Title: A New Radioligand Binding Assay to Measure the Concentration of Drugs in Rodent Brain Ex Vivo

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Running Title: A New Competitive-Saturation Binding Assay

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Abstract

We have developed a new radioligand binding assay method to measure the concentration of non-radiolabeled drugs in the brain \textit{ex vivo}. This new method fuses the concepts of standard competition and saturation binding assays, and utilizes a transformed version of the Cheng-Prusoff equation to calculate the drug concentration. After testing the validity of this method, we demonstrated its utility by measuring the brain concentration of sazetidine-A, a newly developed nicotinic receptor ligand, and its elimination rate after a single subcutaneous administration. Our results indicate that sazetidine-A reaches brain concentrations that are known to occupy and desensitize the majority of nAChR binding sites. Furthermore, using this method, we estimated the half-life of sazetidine-A in the rat brain to be \(\sim\)65 min. It is important to note that the method described here to measure sazetidine-A in brain should be generalizable to other drugs acting at any receptor that can be reliably measured with a radiolabeled ligand.
Sazetidine-A (saz-A) is a recently developed partial agonist that potently desensitizes β2-containing nAChRs (Xiao et al., 2006; Zwart et al., 2008) and produces interesting behavioral effects in rodents. For example it decreases nicotine and alcohol self-administration in rats (Levin et al., 2010; Rezvani et al., 2010; Johnson et al., 2012), improves performance on tasks of attention even in rats treated with the NMDA receptor blocker dizocilpine (MK801) or the muscarinic receptor blocker scopolamine (Rezvani et al., 2011), and decreases anxiety- and depression-like behavior in mice (Kozikowski et al., 2009; Turner et al., 2010; Caldarone et al., 2011).

In contrast to the substantial behavioral pharmacology profile of saz-A that is emerging, little is known about its concentrations in blood and brain or its pharmacokinetic properties in the rat. A recent study of the antidepressant-like effects of saz-A used reverse phase high-performance liquid chromatography (HPLC) to measure the pharmacokinetic properties of saz-A and autoradiography to measure the time course of its receptor occupancy in the mouse (Caldarone et al., 2011). The results indicated that although saz-A was rapidly eliminated from both the brain and plasma following a single intraperitoneal (ip) injection, it occupied the majority of nAChR binding sites for 8 hr or longer. This long-term receptor occupancy is likely due to the high affinity of saz-A at α4β2 nAChR binding sites and its very slow dissociation rate.

Here, to complement the behavioral studies with saz-A that have been carried out in rat models (Levin et al., 2010; Rezvani et al., 2010; Rezvani et al., 2011; Johnson et al., 2012), we describe its pharmacokinetic properties in rats. To measure the concentration and elimination rate of saz-A in blood serum, we used a radioligand binding assay that was originally developed to measure the serum concentrations of dopaminergic drugs (Creese and Snyder, 1977).
measure the concentration of saz-A in brain, we developed a new radioligand receptor binding assay method that combines concepts from competitive and saturation receptor binding. Thus, we called this new assay “competitive-saturation binding”. Furthermore, to actually calculate the concentration of saz-A in this new assay, we derived a transformed version of the Cheng-Prussoff equation (Cheng and Prusoff, 1977). Through our studies, we provide a detailed description of how competitive saturation binding was developed, and demonstrate its accuracy and precision at measuring drug concentrations in brain. Although here we used competitive saturation binding to measure saz-A in rat brain, this method can be easily adapted to measure the concentration in the brain or other tissues of many other drugs of interest that bind to receptors that are measurable by radioligand binding.
Methods

Materials: [3H]Epibatidine ([3H]EB, ~55 Ci/mmol) was purchased from Perkin Elmer Life Science (Boston, MA). 6-(5-(((S)-azetidin-2-yl)methoxy)pyridin-3-yl)hex-5-yn-l-ol (Sazetidine-A) dihydrochloride (Xiao et al., 2006) was synthesized by RTI, International (Research Triangle, NC) and supplied by NIDA. Nicotine hydrogen tartrate was purchased from Sigma-Aldrich (St. Louis, MO).

Animals: Male adult Sprague Dawley rats were purchased from Harlan Laboratories (Frederick, MD). Rats were housed in groups (2 per cage) in AAALAC-approved facilities at Georgetown University (Washington, DC). All rodents were maintained on a 12 hr light/12 hr dark cycle with free access to food and water. Treatment, care, and housing were carried out in accordance with the National Institutes of Health guidelines on animal care. Experimental procedures were approved Georgetown University’s animal care and use committee.

Saz-A administration: Saz-A was dissolved in sterile saline, and the pH was adjusted to 6.5 – 8.0 with NaOH. Drug was delivered by subcutaneous (sc) injections above the shoulders. Doses of sazetidine-A dihydrochloride are reported as the salt.

Preparation of unwashed cerebral cortex homogenates: Ten min after administration of the drug, the rats were anesthetized with isoflurane, decapitated, and the brains were dissected on ice. Dissected cortex was suspended in ~20 volumes of 50 mM Tris HCL buffer (pH 7.4) and then homogenized with a Glass/Teflon homogenizer (6-7 strokes) followed by a 10 sec
sonication. This homogenate was not washed or centrifuged, and thus was called an “unwashed homogenate”.

**Saturation binding assay:** Unwashed homogenates were aliquotted into tubes with increasing concentrations of [3H]EB (5 – 4,000 pM) in a final volume of 500 μL Tris buffer (pH 7.4). Nonspecific binding (NSB) was measured in the presence of 300 μM nicotine. The binding tubes were incubated with gentle shaking at 24 °C for 18 hr to allow equilibrium to come to completion. Bound [3H]EB was collected by vacuum filtration over GF/C filters that were presoaked in 0.5% polyethyleneimine. Radioactivity in the filter was measured by scintillation counting. Specific [3H]EB binding was calculated as the difference between total binding and NSB. Specific [3H]EB binding was normalized to total protein and was plotted against the free (unbound) concentration of [3H]EB in the binding assay. The data was fit with a nonlinear regression saturation curve using Graph Pad Prism 5 (La Jolla, CA), and the Kd of [3H]EB was calculated from the fitted curve.

**Competition Binding:** Unwashed homogenates were aliquotted in a binding assay with 1.0 nM [3H]EB and increasing concentrations of saz-A. Nonspecific binding (NSB) was again measured in the presence of 300 μM nicotine. The assay volume was brought to 500 μL with Tris buffer (pH 7.4) and then incubated with gentle shaking at 24 °C for 18 hr, which allowed equilibrium to come to completion. Bound [3H]EB was collected and measured as described above. The percent specific [3H]EB binding was plotted against the concentration of saz-A. The data was fit with a nonlinear regression curve fit for one-site versus two-sites using Graph Pad
Prism 5. The IC$_{50}$ of saz-A was determined from the fitted curve and the K$_i$ was calculated from the Cheng-Prusoff equation (Cheng and Prusoff, 1973).
Results

We developed a sensitive radioligand binding method that fuses principles of receptor saturation and competition assays to measure the concentration of drugs in brain and/or other tissues that contain receptors that bind radioligands with high affinity. We validated this new method and used it to measure the concentration of saz-A in rat brain.

Competitive-saturation binding. The method to measure saz-A is based on a standard \(^{3}\text{H}\)EB saturation binding assay, but it is carried out in unwashed homogenates of cerebral cortex from rats treated with saline vehicle or different doses of saz-A. In this type of assay the binding affinity of the radioligand (the \(K_d\)) is calculated from the concentration of \(^{3}\text{H}\)EB that binds 50% of the receptors. We hypothesized that the presence of a non-radiolabeled competitive ligand in the tissue would shift the measured \(K_d\) of the radioligand in a saturation binding assay to an apparent \(K_d\) (\(K_{dA}\)), and that this apparent shift in \(K_d\) could be used to calculate the concentration of the non-radiolabeled ligand in the assay.

Before testing these hypotheses, we used a standard competition binding assay to demonstrate that saz-A and \(^{3}\text{H}\)EB bind competitively at cortical nAChRs and to determine the binding affinity (the \(K_i\)) of saz-A at these receptors under these conditions. Increasing concentrations of saz-A (0.01 – 300 nM) and 1.0 nM \(^{3}\text{H}\)EB were added to unwashed cerebral cortical homogenates from untreated rats. The percent specific binding of \(^{3}\text{H}\)EB was then measured as a function of the concentration of saz-A (Fig 1A). The data show that increasing concentrations of saz-A decrease \(^{3}\text{H}\)EB binding at nAChRs in a simple fashion with a Hill coefficient (\(n_H\)) of ~1 and that the higher concentrations of saz-A displace virtually all of the \(^{3}\text{H}\)EB binding. The \(K_i\) of saz-A, which was determined from the IC\(_{50}\) by applying the Cheng-
Prusoff equation (Cheng and Prusoff, 1973), was calculated to be 182 pM. These results in unwashed homogenates from untreated rats are very similar to those obtained in membranes washed twice by centrifugation (data not shown) and demonstrate that saz-A and [\(^3\)H]EB bind competitively at cortical nAChRs.

We next examined how the presence of a fixed concentration of saz-A affects the apparent affinity, \(K_{dA}\), of [\(^3\)H]EB in a saturation binding assay. Known concentrations of saz-A were incubated with unwashed cortical homogenates at 24 °C for 4 hr. Then, without washing these homogenates, aliquots were pipetted directly into tubes for [\(^3\)H]EB saturation binding assays. The specific [\(^3\)H]EB binding in homogenates that had been incubated in the absence or presence of 3 concentrations of saz-A was plotted and fit to saturation binding curves, as shown in figure 1B. Thus, in these assays, the \(K_d\) is the binding affinity of [\(^3\)H]EB in the absence of saz-A, and the \(K_{dA}\) is the binding affinity of [\(^3\)H]EB in the presence of the added saz-A. The \(K_d\) of [\(^3\)H]EB in the absence of saz-A was 61 pM; and as expected, the presence of saz-A in the assay shifted the saturation curves to the right and the measured \(K_{dA}\) of [\(^3\)H]EB to higher values. The increased \(K_{dA}\) values of the [\(^3\)H]EB saturation binding curves are more clearly seen in Scatchard plots (Fig 1B, inset), where the \(K_d\) and \(K_{dA}\) are inversely related to the slopes.

Modifying the Cheng-Prusoff equation for competitive [\(^3\)H]EB saturation binding.

The shift in the \(K_d\) of [\(^3\)H]EB to the higher \(K_{dA}\) values in the presence of saz-A (Fig. 1B) is a direct reflection of the simple competition of the two ligands for the nAChR binding site, in accordance with the law of mass action; thus we refer to this assay format as competitive-
saturation binding. We used this shift from $K_d$ to $K_{dA}$ in a modified version of the Cheng-Prusoff equation to calculate the concentration of saz-A in the assay.

In general, the Cheng-Prusoff equation (Fig. 2A, equation 1) is used to calculate the binding affinity, or $K_i$ value, of a non-radiolabeled ligand by a standard competition binding assay. In the Cheng-Prusoff equation, the $IC_{50}$ is the concentration of saz-A that occupies 50% of the $[^3H]EB$ binding sites; $[L]$ is the fixed concentration of $[^3H]EB$ used in the assay; and $K_d$ is the affinity of $[^3H]EB$ for the receptors, which is pre-determined in a saturation binding assay. This allows the $K_i$ value of saz-A to be calculated from the $IC_{50}$. As an example, the competition binding assay in figure 1A shows that increasing concentrations of saz-A compete for and occupy an increasing percentage of nAChRs, thereby decreasing $[^3H]EB$ binding at cortical nAChRs. The $IC_{50}$ of saz-A competing against 1 nM $[^3H]EB$ in this assay was 3.2 nM, which when inserted into the Cheng-Prusoff equation yields a $K_i$ value of 182 pM.

We derived a transformed version of the Cheng-Prussoff equation to be used for competitive-saturation binding. In a standard competition binding assay, increasing concentrations of a non-radiolabeled ligand compete for binding against a fixed concentration of a radioligand; while in a competitive-saturation binding assay, increasing concentrations of a radioligand compete for binding against a fixed concentration of a non-radioligand, designated “I” (i.e., saturation binding assays are carried out in the absence and presence of a non-radioactive ligand that competes for the receptor, as shown in fig. 1B). Thus, in the original Cheng-Prussoff equation, we substituted values that represent the radioligand with values that represent the non-radioligand, and vice versa. This transformed Cheng-Prussoff equation is designated Equation 2a in figure 2A, and is then rearranged to solve for the concentration of [I]
in the assay (Equation 2b). In this equation, \(K_d\) is the affinity of the radioligand for its receptor in the absence of inhibitor, and \(K_{dA}\) is the shifted affinity of the radioligand in the presence of the inhibitor. \(K_i\) is the affinity of inhibitor for the receptor, which is predetermined by a standard competition binding assay. This leaves the concentration of the inhibitor, \([I]\), in the binding assay, to be solved. To calculate the original concentration of inhibitor in the brain, the concentration of the inhibitor in the binding assay tubes is multiplied by the appropriate dilution factors (DF), which take into account the dilution of the tissue required for homogenization and its further dilution into the assay tubes (Equation 3).

Figure 2B shows a sample calculation with this transformed Cheng-Prusoff equation, using the data from figure 1, as follows: The \(K_i\) value of saz-A was calculated to be 182 pM, as measured in a standard \([3H]EB\) competition binding assay in unwashed cortical homogenates (Fig 1A). The \(K_d\) of \([3H]EB\) in the absence of saz-A, was calculated to be 61 pM in unwashed cortical homogenates (Fig 1B). When 3.5 nM saz-A was added to these homogenates, the apparent affinity of \([3H]EB\), \(K_{dA}\), was calculated to be 540 pM. By using these values in the transformed Cheng-Prusoff equation (Fig 2A, equation 2b), the concentration of saz-A in the assay tubes, \([I]\), was calculated to be 1.43 nM. To calculate the concentration of saz-A in the original homogenate, \([I]\) was multiplied by a dilution factor of 2.5, as 200 \(\mu\)L of the tissue homogenate was diluted in a total volume of 500 \(\mu\)L in the binding assay. Thus, the final concentration of saz-A was calculated to be 3.57 nM, which is close to the 3.5 nM concentration of saz-A that was actually added to the homogenate. Note, in this example a dilution factor for
the homogenization of the tissue was not included because the drug was added directly to the tissue after homogenization.

To further test the accuracy and precision of this method, unwashed cortical homogenates were incubated with 9 different known concentrations of saz-A. [$^3$H]EB saturation binding was then carried in these homogenates, and the concentrations of saz-A were calculated from the fitted competitive-saturation curves, as described above. Figure 2C shows the relationship of the calculated concentrations of saz-A plotted against the known concentrations of saz-A that were actually added to the homogenates. As the plotted data fall close to the line of identity, this figure demonstrates that competitive-saturation binding provides an accurate, precise, and sensitive method for measuring the concentration of saz-A.

**Applications for competitive saturation binding.** Next, we applied competitive-saturation binding to measure the concentration of saz-A in the rat brain *ex vivo*. Adult male rats were sacrificed 10 min following a single sc injection of saline or saz-A (2mg/kg), and dissected cortex was prepared as an unwashed homogenate in 20 volumes of buffer. These homogenates were immediately aliquoted in [$^3$H]EB saturation binding assays. The fitted saturation curves in these unwashed cortical homogenates (Fig. 3A) clearly show that the binding affinity of [$^3$H]EB was shifted in the tissues from saz-A-treated rats. Specifically, [$^3$H]EB displayed a $K_d$ of ~ 65 pM in homogenates from saline-treated rats and a $K_{dA}$ of ~1402 pM in homogenates from rats treated with saz-A. These $K_d$ and $K_{dA}$ values were used in the transformed Cheng-Prussoff equation to calculate the concentration of saz-A in the binding assay ([I]; Fig 2A, equation 2b). The concentration of saz-A in the brain was then calculated by multiplying [I] by the dilution factors (Fig. 2A, equation 3) resulting from dilution of the brain tissues in preparing the
homogenates, and then the further dilution of these homogenates in the binding assay. Using this competitive saturation method, the final concentration of saz-A in the brain was calculated to be ~187 nM.

Serum was also prepared from the same rats that received saline or saz-A (2mg/kg) injections. The concentration of saz-A in the serum was measured by another radioligand binding assay, which was adapted from a method originally developed by Creese and Snyder (1977) to measure the serum concentrations of dopaminergic drugs. In brief, volumes of serum and 1.0 nM [3H]EB were added to unwashed cortical homogenates (source of receptors), much like a standard competition binding assay. The percent specific [3H]EB binding in the presence of serum was then measured. To factor out any displacement of [3H]EB binding by serum alone, all results were normalized to the percent specific [3H]EB binding in the presence of serum from saline-treated rats. Figure 3B shows the normalized percent [3H]EB binding with 10 μL serum from saline treated rats and 1.0 μL serum from saz-A treated rats. Serum from saz-A treated rats displaced more than 50% of the [3H]EB binding, which clearly indicated that saz-A was present in the serum after injection. To calculate the actual concentration of saz-A, the percent [3H]EB binding was compared to competition binding curves with known concentrations of saz-A (Fig. 1A). The results indicate that, 10 min after a single sc injection of (2 mg/kg), saz-A reached a concentration of ~1.3 μM in the serum.

The radioligand binding approaches were also used to measure the elimination rate of saz-A from the brain and serum. Adult male rats were sacrificed at varying time points (10 – 120 min) following a single sc injection of saline or saz-A (2 mg/kg). The concentration of saz-A was then measured in the cortex by competitive saturation binding, and in the serum by competition binding, as described above. The results from this experiment (Fig 4) indicate that
saz-A reached a peak concentration of ~150 nM in the brain and ~1.6 μM in serum. Following the peak concentration, saz-A was eliminated from both the brain and serum with a calculated half life (t1/2) of ~65 min. Taken together, these results indicate that saz-A is not concentrated in brain and, in fact, displays a limited ability to enter the brain; furthermore, it is eliminated relatively rapidly. Nevertheless, the peak concentration of saz-A in the brain after sc injection of 2 mg/kg is ~ 1,000 times its Ki value for α4β2* nAChRs; thus, this concentration of saz-A should occupy virtually all of these receptors in the brain.
Discussion

In this study, we describe a new, relatively simple radioligand receptor binding assay and derived a transformed version of the Cheng-Prussoff equation (Cheng and Prussoff, 1973) to measure the concentration of saz-A in the rodent brain ex vivo. This new method combines the concepts of competition and saturation receptor binding; thus, we named this new method competitive-saturation binding. Competitive-saturation binding is not restricted to measuring the concentration of saz-A or other nicotinic drugs with affinities in the pM range. In fact, theoretically, this method can be used to assess the concentration of any drug in any tissue, as long as the drug reaches a sufficient concentration in the tissue to compete for a receptor that can be accurately and reliably measured with a radioligand. In practice this means that after dilution the drug has to reach a concentration in the binding assay equal to or greater than its dissociation constant, K_i. To some extent, if increased sensitivity is required, tissue can be added to the competitive-saturation binding assay at a higher concentration, which would result in less dilution of the drug of interest. For example, in determining the brain concentration of saz-A after the acute injection done here, the tissue and drug were diluted a total of 50-fold (20-fold dilution in making the tissue homogenate and a 2.5-fold dilution in adding the homogenate to the binding assay), but this total dilution could have been reduced to 12.5-fold by using a lower volume in the initial homogenization and reducing the volume of the binding assay. Together, this change in dilution would result in a 4-fold increase in sensitivity. Thus, this method is potentially sensitive enough to measure the concentrations of certain drugs in the sub-nanomolar range (e.g., Fig. 2C), which in some cases may be below the limits of reliable measurements with other methods.
Here we show that competitive-saturation binding is both accurate and precise at measuring known concentrations of saz-A that were added to brain homogenates (Fig 2C). The utility of this assay was demonstrated by measuring saz-A in the brain after a single sc injection and comparing it to the concentration of saz-A in rat serum, measured by a competitive radioligand binding assay (Fig. 3). Our results indicate that 10 min following a single sc injection (2 mg/kg), saz-A reached brain and serum concentrations of ~190 nM and ~1.3 μM, respectively. This difference in brain versus serum concentrations indicates that saz-A probably does not enter brain easily nor concentrate there. Nevertheless, as indicated above, this concentration of saz-A is ~1000 times its K_i for α4β2* nAChRs, thus it should occupy nearly all of these receptors in the brain. A previous study (Caldarone et al., 2011), which used HPLC methods to measure the concentration of saz-A in mouse brain and plasma, reported that 15 min following a single ip injection of 3 mg/kg, saz-A reached brain and plasma concentrations of ~38 nM and ~240 nM, respectively. Although these values in mice are lower than the values we measured in rats, the ratio of brain to blood concentrations are similar, 0.16 in mice and 0.13 in rats. The differences in concentration values could be a result of differences in drug metabolism between species, differences in drug bioavailability after ip injections compared to sc injections, and/or differences in the time of measurements. Moreover, in that study even the lower concentration of saz-A found in mouse brain occupied ~90% of the nAChRs (Caldarone et al., 2011).

In a companion paper (Hussmann et al., currently under review), we used this competitive-saturation method to measure the brain concentrations of saz-A, nicotine and varenicline 10 min after a series of chronic sc injections, and we compared these concentrations to the steady-state brain concentrations when the drugs were administered via osmotic
minipumps for 2 weeks. The concentrations we measured with this method were similar to the values previously measured with HPLC methods (Ghosheh et al., 1999; Doura et al., 2008; Rollema et al., 2010).

We also used radioligand binding approaches to measure the elimination rate of saz-A from rat brain. We found that after a single sc injection (2mg/kg), saz-A displayed a half-life of ~65 min in brain and serum. Caldarone et al (2011) measured a somewhat faster elimination rate of saz-A from mouse brain and plasma after ip administration, which again might be attributable to species differences and/or to the different routes of administration. Interestingly, in mice, despite the rapid elimination of saz-A from the brain as a whole, receptor occupancy appeared to be > 60% for at least 8 h (Caldarone et al., 2011).

As indicated above, the competitive-saturation binding method is sensitive, accurate and versatile in that theoretically it can be used to measure the concentration of any drug/ligand that competes with high affinity for receptor(s) that can be radiolabeled. A limitation of this method, as with all known analytical methods, is that it does not distinguish between intracellular and extracellular drug concentrations. A conceivable disadvantage of this method is that it doesn’t measure the actual chemical species of the drug/ligand itself but instead reflects the binding of the ligand to the receptor, from which the concentration can be deduced. Thus, the concentration of two or more ligands that competitively bind at the same receptor site in the same tissue would not be distinguishable. For example, if a single drug is metabolized to a compound that also has a high binding affinity for the receptor target, the concentration of the metabolite would be included in, but not distinguished from, the calculated concentration of the parent drug. On the other hand, a potential advantage of competitive-saturation binding is that it allows detection of active metabolites with sufficient binding affinity for the target receptor, even if the parent
compound is inactive. Thus, the assay could be used to screen for unidentified active metabolites. Furthermore, the assay allows the measurements of competitive drug binding at multiple receptor targets in the same tissues.

Finally, an additional advantage of competitive-saturation binding is that it is easy to carry out. Unlike HPLC methods, the drug of interest is not extracted and/or purified from the tissue before measurements. Both the drug and receptor source are provided in the tissue homogenates, so only the radioligand needs to be added to carry out the assay. Again, this method can be used to measure any drug in the brain or other tissue, as long as the drug of interest reaches a concentration equal to or greater than its $K_i$ after dilution in the binding assay, and a competitive radioligand for the target receptor is available. Thus, competitive-saturation binding should be a useful and convenient method for measuring drug concentrations, as well as the pharmacokinetics of drugs in the brain and other tissues.
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Authorship Contributions

Participated in research design: GPH and KJK

Conducted experiments: GPH

Performed data analysis: GPH and KJK

Wrote or contributed to the writing of the manuscript: GPH and KJK
References


Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. _Biochem Pharmacol_ **22**:3099-3108.


Doura MB, Gold AB, Keller AB, Perry DC (2008) Adult and periadolescent rats differ in expression of nicotinic cholinergic receptor subtypes and in the response of these subtypes to chronic nicotine exposure. _Brain Res_ **1215**:40-52.


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Footnotes

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Disclosures: Sazetidine-A was developed by KJK and YX and Georgetown University. Georgetown University currently holds the patent on sazetidine-A.
Legends for Figures

**Figure 1:** Sazetidine-A competition for nAChRs in rat brain. (A) Increasing concentrations of sazetidine-A (Saz-A) competing against 1.0 nM [3H]EB for nAChRs. The percent specific [3H]EB binding was plotted as the mean ± SEM from 3 individual experiments. Data were fit with a nonlinear regression one-site versus two-site competition curve fit. The Ki and Hill slope (nH) values were calculated from the fitted curves and are reported in the figure. (B) Unwashed cortical homogenates were incubated in the absence or presence of saz-A at the indicated concentrations for 4 hr and then the homogenate was aliquotted in a [3H]EB saturation binding assay without prior washing or centrifugation. The data are plotted as the mean ± SEM from 3 – 6 individual experiments and fit with a nonlinear regression saturation curve fit. Figure inset. Saturation data were also plotted as a Scatchard plot and fit by linear regression. The Kd and KdA values (pM) were calculated from the curves and are as follows: Control, 61 ± 6; 0.75 nM Saz-A, 136 ± 17; 2.0 nM Saz-A, 323 ± 20; 3.5 nM Saz-A, 540 ± 46.

**Figure 2:** Derivation and test of the equations used in competitive-saturation binding. (A) Equation 1 is the original Cheng-Prusoff equation. Equation 2a is the modified Cheng-Prusoff equation, where the values that stood for the radioligand were substituted for the non-radiolabeled ligand, and vice versa. Equation 2b is a rearrangement of equation 2a that solves for [I]. Equation 3 is used to account for dilution factors (DF) resulting from dilution of the non-radiolabeled drug ([I]) when preparing the homogenate and adding it in the binding assay. (B) A sample calculation with the modified Cheng-Prusoff equation that uses data from figure 1. (C) Test of the method. Unwashed cortical homogenates were incubated in the absence or presence of 9 different known concentrations of saz-A. Competitive-saturation curves were then carried out in these homogenates and saturation curves were fit to the data. The Kd and KdA values were
then derived from these curves, and the concentration of saz-A was calculated. The calculated concentrations of saz-A in the homogenates were plotted as the mean ± SEM from 3 – 6 individual experiments against the actual concentrations of saz-A that were added. The data were fit by linear regression (solid line) and compared to the line of identity (dashed line).

**Figure 3:** Utilization of the competitive-saturation assay to measure saz-A in brain after acute injection. (A) Rats were sacrificed 10 min following a single sc injection of saline or 2 mg/kg sazetidine-A (Saz-A). An unwashed homogenate was then prepared from dissected cortex and aliquotted in a [3H]EB competitive-saturation binding assay. Specific [3H]EB binding was plotted as the mean ± SEM from 4 individual animals in each treatment group and saturation curves were fit to the data. Scatchard plots were also generated from the data and are shown in the figure inset. The K_d and K_dA values were calculated from the fitted curves and are displayed in the figure. The calculated concentration of saz-A in the brain was calculated to be 187 ± 48 nM. (B) Serum was also collected from these rats and added to a competition binding assay with 1.0 nM [3H]EB and untreated unwashed cortical homogenates as the source of receptors. The percent specific [3H]EB binding was then calculated. All results were normalized to [3H]EB binding in the presence of serum from saline-treated rats. The data were graphed as the mean ± SEM from 4 individual animals in each treatment group. The concentration of saz-A in the serum was calculated to be 1.3 ± 0.1 μM.

**Figure 4:** Utilization of the competitive-saturation assay to measure saz-A elimination rate from brain after sc injection. Rats were given a single sc injection of saline or 2 mg/kg saz-A and were sacrificed after increasing times following the injection. Cerebral cortex and serum were collected and used in [3H]EB competitive-saturation binding and standard [3H]EB competition binding assays, respectively. The concentrations of saz-A in the brain and serum
were calculated from these assays and plotted as the mean ± SEM from 3 individual animals for each time point. A nonlinear regression, exponential decay curve was fit to the data. The half-life (t\(_{1/2}\)) of saz-A in the brain and serum were calculated from these curves and are displayed as the mean ± SEM in the figure.
**Figure 2**

A. **Equation 1: Cheng-Prusoff**

\[
Ki = \frac{IC50}{1 + \frac{[L]}{Kd}}
\]

B. **Equation 2a: Modified Cheng-Prusoff**

\[
Kd = \frac{Kd_A}{1 + \frac{[I]}{Ki}}
\]

**Equation 2b: Modified Cheng-Prusoff (Rearranged)**

\[
[I] = \frac{Ki (Kd_A - Kd)}{Kd}
\]

**Equation 3: Accounting for Dilutions**

\[
[I] = ([I])(DF)
\]

**Example Calculation: 3.5 nM Saz-A Added**

\[
[I] = \frac{Ki (Kd_A - Kd)}{Kd} = \frac{182pM (540pM - 61pM)}{61pM} = 1.43 nM
\]

\[
[I]_{Homogenate} = ([I])(DF) = (1.43 nM)(2.5) = 3.57 nM
\]

C. [Graph showing the relationship between [Saz-A] Added (pM) and Calculated [Saz-A] (pM)]
Figure 3.

A. 

Brain

- Saline
- Saz-A (2mg/kg)

$K_d = 65 \pm 6\text{pM}$

$K_{dA} = 1402 \pm 361\text{pM}$

Specific $[^3H]EB$ Bound (fmol/mg tissue)

Free $[^3H]EB$ (pM)

B. 

Serum

- Saline
- Saz-A (2mg/kg)

% Specific $[^3H]EB$ Binding (Normalized to Saline)

10 µL

1 µL
Figure 4

Elimination Rate of Saz-A

Concentration of Saz-A (nM)

Time Following SC Injection (min)

- Serum: $t_{1/2} = 66 \pm 12$ min
- Brain: $t_{1/2} = 64 \pm 2$ min