

DMD #45682

Title Page

Microdialysis Evaluation of Clozapine and N-desmethylozapine Pharmacokinetics in Rat Brain

Thomas I.F.H. Cremers, Gunnar Flik, Corry Hofland and R.E. Stratford, Jr.

Brains On-Line, South San Francisco, CA, 94080, USA (T.I.F.H.C.)

Department of Biomonitoring and Sensoring, Pharmacy, University of Groningen, Groningen,
The Netherlands (G.F.and C.H.)

Department of Basic Pharmaceutical Sciences, College of Pharmacy, Xavier University of
Louisiana, New Orleans, LA, 70125, USA (R.E.S.)

DMD #45682

Running Title Page

Running Title: Clozapine and N-desmethylozapine Brain Pharmacokinetics

R. E. Stratford, Jr., Xavier University of Louisiana, New Orleans, LA, 70125, USA

Phone: 504-520-5325

Fax: 504-520-7954

Email: rstratfo@xula.edu

Number of text pages: 20

Number of Tables: 3

Number of Figures: 4

Number of References: 31

Abstract (words): 199

Introduction (words): 747

Discussion (words): 1455

Nonstandard abbreviations: CNS, central nervous system; BBB, blood brain barrier; ECF, extracellular fluid; PK, pharmacokinetic(s); PD, pharmacodynamic(s); PET, positron emission tomography; SPECT, single photon emission computed tomography; Pgp, P-glycoprotein; BCRP, breast cancer resistance protein.

DMD #45682

Abstract

A significant barrier to realization of the full potential of clozapine as a therapeutic agent in the treatment of schizophrenia is the substantial inter-patient variability that exists along the therapeutic continuum of no response – efficacious response – adverse response. Genetic polymorphisms that manifest as highly variable pharmacodynamic and pharmacokinetic measures are its expected causes. In order to support investigations that seek to understand these causes, the plasma and CNS pharmacokinetics of clozapine were determined in rats, the latter using microdialysis sampling. Results obtained with clozapine and N-desmethylozapine, a pharmacologically active human metabolite that was administered to a separate group of animals, support a conclusion of net carrier mediated efflux of both compounds across the BBB. These results are supported by the replication of published findings regarding the passive transport and net efflux transport of two model compounds, escitalopram and risperidone, respectively. The results obtained with clozapine and N-desmethylozapine are considered a first step in the development of preclinical PK-PD models that will support deeper mechanistic studies of clozapine in vivo pharmacology, as well as the development of translational models that augment pharmacogenetic investigations that seek to improve the safety and efficacy of clozapine therapeutic intervention in the treatment of schizophrenia.

DMD #45682

Introduction

Schizophrenia is a severe neuropsychiatric disorder characterized by a high degree of morbidity and mortality (Mathers et al., 2006). Over the past 50 years, pharmacotherapy has been an essential component in the management of this disease. Of the several drugs available, clozapine is widely considered as the most efficacious (Horacek et al., 2006); however, its use in therapy is limited due to concerns about its safety (Spina et al., 2000). In particular, seizures, heightened risk of mortality in dementia-related psychosis, prolactin elevation and weight gain are attributed to centrally mediated pharmacology and are consistent with classic dose-exposure-response causality.

The generally accepted aim of antipsychotic drug treatment is to achieve a therapeutic response quickly, in the early stages of symptom manifestation, and then to maintain this response. Unfortunately, in the case of clozapine, use of systemic drug levels to achieve these therapeutic aims while avoiding the adverse effects has met with limited success. This is due in large part to the substantial variability that exists between systemic exposure and clozapine's centrally mediated effects (Spina et al., 2000). Over the past several years, this unpredictable exposure – response relationship has provided compelling justification for research to understand its pharmacokinetic and pharmacodynamic causes (Kane and Correll, 2010). The promise of such research is to develop individualized treatment approaches that are evidence based, with pharmacogenetic evidence being a principal component.

Not surprisingly, given the multiplicity of CNS receptors that clozapine binds to and which are thought to contribute to its effects (Horacek et al., 2006), realization of the goal of individualized therapeutic regimens for clozapine has and will continue to prove challenging. Superimposed on this pharmacodynamic hurdle, genetic differences in clozapine disposition,

DMD #45682

including its metabolism by CYP1A2 and CYP3A4 (Mauri et al., 2007), create pharmacokinetic variability. Given the important role the BBB plays in limiting drug access to the brain through active efflux transport, genetic polymorphisms associated with such transport may also contribute to this pharmacokinetic variability. While there is evidence of Pgp involvement in clozapine absorption across the BBB (Doran et al., 2005), relevancy of this transporter in vivo and the potential for other transporters, such as BCRP, to influence clozapine BBB transport has not been thoroughly evaluated.

In order to support research that probes more deeply into the pharmacodynamic and pharmacokinetic manifestations of genetic polymorphisms that cause the substantial inter-patient variability that exists between clozapine systemic exposure and clinical response, knowledge of clozapine concentrations in the ECF of the brain would be useful. Awareness of these concentrations in the biophase that is in intimate contact with CNS receptors and that is dependent on possible carrier-mediated BBB transport mechanism(s) would support investigations to determine the in vivo relevancy of hypothesized pharmacodynamic and pharmacokinetic causes. Knowledge of ECF concentrations could be compared directly with in vitro derived measures of clozapine binding potency to all relevant receptors, both known and hypothesized. In this regard, we propose the use of quantitative microdialysis to measure ECF concentrations of clozapine in rats. With simultaneous measurement of plasma concentrations, development of a pharmacokinetic model that quantitatively relates systemic exposure to relevant CNS exposure would ensue. Secondly, comparison of unbound clozapine concentrations in plasma to those in brain ECF would provide information regarding the potential involvement of active efflux transport of clozapine across the BBB. Use of microdialysis to fulfill these two objectives has been demonstrated for several CNS drugs

DMD #45682

(deLange et al., 2005). In accord with these objectives, we describe herein an initial pharmacokinetic model that is expected to serve as a substrate to develop pharmacokinetic-pharmacodynamic models of clozapine action in well established rat models of antipsychotic drug action. In addition, application of pharmacokinetic scaling principles to predict human ECF concentrations from plasma exposure could also be explored. This second approach has shown promise for other CNS drugs (Kielbasa and Stratford, 2012).

Since N-desmethylozapine is an important active metabolite of clozapine in humans, we also determined its systemic and ECF pharmacokinetics in rats. Because this metabolite is relatively minor in rats, N-desmethylozapine was administered to a separate cohort of animals to characterize its pharmacokinetics. Finally, we also determined the systemic and CNS pharmacokinetics of escitalopram and risperidone in rats. Work with these two drugs provided context to our clozapine and N-desmethylozapine results with respect to potential involvement of active efflux transport across the BBB, with escitalopram serving as a model drug that exhibits predominantly passive transport (Bundgaard et al., 2007a), while risperidone serving as a model drug in which Pgp mediated efflux occurs (Doran et al., 2005).

DMD #45682

Materials and Methods

Drugs and Chemicals

The four compounds were purchased from Sigma-Aldrich and were used as received. Formulations for administration were prepared on the day of an experiment. Chemicals used in the preparation of microdialysis perfusion buffer and solvents used for HPLC-MS/MS analysis were of reagent grade.

Animal Preparation

For the clozapine and N-desmethyloclozapine experiments, male Wistar rats weighing between 300 – 400 g were purchased from Harlan, Zeist, The Netherlands. For escitalopram and risperidone experiments, male Wistar rats weighing between 280 – 350 g were used and also purchased from Harlan. Rats were individually housed in plastic cages and received food and water *ad libitum*. Experiments were carried out in accordance with the declarations of Helsinki and were approved by the Animal Care Committee of the Department of Mathematics and Natural Science, University of Groningen.

Surgery for implantation of microdialysis guide cannula and venous catheters was conducted under isoflurane anaesthesia (2% with 400 mL/min N₂O and 400 mL/min O₂). A guide cannula was inserted into the medial prefrontal cortex to achieve the following probe tip coordinates: anteroposterior = + 3.3 mm from bregma, mediolateral = – 0.8 mm and dorsoventral = 5.0 mm from dura. Catheters (10 mm silicone tubing) for blood sample collection were inserted into the isolated right jugular vein and exteriorized through an incision at the top of the head. Animals were allowed at least two days to recover from surgery. MetaQuant probes

DMD #45682

(Cellulose membrane, 4 mm (escitalopram and risperidone), 6mm (clozapine and N-desmethylozapine), BrainLink, The Netherlands) were inserted 24 hours before an experiment.

Drug Binding Determination

Unbound plasma ($f_{u,p}$) and unbound brain ($f_{u,b}$) fractions for the four compounds were determined using a 96-well equilibrium dialysis apparatus (HTD Dialysis, Gales Ferry, CT) using a method detailed previously (Kalvass and Maurer, 2002; Kielbasa et al., 2009).

Drug Administration and Sample Collection

On the day of an experiment, rats were connected with flexible PEEK tubing to a CMA 102 microdialysis pump (CMA Microdialysis, Solna, Sweden). Microdialysis probes were perfused with 0.2% (w/v) bovine serum albumin dissolved in a filtered Ringer's buffer containing 147 mM NaCl, 3.0 mM KCl, 1.2 mM CaCl₂ and 1.2 mM MgCl₂ at a flow rate of 0.1 μ L/min (CMA 142 pump). The same buffer, but without albumin, was perfused through the dilution inlet of the probe at a flow rate of 0.8 μ L/min. Under these conditions, compound recovery from probes was > 80%, as determined from in vitro recovery experiments. After initiating flow, probes were allowed to stabilize for 1 hour prior to compound administration. A single dose of each of the 4 compounds was administered by the subcutaneous route. There were 4 – 5 animals per compound. Clozapine and N-desmethylozapine were dosed at 10 mg/kg; escitalopram and risperidone were dosed at 1.6 and 3 mg/kg, respectively. Perfusates from the microdialysis probes were collected every 30 minutes starting one-hour prior to administration and continuing for 270 minutes (risperidone), 360 minutes (escitalopram) or 480 minutes (clozapine and N-desmethylozapine). Dialysate samples were stored at -80°C until time of analysis. Blood samples (250 μ L) were collected at 0, 15, 30, 60, 120, 180, 240, 300 and 360 (escitalopram

DMD #45682

only) minutes following escitalopram and risperidone administration, and at 0, 15, 30, 60, 90, 120, 240, 360 and 480 minutes following clozapine and N-desmethylclozapine administration. Blood samples were collected into microtubes containing 5 μ L heparinized (500 IE/mL) saline and immediately centrifuged at 14,000 rpm for 10 minutes under refrigerated conditions to recover plasma. Plasma samples were stored at -80°C until time of analysis.

Sample Analysis

Concentrations of compounds were measured in dialysate and plasma samples using HPLC with tandem mass spectrometry (MS-MS) detection. The system consisted of an automated sample injector (Shimadzu SIL10, 20 or 30), reverse-phase column, 150 x 2 mm, 5 μ m (Phenomenex, Torrance, CA) and an API 4000 MS/MS with Turbo-Ion Spray interface (Applied Biosystems, The Netherlands) operating in the positive ion mode with multiple reaction monitoring. Standard concentrations in dialysate ranged from 0.05 – 50.0 nM. In plasma, standard concentrations ranged from 1.0 – 20,000 nM for clozapine and N-desmethylclozapine, and from 4.0 – 20,000 nM and from 20.0 – 20,000 nM for escitalopram and risperidone, respectively

Pharmacokinetic Analysis

A pharmacokinetic modeling approach with distribution between plasma and brain ECF was used to describe the pharmacokinetics of the four compounds. The model shown in Figure 1 was fit simultaneously to the unbound plasma and ECF concentration-time data from individual animals using non-linear least squares regression analysis (Phoenix[®] WinNonlin[®] 6.2 Pharsight Corporation, Mountainview, CA). Measured plasma concentrations were converted to unbound concentrations by multiplying by the fraction unbound in plasma, $f_{u,p}$, for each compound (with

DMD #45682

assumption of constant $f_{u,p}$ over the measured concentration range). Unbound brain volume, $V_{u,b}$, was derived from the reciprocal of unbound brain fraction, $f_{u,b}$, obtained from determinations made in brain homogenates (Fridén et al., 2007) and was fixed during the modeling procedure (Tunblad et al., 2003). The time course of ECF-to-plasma_u concentration ratio ($K_{p,u}$) was used to estimate the unbound brain (ECF) equilibration rate constant, k_{eq} , and the steady state ECF-plasma_u ratio, $K_{p,u,ss}$, according to the equation:

$$K_{p,u} = K_{p,u,ss} (1 - e^{-k_{eq} \cdot t})$$

The ECF equilibration half-life, $t_{1/2}$, was calculated from k_{eq} according to the equation:

$$t_{1/2} = \frac{(\ln 2)}{k_{eq}}$$

This analysis approach has been used previously to model the time course of whole brain to plasma ratios of several opiate drugs (Kalvass et al., 2007).

DMD #45682

Results

Subcutaneous administration of a 1.6 mg/kg dose of escitalopram resulted in an average $AUC_{0-\infty}$ of 27776 ± 3361 nM (mean \pm s.d.). Following this same route of administration, a dose of 3 mg/kg of risperidone, or 10 mg/kg of either clozapine or N-desmethylclozapine resulted in $AUC_{0-\infty}$ values of 150138 ± 27025 , 323475 ± 158503 or 293600 ± 99824 nM, respectively. Correcting for differences in plasma protein binding, unbound systemic exposures were similar for the four compounds, being within a 2.5 fold range (Table 1). Also shown in Table 1 are corresponding compound exposures in brain ECF. Unbound plasma concentration time courses are summarized in Figure 2; also shown are measured brain ECF concentrations. In the case of clozapine administration, N-desmethylclozapine plasma concentrations are also shown. Consistent with previous findings in rats (Olsen et al., 2008), systemic exposure averaged only approximately 10% of clozapine $AUC_{0-\infty}$ (31165 vs. 323476 nM min). There was no detectable N-desmethylclozapine in the ECF following this dose of clozapine (lower quantitation limit = 0.05 nM).

Plasma and ECF pharmacokinetic parameter estimates derived from simultaneous fitting of unbound plasma and ECF concentrations in individual animals to the model specified in Figure 1 are summarized in Table 2. A 1-compartment model with first-order absorption was used to describe the plasma concentration time course data for clozapine, N-desmethylclozapine and risperidone. Consistent with previous studies (Bundgaard et al., 2007), escitalopram systemic exposure, subsequent to first-order absorption, was best described using a 2-compartment approach. Precision (% CV) of the various parameter estimates in each animal was typically < 25% across the four compounds. Figure 3 summarizes model predicted concentrations vs. observed concentrations. For the four compounds, these data were close to and randomized

DMD #45682

across the line of unity, thus indicating an acceptable fit to the data. Relative to escitalopram, uptake clearance (Cl_{in}) into the brain of the other compounds ranged from 8% (N-desmethylclozapine) to 35% (clozapine). Conversely, efflux clearance (Cl_{out}) from the brain was greater for these three compounds vs. escitalopram, ranging from 1.7- (risperidone) to 2.7-fold (clozapine).

Table 3 summarizes various measures of the extent of ECF exposure relative to unbound plasma exposure for the four compounds. For escitalopram, the ratio of Cl_{in}/Cl_{out} of 1.24 ± 0.309 (mean \pm s.d.) agrees with the previously reported finding of 0.80 (Bundgaard et al., 2007) and supports an interpretation of no net carrier-mediated uptake or efflux transport across the rat BBB. For each of the four compounds, this model derived measure of BBB transport was in good agreement with the non-compartmental derived (AUC ratio) and the steady-state ECF-to-plasma_{unbound} ratio ($K_{p,u,ss}$) derived from fitting this ratio vs. time. The time course of $K_{p,u}$ for the four compounds is summarized in Figure 4. Compared to escitalopram, these various measures for risperidone, which, based on Pgp mouse KO studies (Doran et al., 2005), is considered an ABCB1 (Pgp) substrate, were below unity and statistically lower ($p < 0.01$). These results are consistent with in vivo functional presence of carrier-mediated risperidone efflux across the blood-brain barrier. Similar to risperidone, the three measures of blood-brain barrier transport obtained for clozapine and N-desmethylclozapine were below unity and statistically lower than escitalopram ($p < 0.01$). Therefore, at the plasma exposures obtained in this study, operation of carrier-mediated efflux across the blood-brain barrier is implicated for clozapine and its principal human pharmacologically active metabolite.

DMD #45682

Discussion

Our goals in this work were to measure biophase exposure of clozapine and N-desmethylozapine in the rat using brain microdialysis and to develop a PK model that describes the relationship between this pharmacologically relevant CNS exposure and plasma exposure. Surprisingly, while there is one report (Liu et al., 2009) of measured N-desmethylozapine in rat brain ECF derived from microdialysis measures, there are no reported studies regarding clozapine ECF exposure using this technique. We believe the stated goals are important because they represent the initial step in the development of a rat-to-human translatable PK-PD model that quantifies the relationship between brain biophase concentrations to those in plasma. Given that clozapine binds to a multiplicity of receptors (Horacek et al., 2006), such a model has the potential to be more robust in advancing our understanding of preclinical and clinical antipsychotic drug action compared to a receptor occupancy-based PK-PD model that is based on one or two receptors.

Quantitative microdialysis has been used to evaluate escitalopram CNS pharmacokinetics in rats following intravenous administration (Bundgaard et al., 2007a, 2007b). Based on model-independent (AUC) and -dependent (Cl_{in} , Cl_{out}) analyses in these studies, there was no evidence of carrier-mediated uptake or efflux of escitalopram across the BBB, and it was concluded that transport of this drug occurs predominantly by a passive mechanism. In these studies, quantitative microdialysis was achieved using dynamic-no-net flux and/or retrodialysis. Herein, we describe results obtained with MetaQuant microdialysis probes. These probes rely on the principle of ultraslow flow microdialysis (< 200 nL/min) to achieve quantitative recovery of analyte (Cremers et al., 2009) while maintaining temporal resolution typical of standard microdialysis probes. Based on the ECF exposures attained with this approach, and achieving

DMD #45682

plasma exposures similar to those reported by Bundgaard et al. (2007a), we arrive at the same conclusion; namely, that there is no evidence that escitalopram transport across the BBB occurs by active carrier-mediated transport processes. These similar findings provide additional verification with respect to the use of MetaQuant technology for quantitative microdialysis, which is a more facile approach, requiring fewer experiments and animals, than either the dynamic-no-net flux or retrodialysis approaches.

As with escitalopram, risperidone was selected as a model drug to support and add context to interpretations made regarding the clozapine and N-desmethylclozapine results. Specifically, risperidone was selected as a drug in which net efflux across the BBB was expected. There are several reports based on *mdr1a* KO models demonstrating that Pgp reduces risperidone uptake into the brains of both mice (Doran et al., 2005; Summerfield et al., 2006; Wang et al., 2004) and rats (Bundgaard et al., 2011). Using plasma and brain homogenate free fraction analysis, efflux asymmetry was observed in mice (Maurer et al., 2005) and rats (Watson et al., 2009). In a study involving direct measurement by microdialysis of risperidone in brain ECF of rats and comparison to unbound plasma concentrations following intravenous infusion over six hours, Liu et al. (2009) reported a risperidone steady state ECF/plasma_u ratio of 0.53. Since this value was within 3-fold of a ratio of 1, the authors concluded that there was no clear evidence in vivo of risperidone efflux across the BBB. Based on the three measures of extent of brain ECF exposure relative to unbound plasma concentration (AUC, model-derived bidirectional clearance ratio and $K_{p,u,ss}$) summarized in Table 3, our results support a conclusion of net efflux of risperidone across the BBB and are consistent with a Pgp-mediated mechanism. Our study and the Liu et al. study, while both in rats, used different routes of administration (subcutaneous vs. intravenous infusion), so it is not possible to directly compare plasma exposures and comment regarding the

DMD #45682

potential for saturation of efflux. Both studies were at a single dose level. In view of the several studies that have been performed on this drug in relation to its BBB transport and Pgp role in such transport, a focused study with a full plasma and ECF time course at different doses would seem worthwhile and represent a comprehensive *in vivo* analysis of the potential saturability of Pgp-mediated efflux at this site.

The ability to discriminate between a drug with predominantly passive BBB transport (escitalopram) and one with net efflux mediated transport (risperidone) augments our ability to interpret results obtained with clozapine and N-desmethylclozapine. As with risperidone, the various measures of brain distribution we observed support a conclusion for clozapine and its metabolite that net efflux occurs across the rat BBB (Table 3). The results for clozapine are somewhat surprising. In *mdr1a* mouse KO studies (Doran et al., 2005), clozapine brain concentrations were increased in KO relative to WT animals, but the drug was unlike risperidone in strongly differentiating as a Pgp substrate. As well, no support for clozapine net efflux asymmetry was obtained using plasma and brain free fraction analysis in mice (Maurer et al., 2005) or rats (Watson et al., 2009). Interestingly, clinical studies have shown a relationship between MDR1a polymorphisms and efficacious clozapine systemic exposure (Consoli G et al., 2009; Jaquenoud Sirot et al., 2009). Thus, in view of the above discrepancies, additional microdialysis studies over a range of doses and systemic exposures are warranted, particularly at higher exposures to determine if the net efflux we observed is saturable, as would be expected based on the aforementioned preclinical findings of others. While clozapine has been shown to be a weak Pgp substrate in *caco-2* cells (El Ela et al., 2004), there have been no reports that its transport is influenced by BCRP. Thus, evaluation of clozapine transport dependency with concentration using transfected *in vitro* models that isolate Pgp (ABCB1) and BCRP (ABCG2)

DMD #45682

function would also seem useful. In order to support extrapolations from rats to humans, these detailed transport analyses would need to be conducted with the rat and human transporters. The potential for expression differences between rodents and humans also needs to be considered in light of the findings of Uchida et al. (2011), which demonstrated higher BCRP expression in human BBB relative to mouse and just the opposite for Pgp.

The net efflux asymmetry we observed with N-desmethylozapine is consistent with the findings of Liu et al. (2009), which were also based in rats using microdialysis. As recommended above for clozapine, additional studies, particularly in vitro transport analyses using rat and human Pgp and BCRP transfected cells, and considering possible expression differences, would be useful to characterize the mechanism of the net efflux observed and its relevancy in humans. Clozapine is a weak inhibitor of both Pgp (Wang et al., 2006) and BCRP (Wang et al., 2008); however, the potential for N-desmethylozapine to inhibit these two transporters should be evaluated. Furthermore, genetic polymorphisms in clozapine metabolism, and the transporters responsible for its carrier-mediated efflux and that of N-desmethylozapine, could be significant contributors to the substantial inter-patient variability that exists between the combined clozapine/N-desmethylozapine exposure and clinical response (Llorca et al., 2002; Mauri et al., 2007; Couchman et al., 2010).

Our estimate of escitalopram Cl_{in} of 0.19 ± 0.074 mL/min/g brain (mean \pm s.d.) is within 3-fold of 0.54 mL/min/g brain reported previously (Bundgaard et al., 2007a) indicating reasonable agreement between the two estimates. The half-life for unbound escitalopram to reach equilibrium across the BBB ($t_{1/2,eq}$) was about one hour (Table 3). In contrast, this time was about 3 hours for the antipsychotics. Based on physicochemical properties (cLogD, pH 7.4 ranging from 0.74 for escitalopram to 3.28 for clozapine), high permeability by passive diffusion

DMD #45682

would be expected for the compounds. Thus, the observed difference in equilibration rate is consistent with the role that Cl_{in} and Cl_{out} play in determining this rate (Liu et al., 2005). The three-fold difference is believed to be due to the balance of a larger reduction in Cl_{in} of the antipsychotics (up to 10-fold vs. escitalopram) that is partially offset by their 2 to 3-fold higher Cl_{out} relative to escitalopram.

In conclusion, compartmental modeling has been used to describe the relationship between brain ECF and unbound plasma concentrations of clozapine and N-desmethylozapine in rats. This approach characterizes the extent of distribution of pharmacologically relevant unbound concentrations across the BBB, as well as the rate of this transport and equilibration process. Results demonstrate the existence of net efflux of both molecules across the BBB; they are qualitatively similar to risperidone in this regard. Additional in vitro and in vivo experiments will be important to substantiate these findings, which, for clozapine, are novel. Given the complexities inherent to the use of in vitro models to predict in vivo relevancy, development of a model that predicts human biophase concentrations of clozapine and N-desmethylozapine will be a challenge. However, the investment is considered worthwhile, as it will support a deeper understanding of clozapine's mechanism of action and underlying causes of the substantial inter-patient variability in clinical response.

DMD #45682

Acknowledgements

We would like to thank Dr. Maciej Zamek-Gliszczynski of Eli Lilly and Company for his helpful advice regarding the protein binding determinations.

Authorship Contributions

Participated in research design: Cremers, Flik, and Stratford

Conducted experiments: Flik and Hofland

Performed data analysis: Stratford

Wrote or contributed to the writing of the manuscript: Stratford

DMD #45682

References

Bundgaard C, Jørgensen M, and Larsen F (2007a) Pharmacokinetic modeling of blood-brain barrier transport of escitalopram in rats. *Biopharm Drug Disp* **28**: 349-360.

Bundgaard C, Jørgensen M, and Mørk A (2007b) An integrated microdialysis rat model for multiple pharmacokinetic/pharmacodynamic investigations of serotonergic agents. *J Pharmacol Toxicol Methods* **55**: 214-223.

Bundgaard C, Nyberg CJ, and Garmer M (2011) Species comparison of in vivo P-glycoprotein mediated efflux using *mdr1a* deficient rats and mice. *Drug Metab Disp Fast Forward* **doi:10.1124/dmd.111.043083**: November 2011.

Consoli G, Lastella M, Ciapparelli A, Catena Dell'Osso M, Ciofi L, Guidotti E, Danesi R, Dell'Osso L, Del Tacca M, DiPaolo A (2009) ABCB1 polymorphisms are associated with clozapine plasma levels in psychotic patients. *Pharmacogenomics* **10**: 1267-1276.

Couchman L, Morgan PE, Spencer EP, and Flanagan RJ (2010) Plasma clozapine, norclozapine and the clozapine:norclozapine ratio in relation to prescribed dose and other factors: Data from a therapeutic drug monitoring service, 1993-2007. *Ther Drug Monit* **32**: 438-447.

Cremers TIFH, de Vries, MG, Huinink KD, Van Loon, JP, Hart MVD, Ebert B, Westerink BHC, and de Lange ECM (2009) Quantitative microdialysis using modified ultraslow microdialysis: Direct rapid and reliable determination of free brain concentrations with the MetaQuant technique. *J Neurosci Methods* **178**: 249-254.

De Lange ECM, Ravenstijn PGM, Groenendaal D, and van Steeg TS (2005) Towards the prediction of CNS drug effect profiles in physiological and pathological conditions using

DMD #45682

microdialysis and mechanism-based pharmacokinetic-pharmacodynamic modeling. *AAPS J* **7** : E532 - E543.

Doran A, Obach RS, Smith BJ, Hosea NA, Becker S, Callegari E, Chen C, Chen X, Choo E, Cianfrogna J, et al. (2005) The impact of P-glycoprotein on the disposition of drugs targeted for indications of the central nervous system: evaluation using the MDR1A/1B knockout mouse model. *Drug Metab Dispos* **33**: 165-174.

El Ela AA, Härter S, Schmitt U, Hiemke C, Spahn-Langguth H, and Langguth P (2004) Identification of P-glycoprotein substrates and inhibitors among psychoactive compounds – implications for pharmacokinetics of selected substrates. *J Pharm Pharmacol* **56**: 967-975.

Fridén M, Gupta A, Antonsson M, Bredberg U, and Hammarlund-Udenaes M (2007) In vitro methods for estimating unbound drug concentrations in brain interstitial and intracellular fluids. *Drug Metab Dispos* **35**: 1711-1719.

Horacek J, Bubenikova-Valesova V, Kopecek M, Palenicek T, Dockery C, Mohr P, and Höschl C (2006) Mechanism of action of atypical antipsychotic drugs and the neurobiology of schizophrenia. *CNS Drugs* **20**: 389-409.

Jaquenoud-Sirot E, Knezevik B, Morena GP, Harenberg S, Oneda B, Crettol S, Ansermot N, Baumann P, and Eap CB (2009) ABCB1 and cytochrome P450 polymorphisms: clinical pharmacogenetics of clozapine. *J Clin Psychopharmacol* **29**: 319-326.

Kalvass JC and Maurer TS (2002) Influence of nonspecific brain and plasma binding on CNS exposure: implications for rational drug discovery. *Biopharm Drug Dispos* **23**: 327-338.

DMD #45682

Kalvass JC, Olson ER, Cassidy MP, Selley DE, and Pollack GM (2007) Pharmacokinetics and pharmacodynamics of seven opioids in P-glycoprotein competent mice: Assessment of unbound brain $EC_{50,u}$ and correlation of in vitro, preclinical and clinical data. *J Pharmacol Exp Ther* **323**: 346-355.

Kane JM and Correll CU (2010) Past and present progress in the pharmacologic treatment of schizophrenia. *J Clin Psychiatry* **71**: 1115-1124.

Kielbasa W, Kalvass JC, and Stratford R (2009) Microdialysis evaluation of atomoxetine brain penetration and central nervous system pharmacokinetics in rats. *Drug Metab Dispos* **37**: 137-142.

Kielbasa W and Stratford RE (2012) Exploratory translational modeling approach in drug development to predict human brain pharmacokinetics and pharmacologically relevant clinical doses. *Drug Metab Dispos* **40**: 1-12.

Liu X, Smith BJ, Chen C, Callegari E, Becker SL, Chen X, Cianfrogna J, Doran AC, Doran SD, Gibbs JP, Hosea N, Liu J, Nelson FR, Szewc MA, and Van Deusen J (2005) Use of a physiologically based pharmacokinetic model to study the time to reach brain equilibrium: An experimental analysis of the role of blood-brain barrier permeability, plasma protein binding, and brain tissue binding. *J Pharmacol Exp Ther* **313**: 1254-1262.

Liu X, Van Natta K, Yeo H, Vilenski O, Weller, PE, Worboys PD, and Monshouwer M (2009) Unbound drug concentration in brain homogenate and cerebral spinal fluid at steady state as a surrogate for unbound concentration in brain interstitial fluid. *Drug Metab Dispos* **37**: 787-793.

DMD #45682

Llorca PM, Lancon C, Disdier B, Fariße J, Sapin C, and Auquier P (2002) Effectiveness of clozapine in neuroleptic-resistant schizophrenia: Clinical response and plasma concentrations. *J Psychiatry Neurosci* **27**: 30-37.

Mathers CD, Lopez AD and Murray CJL (2006) The burden of disease and mortality by condition: Data, methods and results, Chapter 3. In: Lopez AD, Mathers CD, Ezzati M, Jamison DT and Murray CJL, eds. *Global Burden of Disease and Risk Factors*. New York, NY: The World Bank and Oxford University Press.

Maurer TS, DeBartolo DB, Tess DA, and Scott DO (2005) Relationship between exposure and nonspecific binding of thirty-three central nervous system drugs in mice. *Drug Metab Dispos* **33**: 175-181.

Mauri MC, Volonteri LS, Colasanti A, Fiorentini A, De Gaspari IF, and Bareggi SR (2007) Clinical pharmacokinetics of atypical antipsychotics: A critical review of the relationship between plasma concentrations and clinical response. *Clin Pharmacokinet* **46**: 359-388.

Olsen CK, Brennum LT, and Kreilgaard M (2008) Using pharmacokinetic-pharmacodynamic modelling as a tool for prediction of therapeutic effective plasma levels of antipsychotics. *Eur J Pharmacol* **584**: 318-327.

Spina E, Avenoso A, Facciola G, Scordo MG, Ancione M, Madia AG, Ventimiglia A, and Perucca E (2000) Relationship between plasma concentrations of clozapine and norclozapine and therapeutic response in patients with schizophrenia resistant to conventional neuroleptics. *Psychopharmacol* **148**: 83-89.

DMD #45682

Summerfield SG, Stevens AJ, Cutler L, Osuna MC, Hammond B, Tang SP, Hersey A, Spaldig DJ, and Jeffrey P (2006) Improving the in vitro prediction of in vivo central nervous system penetration: Integrating permeability, P-glycoprotein efflux, and free fractions in blood and brain. *J Pharmacol Exp Ther* **316**: 1282-1290.

Tunblad K, Jonsson EN, Hammarlund-Udenaes M (2003) An integrated model for the analysis of pharmacokinetic data from microdialysis experiments. *Pharm Res* **21**: 1698-1707.

Uchida Y, Ohtsuki S, Katsukura Y, Ikeda C, Suzuki T, Kamiie J, and Terasaki T (2011) Quantitative targeted absolute proteomics of human blood-brain transporters and receptors. *J Neurochem* **117**: 333-345.

Wang J-S, Ruan Y, Taylor RM, Donovan JL, Markowitz JS, and DeVane CL (2004) The brain entry of risperidone and 9-hydroxyrisperidone is greatly limited by P-glycoprotein. *Intl J Neuropsychopharm* **7**: 415-419.

Wang J-S, Zhu HJ, Markowitz JS, Donovan JL, and DeVane CL (2006) Evaluation of antipsychotic drugs as inhibitors of multidrug resistance transporter P-glycoprotein. *Psychopharmacology (Berl)*. **187**: 415-423.

Wang J-S, Zhu HJ, Markowitz JS, Donovan JL, Yuan HJ, and DeVane CL (2008) Antipsychotic drugs inhibit the function of breast cancer resistance protein. *Basic Clin Pharmacol Toxicol* **103**: 336-341.

Watson J, Wright S, Lucas A, Clarke KL, Viggers J, Cheetham S, Jeffrey P, Porter R, and Read KD (2009) Receptor occupancy and brain free fraction. *Drug Metab Dispos* **37**: 753-760.

DMD #45682

Footnotes

This work was sponsored by Brains On-Line, South San Francisco, CA, USA.

This work was not previously presented.

R. E. Stratford, Jr., Xavier University of Louisiana, New Orleans, LA, USA; reprint;

rstratfo@xula.edu

There are no numbered footnotes in this manuscript.

Generic Drug and Chemical Names

Escitalopram; Chemical name: (1S)-1-[3-(dimethylamino)propyl]-1-(4-fluorophenyl)-1,3-dihydro-2-benzofuran-5-carbonitrile

Clozapine; Chemical name: 6-chloro-10-(4-methylpiperazin-1-yl)-2,9-diazatricyclo[9.4.0.0^{3,8}]pentadeca-1(11),3(8),4,6,9,12,14-heptaene

N-desmethylozapine; Chemical name: 8-Chloro-11-(1-piperaziny)-5H-dibenzo(b,e)(1,4)diazepine

Risperidone; Chemical name: 3-{2-[4-(6-fluoro-1,2-benzoxazol-3-yl)piperidin-1-yl]ethyl}-2-methyl-4H,6H,7H,8H,9H-pyrido[1,2-a]pyrimidin-4-one

DMD #45682

Figure Legends

Figure 1. Pharmacokinetic model for escitalopram, clozapine, N-desmethylozapine and risperidone disposition in plasma and brain ECF. Pharmacokinetic parameters were obtained by simultaneous fitting of unbound plasma and brain (medial prefrontal cortex) ECF concentrations in rats following subcutaneous administration. The absorption rate constant (k_a), systemic clearance (Cl), central volume (V_c), peripheral volume (V_p , escitalopram only), distributional clearance (Q_d , escitalopram only), brain ECF uptake clearance (Cl_{in}), brain ECF efflux clearance (Cl_{out}) were estimated in individual rats for each compound. The unbound brain volume ($V_{b,u}$) was fixed during the PK analysis.

Figure 2. Time course of unbound plasma (triangles) and brain ECF (squares) concentrations in individual animals for escitalopram, clozapine, N-desmethylozapine and risperidone following single subcutaneous administration of 1.5, 10, 10 and 3 mg/kg, respectively. Corresponding average (mean \pm s.d., $n = 4$ rats per compound) concentrations are shown as solid lines (plasma) or dashed lines (ECF). Average N-desmethylozapine unbound plasma concentrations following clozapine administration are also shown (dotted line).

Figure 3. Individual PK model predicted unbound plasma (closed triangles) and brain ECF (open squares) concentrations vs. observed concentrations for escitalopram, clozapine, N-desmethylozapine and risperidone.

Figure 4. Brain ECF to unbound plasma concentration ratio ($K_{p,u}$) vs. time data in individual rats (diamonds). Corresponding average (mean \pm s.d., $n = 4$ rats per compound) concentrations are shown as solid lines. Data are derived from the concentration data presented in Figure 1.

Tables

TABLE 1

Unbound fractions in plasma and non-compartmental pharmacokinetic parameter estimates of unbound plasma concentrations and brain ECF concentrations. Data are presented as mean (% CV), n = 3 replicates for unbound fraction determinations and n = 4 for rats.

Drug	$f_{u,p}$	$AUC_{0-\infty,p,u}$	$C_{max,p,u}$	$T_{max,p,u}$	$AUC_{0-\infty,ecf}$	$C_{max,ecf}$	$T_{max,ecf}$
	%	nM min	nM	minutes	nM min	nM	minutes
Escitalopram	50*	13888 (12)	131 (27)	26 (29)	11815 (10)	62 (23)	120 (35)
Clozapine	6.1 (4)	19732 (49)	77 (41)	60 (41)	2659 (49)	6 (33)	240 (18)
N-desmethylozapine	11 (8)	32296 (34)	90 (77)	63 (157)	805 (55)	2 (64)	278 (47)
Risperidone	16 (7)	24022 (18)	181 (32)	53 (29)	3543 (17)	20 (23)	75 (23)

*Escitalopram $f_{u,p}$ estimate is from Bundgaard et al., 2007a.

TABLE 2

Compartmental pharmacokinetic parameter estimates based on simultaneous fitting of concentrations in brain ECF and unbound concentrations in plasma. The mean (%CV) calculated from the estimates obtained from fitting of individual animal data are presented, n = 4 rats for each compound.

Drug	V _c	Cl	k _a	V _p	Q _d	V _{b,u} *	Cl _{in}	Cl _o
	L/kg	mL/min/kg	min ⁻¹	L/kg	mL/min/kg	mL/g brain	mL/min/g	mL/min/g
Escitalopram	4.1 (34)	277 (32)	0.02 (43)	17 (39)	117 (58)	18	0.19 (39)	0.16 (52)
Clozapine	275 (12)	2006 (49)	0.04 (97)			70	0.07 (50)	0.44 (38)
N-desmethylclozapine	607 (83)	1597 (83)	0.50 (68)			82	0.02 (59)	0.38 (68)
Risperidone	21 (50)	307 (21)	0.03 (53)			10	0.05 (25)	0.32 (12)

*V_{b,u} estimates = 1/f_{u,b} and were fixed during the modeling. For escitalopram, f_{u,b} was 3.1% and taken from Bundgaard et al. (2007a). For clozapine, N-desmethylclozapine and risperidone, f_{u,b} estimates were 0.8% (2), 0.7% (10) and 6.5% (8), respectively [mean (%CV)].

TABLE 3

Extent of brain ECF exposure (AUC and Cl ratios, $K_{p,u,ss}$) and rate of unbound compound equilibration between plasma and brain ECF (k_{eq} and $t_{1/2}$). Results are presented as mean (% CV), $n = 4$ rats for each compound except escitalopram, where $n = 3$ rats.

Drug	AUC _{ecf} /AUC _{p,u}	Cl _{in} /Cl _{out}	$K_{p,u,ss}$	k_{eq} (min ⁻¹)	$t_{1/2,eq}$ (min)
Escitalopram	0.86 (13)	1.24 (25)	1.06 (42)	0.015 (42)	54 (56)
Clozapine*	0.15 (37)	0.16 (36)	0.13 (33)	0.004 (26)	194 (23)
N-desmethylclozapine*	0.03 (74)	0.05 (60)	0.04 (85)	0.006 (87)	165 (48)
Risperidone*	0.15 (9)	0.14 (16)	0.21 (39)	0.006 (72)	165 (63)

* AUC and Cl ratios, and $K_{p,u,ss}$ significantly different ($p < 0.01$) vs. corresponding escitalopram values according to 1-way ANOVA followed by the Tukey t-test for multiple comparisons.

Figure 1

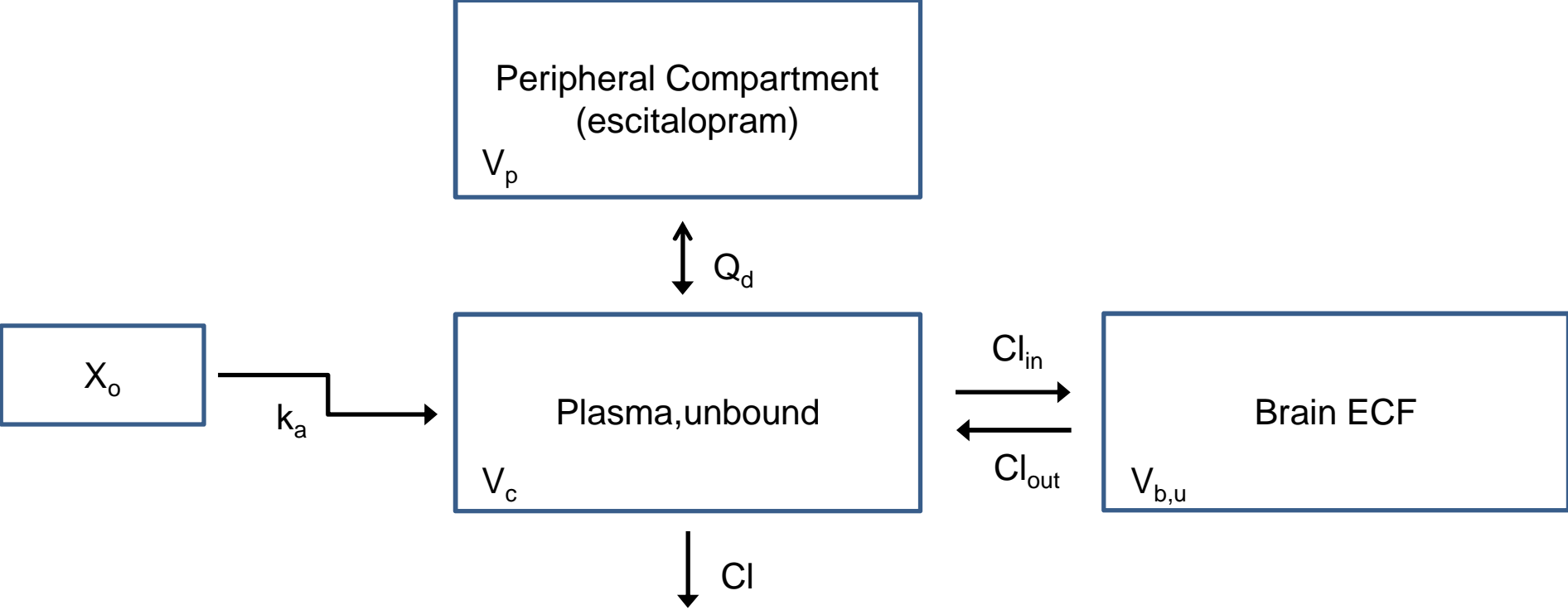
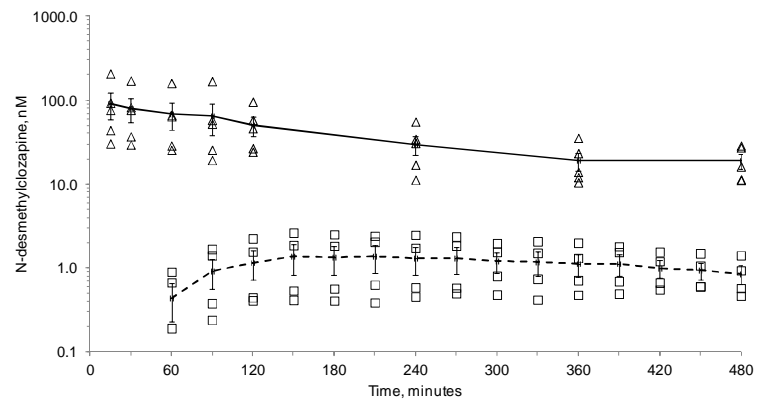
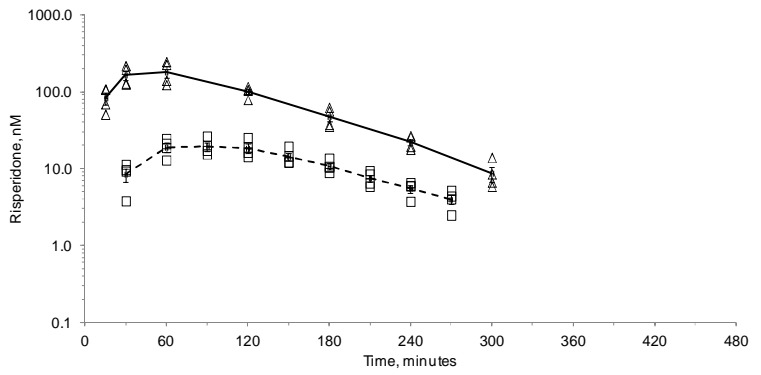
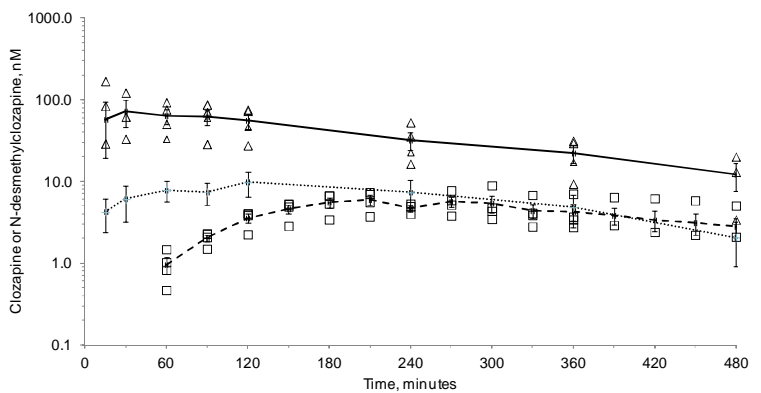
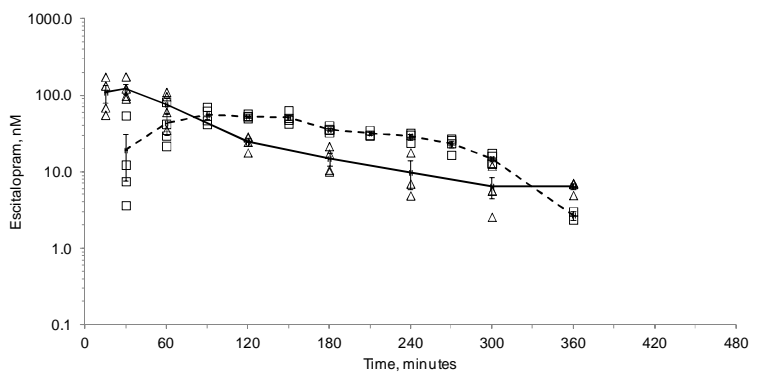


Figure 2



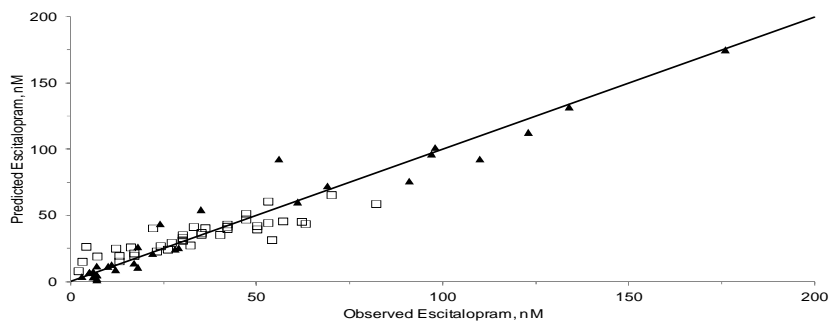


Figure 3

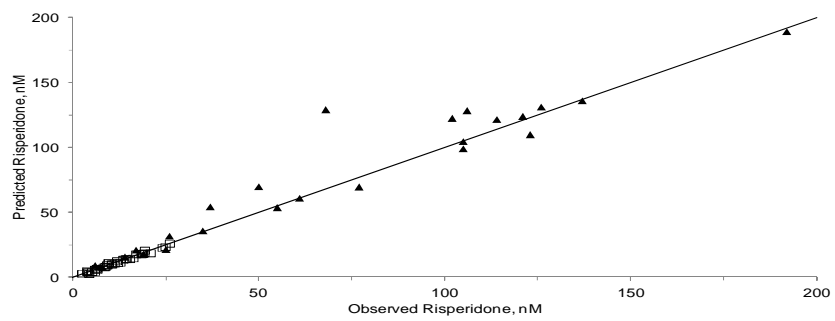
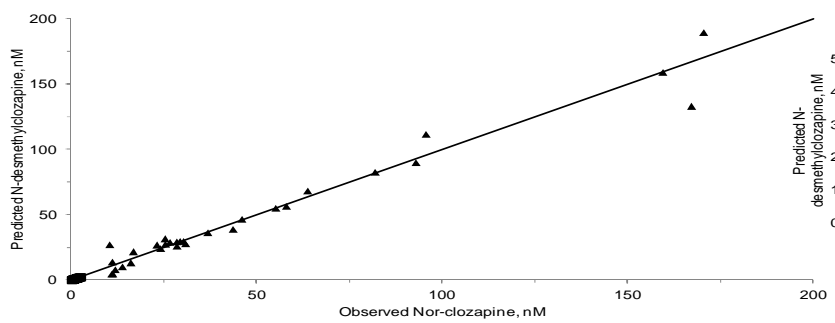
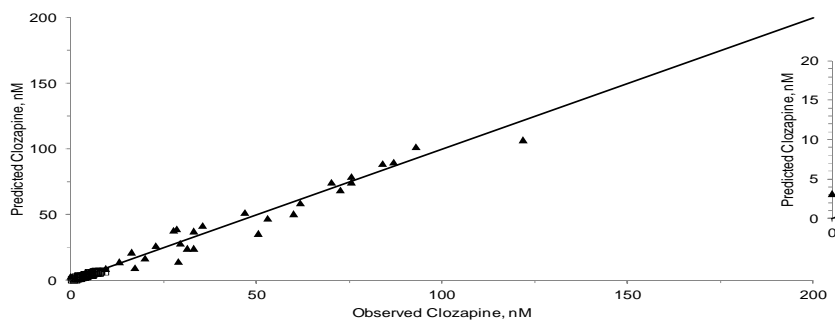


Figure 4

