific enzymes responsible for breaking down the drug, the kinetic parameters of enzyme interactions, and the products of the reactions [2,3]. *In vitro* pharmacokinetic studies, and specifically metabolism studies, have always been an important part of the drug discovery and development process [2]. Understanding the metabolism of a new drug candidate is important for predicting *in vivo* clearance, and assessing potentially toxic or biologically active metabolites is also necessary before *in vivo* testing of any new drug candidate can begin [2]. *In vitro* methods can also be used to assess potential drug inhibition and drug–drug interactions. Multi-drug use is common for the treatment of many diseases [4] and among illicit drug users [5].

Drugs that are analogs of amphetamine, in particular 3,4-methylenedioxy-methamphetamine (MDMA), have received a great deal of attention in recent years due to the increasing incidence of abuse among young people [6]. Potential interactions between amphetamine analogs and selective serotonin reuptake inhibitor drugs (SSRIs), such as fluoxetine (Prozac™) are of particular interest because these popular antidepressant drugs are often taken in combination with, or the day after, amphetamine analogs [5]. This combination is also of concern to those patients being treated for attention deficit – hyperactivity disorder (ADHD). Psychostimulants including amphetamine are frequently used to treat ADHD in children and adolescents, and may be prescribed in conjunction with SSRIs and tricyclic antidepressants for the treatment of concurrent psychiatric disorders [7].

The amphetamine analog *p*-methoxymethamphetamine (PMMA) is a relatively new designer drug that is not as widely abused, but which has similar physiological effects as MDMA, the drug usually sold as 'Ecstasy'. Studies of trained rats indicate that PMMA has similar response properties as MDMA, but without the amphetamine-like stimulant character of MDMA [8]. Both PMMA and a close structural analog,

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p-methoxyamphetamine (PMA) have been blamed for fatalities around the world. In one particular report, three case histories are cited in which the users were taking 'Ecstasy' tablets and died days later of complications believed to be from an overdose of PMMA or PMA [9].

1.2. Cytochrome P450

Cytochromes P450 (CYPs) are a group of membrane bound enzymes present in the liver that are responsible for the catalysis of numerous oxidative reactions involving carbon, oxygen, nitrogen, and sulfur atoms in thousands of different substrates with many diverse structures [10]. CYPs have characteristics that allow a vast number of compounds to be metabolized by a limited number of isozymes (about 100), including broad substrate specificity and broad regio- and stereoselectivity [10]. The isozyme CYP2D6 is reported to be involved in the metabolism of about 12% of the most commonly prescribed pharmaceuticals [4], despite the fact that it accounts for only a small percentage (about 2%) of the CYPs found in the liver [11]. Some common CYP2D6 substrates include debrisoquine, tricyclic antidepressants, SSRIs including fluoxetine, various amphetamine analogs, and dextromethorphan, an over the counter cough suppressant [12]. CYP2D6 is a particularly interesting isozyme to study because it is known to be underexpressed in certain populations, which can lead to differences in drug metabolism between individuals. There are several alleles of CYP2D6 with varying activity, as well as some that are not active at all, resulting in a range of diverse phenotypes [1]. The development of rapid and robust *in vitro* methods for characterizing CYP2D6 and other enzymatic reactions will help to pave the way for understanding common drug interactions and help to identify or predict unknown interactions.

1.3. Enzyme kinetics and in vitro methods

The simplest model for an enzyme-catalyzed reaction consists of a reversible binding of a substrate (S) to an enzyme (E) to form an enzyme–substrate complex (ES). This complex can break down irreversibly to form product (P). The reaction is shown in Eq. (1):

$$\mathrm{E} + \mathrm{S} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \xrightarrow{k_3} \mathrm{P} \tag{1}$$

where k_1, k_2, and k_3 are the micro-rate constants for each of the reactions. Because this model is not always sufficient to describe the experimental data, more complex models are often required. Methods for describing and fitting data to these models are useful in pharmacokinetic studies.

The most common type of experiment to determine *in vitro* pharmacokinetic parameters is the steady-state (SS) approach [13]. The Michaelis-Menten (MM) equation for the initial velocity of the reaction, v_0, is used to describe the data obtained in a SS experiment:

$$\left(\frac{\mathrm{d[P]}}{\mathrm{d}t}\right)_{t=0} = v_0 = \frac{v_{\max}[\mathrm{S}]_0}{K_\mathrm{m} + [\mathrm{S}]_0} \tag{2}$$

where K_m is the Michaelis constant and $v_{\max}$ is the maximum velocity of the reaction. The constants in the MM equation are related to the micro-rate constants by Eqs. (3) and (4),

$$K_\mathrm{m} = \frac{k_2 + k_3}{k_1} \tag{3}$$

$$v_{\max} = k_3[\mathrm{E}]_0 \tag{4a}$$

$$v_{\max} = k_3 \tag{4b}$$

where $[\mathrm{E}]_0$ is the initial concentration of the enzyme species in the reaction mixture [14]. Eq. (4b) is used if the initial velocity, v_0, is normalized by the initial enzyme concentration. Because the *in vivo* substrate concentrations are usually likely to be much less than K_m, $[\mathrm{S}]_0$ can be neglected in the denominator of Eq. (2) [13], which allows the prediction of *in vivo* intrinsic clearance using the following equation

$$\mathrm{CL_{int}} = \frac{v_{\max}}{K_\mathrm{m}} \tag{5}$$

where $\mathrm{CL_{int}}$ is the intrinsic clearance. Multiplying Eq. (5) by the substrate concentration, along with the appropriate scaling factors, permits the estimation of the rate of metabolism *in vivo* from *in vitro* parameters [13].

Recently published alternatives to the standard SS experiment include a direct injection LC/MS/MS technique [15], a pseudo-first-order kinetics method [16] and a substrate depletion approach [17]. Bhoopathy et al. [15] suggested that $\mathrm{CL_{int}}$ can be estimated using a direct injection technique with no stirring of the reaction mixture in a temperature-controlled LC autosampler tray. Their method requires the concentration of the probe drug to be much less than K_m in order to determine the first-order rate constant of elimination. They were able to determine $\mathrm{CL_{int}}$ by monitoring the depletion of substrate only. Schnell and Mendoza [16] discussed the validity of such an approach by examining the mathematical derivation of pseudo-first-order (PFO) kinetics. They stated that approximating PFO conditions experimentally requires only that $[\mathrm{S}]_0 \ll K_\mathrm{m}$ and is independent of the initial enzyme concentration, contrary to previous reports [18,19] that claimed that one of the reactants, $[\mathrm{E}]_0$ or $[\mathrm{S}]_0$, should be in large excess. Jones and Houston reported the application of a substrate depletion method, which has the advantage that the specific metabolic pathways of the drug do not need to be known [17].

A more general way to express the change of any of the species in a chemical reaction is by using the rate laws for the elementary reaction steps and an ordinary differential equation solver to find mathematical solutions to the rate laws [20]. For example, for the enzyme reaction mechanism shown in Eq. (1), the change in product with time is expressed generally as

$$\frac{\mathrm{d}[P]}{\mathrm{d}t} = k_3[\mathrm{ES}] \tag{6}$$

Analogous differential equations can be written for each of the other species (E, S, and ES) involved in the general enzyme reaction shown in Eq. (1). The SS approach (where d[ES]/dt is assumed to equal zero) is usually applied in order to obtain the expression given by Eq. (2). Using the basic rules of chemical

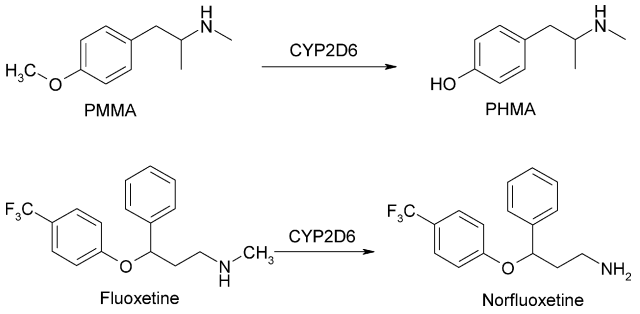

Fig. 1. CYP2D6 mediated demethylation of PMMA and fluoxetine to PHMA and norfluoxetine, respectively.

kinetics, any enzyme mechanism can be modeled by representing the rate of each step in the mechanism with a differential equation [21] and by monitoring only one or two of the species participating in the reaction. Bezemer and Rutan have previously described an approach for the fitting of kinetic data to any model [20], and more recently presented the method specifically for the fitting of enzyme kinetic data to the general kinetic model [22]. This approach uses the ordinary differential equation solver in Matlab® to find numerical solutions to the differential rate equations.

In this work, we present a general enzyme (GE) kinetics experiment that can be carried out by monitoring the depletion of substrate and/or the formation of product as a function of time. We use liquid chromatography mass spectrometry data (LC–MS) and compared the calculated intrinsic clearances from the GE method to the traditional SS approach. The two drug systems studied are shown in Fig. 1. The O-demethylation of PMMA to *p*-hydroxymethamphetamine (PHMA) and the N-demethylation of fluoxetine to norfluoxetine by CYP2D6 are investigated as model reactions to validate the new method. We also show that the new method can be used to carry out inhibition studies.

We use previously characterized systems to validate this approach, and we believe the methods presented in this work will generalize well to unknown drugs even without previous knowledge of the metabolic reactions. For example, if the structure of a product is unknown, the data can be fit by measuring only the depletion of substrate or by the formation of unidentified products. The utility of this method lies in the enhanced ability to screen new drugs from the same class (i.e., phenylalkylamines, or SSRI's) for activity with a certain CYP enzyme, or for all possible CYP catalyzed reactions if liver microsomes or hepatocytes are used.

2. Experimental

2.1. Materials

The authentic standards for PMMA, fluoxetine, and norfluoxetine were obtained from Alltech (State College, PA, USA) as unscheduled chromatographic standard solutions of 1 mg/mL of the free base in methanol. PHMA and nicotinamide adenine dinucleotide phosphate (NADPH) tetrasodium salt were obtained from Sigma (St. Louis, MO, USA). Formic acid, 98%, was obtained from Fluka (Steinheim, Germany), 6 M ammonium hydroxide was obtained from Ricca Chemical Company (Arlington, TX, USA), and acetonitrile was obtained from EMD (Gibbstown, NJ, USA). Sodium hydrogen phosphate was obtained from EM Science (Cherry Hill, NJ, USA), and phosphoric acid (85%) was obtained from Fisher Scientific (Pittsburgh, PA, USA). Ultrapure 18 MΩ-cm water dispensed in house was used to prepare all chromatographic eluents and buffers. For the enzyme incubation experiments, CYP2D6*1 Supersomes™, (baculovirus-insect cell expressed with coexpression of CYP450 reductase) and control Supersomes™ (from wild-type baculovirus-insect cells) and an NADPH regenerating system (solutions A and B) were all obtained from BD Biosciences (Bedford, MA, USA).

2.2. Incubation experiments

2.2.1. Steady-state incubations

A 0.10 M phosphate buffer was prepared with Na_2HPO_4, and H_3PO_4 was used to adjust the pH to 7.4. A 10 mM solution of NADPH was prepared on the day of analysis by dissolving the appropriate amount of the tetrasodium salt in phosphate buffer. Stock solutions of PHMA, PMMA, fluoxetine and norfluoxetine were prepared in phosphate buffer and diluted to make calibration standards as outlined in Table 1. The reaction components were added to a 1.5 mL microcentrifuge tube in the following order: enzyme, substrate (PMMA or fluoxetine), phosphate buffer, and then NADPH to start the reaction. This particular order of addition of the reactants was based on previously published incubation procedures [23–25]. The total incubation volume was brought to 500 μL with phosphate buffer. The concentration of NADPH in the final reaction mixture was 600 μM, and the concentrations of the other reactants were as shown in Table 1. The tubes were placed in a Precision metabolic shaker (Winchester, VA, USA) at 37 °C immediately after the NADPH was added. After the time indicated in Table 1, the samples tubes were placed on ice, and 750 μL of ice cold acetonitrile was added to stop the reaction. Samples were centrifuged on a Biofuge 17R centrifuge from Baxter Scientific Products (West Chester, PA, USA) at 9300 × *g* (12,000 rpm) for 10 min. The supernatant was filtered through a 0.2 μm nylon filter, and placed into an autosampler vial for HPLC analysis. Unanalyzed portions were stored in the freezer.

Table 1
Conditions for the SS experiments with PMMA and fluoxetine

Analyte	Calibration range (μM)	Incubation concentrations (μM)	Enzyme concentration (μM)	Incubation time (min)
PMMA	2–500	0–700[a]	0.02[a]	45
PHMA	5–80	0–250[b]	–	–
Fluoxetine	0.2–10	0–500[a]	0.02[a]	120
Norfluoxetine	0.05–5	0–20[b]	–	–

[a] Concentration added to incubation mixture.
[b] Concentrations detected by LC–MS.

Table 2
Conditions for the GE experiments with PMMA and fluoxetine

Analyte	Calibration range (μM)	Incubation concentration (μM)	Enzyme concentration (μM)	Incubation times (min)
PMMA	0.4–12	5[a]	0.02[a]	0–75
PHMA	0.4–12	0–5[b]	–	–
Fluoxetine	2–10	10[a]	0.05[a]	0–150
Norfluoxetine	2–10	0–4[b]	–	–

[a] Concentration added to incubation mixture.
[b] Concentrations detected by LC–MS.

2.2.2. General enzyme kinetics incubations

Standard curves for all analytes were prepared as shown in Table 2. NADPH regenerating solution A and solution B were mixed in a ratio of 25:5 and kept at 37 °C until added to the reaction vessel. The following components were mixed in an HPLC autosampler vial: enzyme, substrate (PMMA and/or fluoxetine), and phosphate buffer for a total incubation volume of 400 μL. The incubation mixtures were injected directly into the LC–MS system without further preparation. One injection was made before the NADPH mixture was added, and then an injection was made every 4 (for PMMA) or 6 (for fluoxetine) min after the NADPH (30 μL of the mixed regenerating solution) was added to the mixture. The autosampler tray was held at 37 °C for the duration of the incubation. For the inhibition experiment, 5 μM of fluoxetine was added to the reaction mixture before adding the NADPH.

2.3. LC–MS analysis

All chromatographic separations were carried out on a Waters Alliance 2795 LC system equipped with a heated autosampler and column compartment (Waters Corp., Milford, MA, USA). The column was thermostated to 40 °C unless otherwise noted. A guard column and an in-line filter were used for all chromatographic separations. All mobile phases were filtered through 0.45 μm membrane filters before use. The injection volume from the autosampler was 10 μL. Detection was accomplished with a Thermo LCQ XP Deca Plus ion trap mass spectrometer equipped with an electrospray ionization source (ESI) (Thermo Electron Corp., Waltham, MA, USA). A divert valve was used in front of the electrospray source to avoid contamination from buffer salts in the chromatographic dead volume. Calibration curves for each analyte were constructed as described in the following sections. Standards were made in both "clean" phosphate buffer matrix, and in matrix containing 0.020 μM of insect control enzyme. The insect control standards were used for quantification of the analytes in the incubation mixtures. Levallorphan was used as an internal standard for the fluoxetine incubation (added to the mixtures), and a post-column infusion [26] of *d*-amphetamine was used as an internal standard for the PMMA incubations. However, the use of the internal standard for the quantification of the components resulted in a degradation of the precision of the calibration parameters; therefore direct calibration in conjunction with the resolved responses from the curve fitting analysis was employed for quantification.

2.3.1. Analysis of steady-state incubations of PMMA

The LC conditions for the SS incubations of PMMA (PMMA-SS method) were as follows: mobile phase A was 98% deionized water, 2% acetonitrile, and 0.01% formic acid; mobile phase B was 98% acetonitrile, 2% water, and 0.01% formic acid. The pH of the aqueous phase was approximately 3. The flow rate used was 250 μL/min and the column output went into the electrospray source via the divert valve on the mass spectrometer. The mobile phase gradient was from 8 to 20% mobile phase B from 2 to 12 min, then from 20 to 30% B from 12 to 14 min, then from 30% back to 8% B from 14 to 18 min. The total run time was 25 min, including column re-equilibration time. The chromatographic column used was a 50 mm × 4.6 mm Phenomenex Luna C18(2) stationary phase, with 5 μm particles (Phenomenex, Torrance, CA, USA). The ESI settings were as follows: the spray voltage was set at 5.50 kV, and the capillary temperature was set at 275 °C. Nitrogen was used for the drying gas and the auxiliary gas. Full scan mode was used for detection of analytes.

2.3.2. Analysis of general enzyme kinetics incubations of PMMA

The LC conditions for the GE incubations of PMMA alone and the inhibition experiment (PMMA-GE method) were as follows: mobile phase A was a 10 mM ammonium formate buffer, prepared gravimetrically with appropriate amounts of formic acid and ammonium hydroxide to achieve a pH of 3.6. Mobile phase B was 100% acetonitrile. The flow rate used was 650 μL/min and the effluent from the column went to the ionization source via the divert valve on the mass spectrometer. The column was thermostated to 50 °C. The gradient was from 3 to 33% mobile phase B from 0.6 to 0.7 min, stayed at 33% B until 1.9 min, and then went from 33 to 3% B from 1.9 to 2.0 min. The total run time was 3 min, including column re-equilibration time. The chromatographic column used was a 20 mm × 2.1 mm Betasil C18 DASH HTS (Thermo Electron Corp. Waltham, MA, USA) with 5 μm particles. The mass spectrometer was used in selected reaction monitoring (SRM) mode to select the fragmentation products of PMMA and PHMA (*m/z* 150 and 135, respectively). The ESI settings were as follows: the spray voltage was set at 4.5 kV, and the capillary temperature was set at 200 °C. Nitrogen was used for the drying gas and the auxiliary gas.

2.3.3. Analysis of fluoxetine incubations

Both the SS and GE kinetics incubations of fluoxetine and CYP2D6 were analyzed as follows (FLX method): The mobile phases were the same as described in Section 2.3.2 and the column was as described in Section 2.3.1. The separation was carried out under isocratic conditions with 33% mobile phase B. The total run time was 6 min. The mass spectrometer was used in full scan mode for the detection of analytes. The ESI settings were as follows: the spray voltage was set at 4.5 kV, and the capillary temperature was set at 200 °C. Nitrogen was used for the drying gas and the auxiliary gas.

2.4. Data analysis

The XCalibur® software program (Thermo Electron Corp., Waltham, MA, USA) was used to determine the peak areas of the analytes from the LC–MS chromatograms. The file converter tool in XCalibur® was used to convert collected chromatograms into text files and a Pascal program written in house for MS-DOS was used to convert the text files into a matrix format suitable for analysis in Matlab®. Kinetic analysis of all collected data was carried out in the Matlab programming environment, using Matlab®, ver. 7.0.4 (Mathworks, Natick, MA, USA). An alternating least squares (ALS) fitting program that has previously been developed in our laboratory [27] was used to resolve the chromatographic, spectral, and concentration profiles from the LC–MS data. A least-squares fitting routine with a built in steady-state constraint [28] was used to fit the curves obtained from the SS experiments, and a general kinetic fitting function [20] was used to fit the curves obtained from the GE experiments.

3. Results and discussion

Rather than following the classical steady-state approach, wherein multiple substrate levels are monitored after a fixed incubation time (verified by previous experiments to ensure that the time point is within the linear velocity regime) [1], the new method presented in this work uses a general kinetic approach. The change in concentration of a single substrate level is measured as a function of time, and the resulting data is fit using an ordinary differential equation solver and a kinetic fitting routine previously developed in our lab. The advantages to this method are that fewer raw materials are needed (including costly enzyme preparations), sample preparation time is significantly less, and the concentrations of all reactants can be tailored to suit the detection limits of the instrument being used. We use a fast LC–MS method to quantify the substrates and product(s) present in enzyme incubation samples. The results discussed below show that we were able to obtain consistent results for the intrinsic clearance of fluoxetine and PMMA using both the GE and SS methods.

The fit constants (the micro-rate constants for the GE method and the K_m and v_{max} values for the SS method) cannot be calculated with very much precision by either fitting approach due to the fact that they all co-vary significantly. This covariance indicates that there are many combinations of the constants that will lead to a satisfactory fit of the data. However, the intrinsic clearance can be calculated using Eqs. (3), (5), and (6), and Monte Carlo error estimation methods confirm that the error in CL_{int} is relatively low.

One issue that was not addressed experimentally, but that has received a good deal of attention in the literature is that of enantiomeric specificity of enzymatic reactions. Both PMMA and fluoxetine have chiral centers, therefore the possibility of differential *in vitro* metabolism of the stereoisomers exists. The drugs used in this study were racemic mixtures (as are the corresponding street and marketed drugs) and no attempt at differentiating the metabolism of the steroisomers was made. However, it should be pointed out that several studies have been published discussing the possibility of chirality playing a role in the metabolism of chiral drugs [29–32]. Caldwell's thorough review article described the effect of enantiomeric discrimination in drug metabolism for several systems at both the substrate and product level. We are not aware of any studies specifically on the stereoselective metabolism of PMMA; however, several sources have published values for K_m and v_{max} of the *R* and *S* isomers of MDMA. Tucker et al. reported a K_m of 1.72 ± 0.12 μM and 2.90 ± 0.10 for (+)-MDMA and (−)-MDMA, respectively. They also cited several older reports that the neurotoxic effects of MDMA were isomer specific and that the enantiomers of MDMA and MDA may have different behavioral effects, however they did note that the differences in the *in vitro* metabolism parameters were small.

Based on these published reports, it would not be unexpected to find some difference in the metabolism of the enantiomers of PMMA and fluoxetine. Simulations of the kinetic results for racemic mixtures of MDMA (based on the reports of the enantioselective pharmacokinetic constants) show that it is unlikely that the clearance values for the isomers could be resolved in practice. The simulations of the MDMA *in vitro* reactions show that a racemic mixture would result in a K_m and v_{max} value that are approximately averages of the (+) and (−) isomers and within the standard error of the measurement. It suffices to say that a detailed analysis of stereospecific metabolism is beyond the scope of the simple screening method described here.

3.1. PMMA incubations

Carrying out *in vitro* metabolism experiments under classical SS conditions allows for the calculation of the constants K_m and v_{max} by fitting the data to a steady-state model as described in reference [28], which then allows for an estimation of intrinsic clearance (CL_{int}, Eq. (5)). Fig. 2 shows the results of the SS experiment used to characterize the *in vitro*

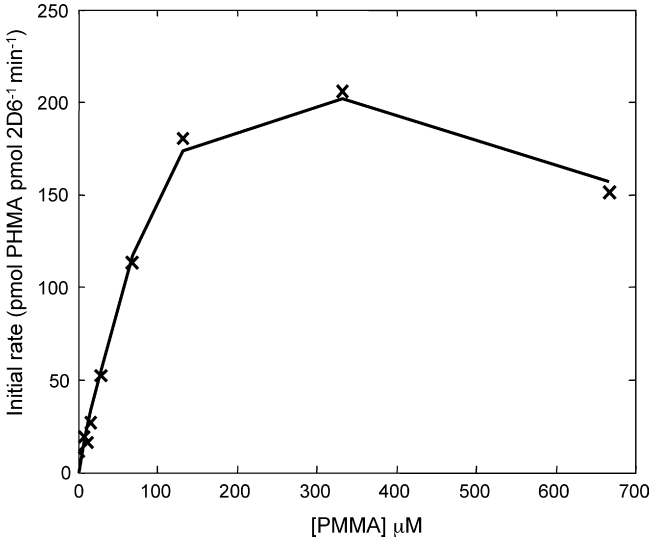

Fig. 2. Results of the SS experiment for PMMA, fit to a biphasic profile. $CL_{int} = 2.7 \pm 0.2$ μL pmol 2D6^{-1} min^{-1}, $SE_{fit} = 10.2$ pmol PHMA pmol 2D6^{-1} min^{-1}.

metabolism of PMMA. The LC–MS data were resolved using the ALS algorithm described in reference [27] which allows for flexible implementation of the constraints on a component-by-component basis. In this work, spectral selectivity and unimodality constraints were used for the analyte components.

The results from the SS experiment with PMMA and CYP2D6 showed an atypical kinetic profile, with the concentration of PHMA decreasing at the highest substrate concentration. Atypical kinetic profiles for *in vitro* pharmacokinetic studies have been extensively discussed in the literature [13,33–37], particularly for the CYP2D6 isozyme. The pattern observed in the SS plot for this experiment is characteristic of a substrate inhibition model [13], and forcing a fit to the MM equation resulted in an overestimation of the CL_{int} and a poor fit quality. The data were instead fit to the substrate inhibition model suggested by Tracy [13] and the intrinsic clearance was calculated to be 2.7 ± 0.2 μL pmol $2D6^{-1}$ min^{-1}. The intrinsic clearance values determined from the data of Staack et al. [38] were approximately 10-fold higher than our results. We believe that the discrepancy is due to the fact that they used 5 mM Mg^{+2} in their incubations, while we did not employ any Mg^{+2}. Obviously, some experimental evidence will be required in order to confirm this suspicion. Adjustment of the reaction parameters (e.g., concentration, ionic strength, order of addition of reactants) will be the subject of future studies. In addition, without knowledge of the covariance of their parameters, we were not able to calculate a propagated error for their results. Their published data did not employ substrate concentrations greater than 400 μM, and the data were fit to a MM model, which also may have led to significant differences in the reported kinetic parameters.

The results from the GE incubation of PMMA with CYP2D6 are shown in Fig. 3. These results show a general case where the depletion of substrate and the formation of product are measured as a function of time. These data are similar to a general first-order kinetics experiment, and carried out without regard to the relative concentrations of enzyme and substrate. The data were fit to the general enzyme model shown in Eq. (1) to determine intrinsic clearance. For the GE kinetics experiment, the CL_{int} of PMMA was determined to be 3.0 ± 0.6 μL pmol $2D6^{-1}$ min^{-1}, which is within experimental error of the value calculated from the SS experiment. Atypical (i.e., substrate inhibition) kinetic profiles did not need to be modeled for the GE experiment because the substrate concentration was low (5 μM) and substrate inhibition only occurs at high substrate concentrations. These results show that consistent estimations of intrinsic clearance can be obtained using the GE method as compared to the SS method.

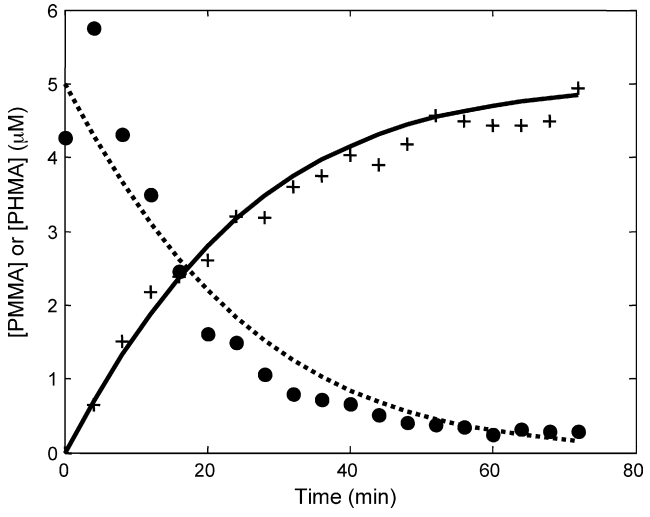

Fig. 3. Results of GE experiment for PMMA. The fit for the model shown in Eq. (1) is shown for the formation of product (solid line and +) and the depletion of substrate (dotted line and ●). $CL_{int} = 3.0 \pm 0.6$ μL pmol $2D6^{-1}$ min^{-1}, $SE_{fit} = 0.40$ μM.

3.2. Fluoxetine incubations

The results from both the SS and GE experiments with fluoxetine and CYP2D6 strongly indicated the existence of atypical kinetic profiles. Atypical kinetic profiles have been previously observed for the metabolism of fluoxetine in the literature. Margolis et al. [39] showed that fluoxetine follows MM kinetics at relatively low concentrations, but Ring et al. [40] have shown that the pure *R*-fluoxetine enantiomer follows an atypical kinetic profile, specifically substrate inhibition. There have also been previous reports that fluoxetine can be metabolized into multiple other metabolites including hippuric acid and *p*-trifluoromethylphenol [41]. The *m/z* values for hippuric acid and *p*-trifluoromethylphenol are 179 and 162 amu, respectively; these two compounds were not detected in the present experiments because the ion intensity data was only collected for *m/z* values ranging from 250 to 350 amu. The flexibility of the fitting algorithms used for both the SS experiment [28] and for the GE experiments [20] allowed us to design a model that best described the experimental data and was consistent with previous reports of atypical kinetics observed in the fluoxetine and CYP2D6 system.

A plot of v_0 versus $[S]_0$ for the fluoxetine SS experiment is shown in Fig. 4. The curve that we obtained from this experiment was consistent with the biphasic kinetic model suggested by Korzekwa et al. [34] where the enzyme has two binding sites for fluoxetine. This particular profile indicates that one of the binding sites has a much higher affinity (i.e., a lower K_m) than the other. Incorrectly forcing the data to fit to the MM model poorly predicted the norfluoxetine concentration at both the low and high fluoxetine concentrations. The data obtained in this experiment also had a high level of noise, which compromised the quality of the fit obtained and the calculated clearances. However, three points (indicated in grey in Fig. 4) could be omitted as outliers based on a plot of the known concentration of fluoxetine versus the resolved concentration profiles of fluoxetine. This plot (not shown) indicated that the resolved profiles of these three points were not consistent with the known concentration of fluoxetine in the system and thus they were omitted.

Fitting the data from the GE experiment for the metabolism of fluoxetine by CYP2D6 to the general enzyme reaction shown in Eq. (1) did not model the data well and had a high fit error. The data and the fit are shown in Fig. 5. The fluoxetine concentration continued to decrease after the concentration of norfluoxetine

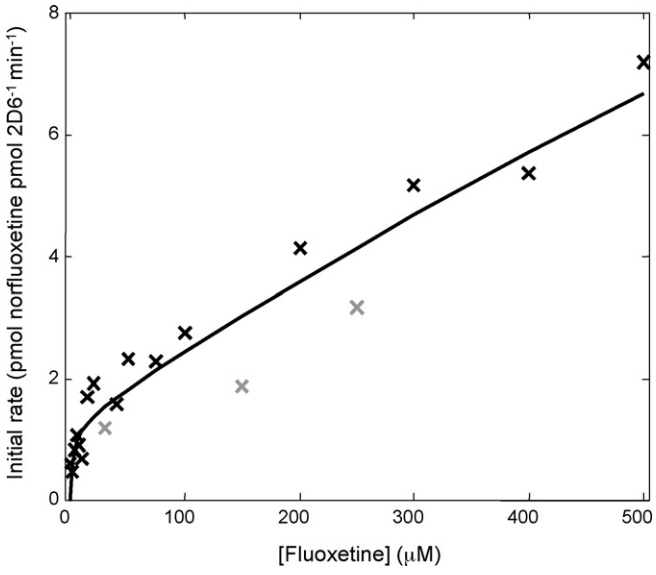

Fig. 4. Results of the SS experment for fluoxetine and CYP2D6. The data were fit to the model shown in Fig. 6. $CL_{int} = 0.33 \pm 0.17$ μL pmol $2D6^{-1}$ min^{-1}, $SE_{fit} = 0.33$ pmol norfluoxetine pmol $2D6^{-1}$ min^{-1}. The points indicated in grey are those that were omitted as outliers.

leveled off, suggesting the possibility that another product was being formed that contributed to the overall clearance of fluoxetine. Also, the rate of decrease of the fluoxetine concentration appeared to slow down toward the end of the incubation, which indicated that the enzyme was losing activity over the rather long time course of the experiment (this phenomenon has been reported previously [42]).

A modified mechanism was developed to incorporate the atypical kinetics observed in both the SS and GE experiments. The modified mechanism is shown in Fig. 6. The mechanism modeled the biphasic kinetics (6a and 6b) seen in the SS experiment, and the formation of a second product (6c) and the loss of

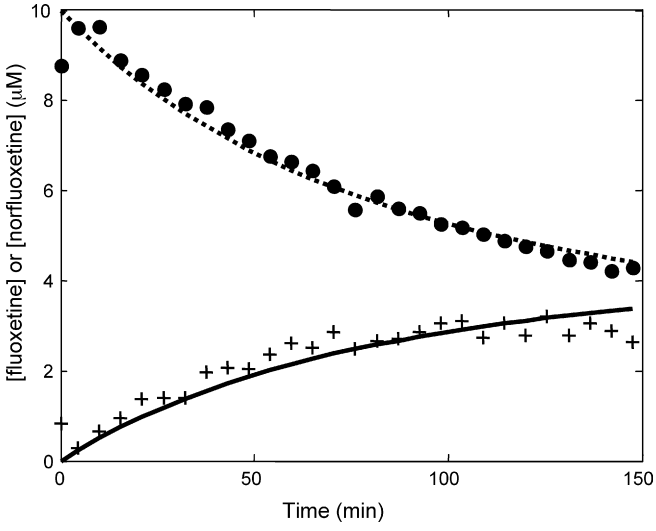

Fig. 5. Results of GE experiment for fluoxetine. The fit is shown for the model in Fig. 6 as the formation of product (solid line and +) and the depletion of substrate (dotted line and ●). $CL_{int} = 0.188 \pm 0.013$ μL pmol $2D6^{-1}$ min^{-1}, $SE_{fit} = 0.34$ μM.

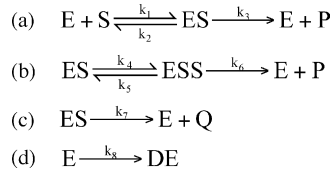

Fig. 6. Modified enzyme mechanism for fluoxetine experiments. S is fluoxetine, P is norfluoxetine, Q is a second product, and DE is the deactivated enzyme. (a) General enzyme reaction; (b) second active (low affinity) site; (c) formation of second product; and (d) decay of enzyme activity.

activity of the enzyme (6d) observed in the GE experiment. The CL_{int} from each pathway can be combined in order to obtain an overall CL_{int} for the substrate as shown in Eq. (7) [43]

$$CL_{int} = \frac{v_{max\,1}}{K_{m1}} + \frac{v_{max\,2}}{K_{m2}} + \frac{v_{max,Q}}{K_{m1}} \quad (7)$$

where $v_{max\,1}$, K_{m1}, $v_{max\,2}$, and K_{m2} are the maximum rates and the Michaelis constants of the two enzyme binding sites, and $v_{max,Q}$ is the maximum rate for the reaction of the enzyme–substrate complex (ES) forming product Q. Eqs. (3) and (4) were used to calculate the constants in Eq. (7) for each pathway. The formation of the second product Q was not included in the calculation of intrinsic clearance; for both experiments the reported clearance is with respect to the formation of norfluoxetine only, since it was the only product measured. For the GE experiment, the biphasic portion (Fig. 6b) of the model shown in Fig. 6 was not included in the fitting of the GE experimental data, because the low substrate concentrations employed in this experiment did not warrant it. The error in the value of CL_{int} was calculated using Monte Carlo methods because the individual rate constants displayed a high degree of covariance [44]. The CL_{int} for the GE experiment with respect to the formation of norfluoxetine was calculated to be 0.188 ± 0.013 μL pmol $2D6^{-1}$ min^{-1} and the CL_{int} for the SS experiment was calculated to be 0.33 ± 0.17 μL pmol $2D6^{-1}$ min^{-1}.

Although the GE and SS fluoxetine incubations required a modified mechanism to fit the data, it can be shown that the *overall* clearance of fluoxetine from a system with respect to norfluoxetine is consistent (within experimental error) regardless of the experimental method. The inclusion of the low affinity site (Fig. 6b) in the GE experiment was not necessary because of the different incubation conditions. This difference in the applied model was also true for the PMMA systems: at lower substrate concentrations, the PMMA followed typical general enzyme kinetics, while at the higher concentrations used in the SS experiment, a substrate inhibition model was required to fit the data.

3.3. Inhibition of PMMA by fluoxetine

In the inhibition experiment, we show that the inhibition of PMMA metabolism by fluoxetine could be modeled using the GE approach. The results of a GE incubation of PMMA with CYP2D6 in the presence of 5 μM fluoxetine are shown in Fig. 7. The intrinsic clearance for PMMA in this system was 0.40 ± 0.14 μL pmol $2D6^{-1}$ min^{-1}. There is nearly an order of magnitude decrease in the clearance for PMMA in the presence

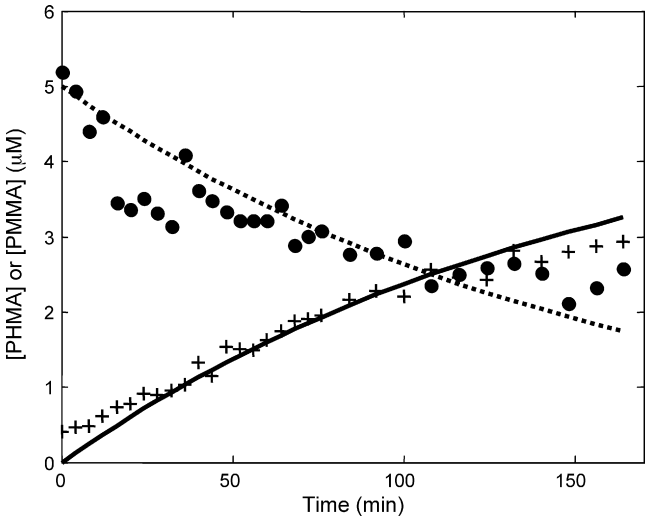

Fig. 7. Inhibition of PMMA by fluoxetine. The fit is shown for the formation of product (solid line and +) and the depletion of substrate (dotted line and ●). $CL_{int} = 0.40 \pm 0.14$ μL pmol $2D6^{-1}$ min^{-1}, $SE_{fit} = 0.37$ μM.

of an equimolar amount of fluoxetine. Fluoxetine has previously been shown to be a potent inhibitor of CYP2D6 [45], and we have shown with a relatively simple experiment that the change in intrinsic clearance can be determined.

4. Conclusions

PMMA and fluoxetine both have well characterized metabolic profiles, and thus make suitable models to validate a new method of screening intrinsic clearance values from *in vitro* data [38,45]. The SS experiments that were carried out served as test cases to compare the "traditional" method for determining intrinsic clearances, and a steady-state kinetics curve fitting algorithm was used to fit the data [28]. This algorithm allowed the data to be fit to atypical kinetic profiles to account for deviations from the MM equation. Both PMMA and fluoxetine showed evidence of atypical kinetic profiles.

The results of the GE experiments showed that a simple kinetics method can be used to predict intrinsic clearance with similar precision to the longer SS experiment. By simply monitoring the formation of product or the depletion of substrate as a function of time, the micro-rate constants of any kinetic model can be calculated and used to estimate the intrinsic clearance. In this work, we investigated the kinetics of previously characterized systems to validate the approach. The extension of this technique to new drug entities (whether licit or illicit) should be possible with the following caveat: the mass spectrometer used for detection should have adequate sensitivity in the full scan mode to detect substrate and products without prior knowledge of their structures or masses. Time-of-flight mass spectrometry would be ideal for this purpose. In this work we used an ion trap spectrometer which necessitated the use of SRM detection in some cases to obtain adequate detection sensitivity (which required knowledge of the masses of the analytes). As with the steady-state algorithm, the kinetic fitting routine used for the general kinetics experiments allowed flexibility when selecting a model. The PMMA incubation fit the general enzyme model at the low substrate level studied, but the fluoxetine required a more complex model to adequately fit the data and to determine intrinsic clearance. The clearances for both the PMMA and fluoxetine were in agreement between the two different methods.

It should be noted that the data were fit to models with the lowest complexity that could fit the data with acceptable precision. It is highly likely that the actual mechanisms are much more complex than the ones represented by the models used in this work [46]. However, to adequately elucidate these mechanisms, multiple experiments at multiple time-scales are required, whereas the method presented here is intended for the screening of intrinsic clearance values and drug reactions. Additionally, the goal of these experiments is the estimation of the CL_{int}, which is the key descriptor of the reaction rate at the low substrate concentrations expected *in vivo*. It has been shown previously that while the model parameters for increasingly complex models may not be well-defined, the CL_{int} parameter can still be precisely determined [22,28], and this is supported by the results of the present work.

Finally, the GE method was modified for a study of the inhibition of PMMA by fluoxetine. By adding an equimolar amount of fluoxetine to the mixture, the clearance of PMMA was reduced by nearly an order of magnitude. The results of this experiment confirm previous reports that fluoxetine is a potent inhibitor of CYP2D6. Future studies will focus on characterizing other drug–drug interactions using similar experiments and using multiple inhibitor concentrations to calculate inhibition constants.

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