

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

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ABSTRACT

We have developed a new radioligand binding assay method to measure the concentration of nonradiolabeled drugs in the brain ex vivo. This new method fuses the concepts of standard competition and saturation binding assays and uses a transformed version of the Cheng-Prusoff equation (*Biochem Pharmacol* 22:3099–3108, 1973) 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 neuronal nicotinic acetylcholine receptor binding sites. Furthermore, using this method, we estimated the half-life of sazetidine-A in the rat brain to be ~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.

Introduction

Sazetidine-A (saz-A) is a recently developed partial agonist that potently desensitizes $\beta 2$ -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 *N*-methyl-D-aspartate receptor blocker dizocilpine [(5*S*,10*R*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine (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 pharmacoki-

netic 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 after a single intraperitoneal injection it occupied the majority of nAChR binding sites for 8 h or longer. This long-term receptor occupancy is probably caused by the high affinity of saz-A at $\alpha 4\beta 2$ 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, 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). To 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-Prusoff equation (Cheng and Prusoff, 1973). 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

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Sazetidine-A was developed by K.J.K. and Yingxian Xiao. Georgetown University currently holds the patent on sazetidine-A.

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ABBREVIATIONS: saz-A, sazetidine-A; nAChR, neuronal nicotinic acetylcholine receptor; [3 H]EB, [3 H]epibatidine; HPLC, high-performance liquid chromatography; MK801, (5*S*,10*R*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine; NSB, nonspecific binding; DF, dilution factors.

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.

Materials and Methods

Materials. [^3H]epibatidine ([^3H]EB; ~ 55 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). 6-(5-(((S)-azetid-2-yl)methoxy)pyridin-3-yl)hex-5-yn-1-ol (sazetidine-A) dihydrochloride (Xiao et al., 2006) was synthesized by RTI International (Research Triangle, NC) and supplied by the National Institute on Drug Abuse (Rockville, MD). Nicotine hydrogen tartrate was purchased from Sigma-Aldrich (St. Louis, MO).

Animals. Male adult Sprague-Dawley rats were purchased from Harlan (Indianapolis, IN). Rats were housed in groups (two per cage) in Association for Assessment and Accreditation of Laboratory Animal Care-approved facilities at Georgetown University (Washington, DC). All rodents were maintained on a 12-h light/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 (Institute of Laboratory Animal Resources, 1996). Experimental procedures were approved by 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 to 8.0 with NaOH. Drug was delivered by subcutaneous injections above the shoulders. Doses of sazetidine-A dihydrochloride are reported as the salt.

Preparation of Unwashed Cerebral Cortex Homogenates. Ten minutes after administration of the drug, the rats were anesthetized with isoflurane and 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 (six-seven strokes) (Thomas Scientific, Swedesboro, NJ) followed by a 10-s sonication. This homogenate was not washed or centrifuged and thus was called an unwashed homogenate.

Saturation Binding Assay. Unwashed homogenates were aliquoted into tubes with increasing concentrations of [^3H]EB (5–4000 pM) in a final volume of 500 μl of 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 h 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 plotted against the free (unbound) concentration of [^3H]EB in the binding assay. The data were fit with a nonlinear regression saturation curve by using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA), and the K_d of [^3H]EB was calculated from the fitted curve.

Competition Binding. Unwashed homogenates were aliquoted in a binding assay with 1.0 nM [^3H]EB and increasing concentrations of saz-A. 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 h, which allowed equilibrium to come to completion. Bound [^3H]EB was collected and measured as described above. The percentage of specific [^3H]EB binding was plotted against the concentration of saz-A. The data were fit with a nonlinear regression curve fit for one site versus two sites by using GraphPad 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 [^3H]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 [^3H]EB that binds 50% of the receptors. We hypothesized that the presence of a nonradiolabeled 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 this apparent shift in K_d could be used to calculate the concentration of the nonradiolabeled ligand in the assay.

Before testing these hypotheses, we used a standard competition binding assay to demonstrate that saz-A and [^3H]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 [^3H]EB were added to unwashed cerebral cortical homogenates from untreated rats. The percentage of specific binding of [^3H]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 [^3H]EB binding at nAChRs in a simple fashion with a Hill coefficient (n_H) of ~ 1 , and the higher concentrations of saz-A displace virtually all of the [^3H]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 were very similar to those obtained in membranes washed twice by centrifugation (data not shown) and demonstrate that saz-A and [^3H]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 [^3H]EB in a saturation binding assay. Known concentrations of saz-A were incubated with unwashed cortical homogenates at 24°C for 4 h. Then, without washing these homogenates, aliquots were pipetted directly into tubes for [^3H]EB saturation binding assays. The specific [^3H]EB binding in homogenates that had been incubated in the absence or presence of three concentrations of saz-A was plotted and fit to saturation binding curves, as shown in Fig. 1B. Thus, in these assays, the K_d is the binding affinity of [^3H]EB in the absence of saz-A, and the K_{dA} is the binding affinity of [^3H]EB in the presence of the added saz-A. The K_d of [^3H]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 [^3H]EB to higher values. The increased K_{dA} values of the [^3H]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 [^3H]EB Saturation Binding. The shift in the K_d of [^3H]EB to the higher K_{dA} values in the presence of saz-A (Fig.

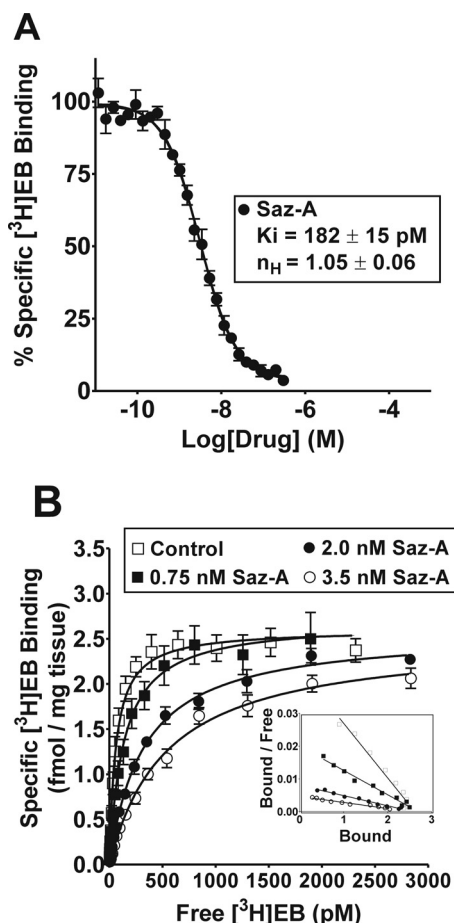


Fig. 1. Sazetidine-A competition for nAChRs in rat brain. **A**, increasing concentrations of saz-A competing against 1.0 nM [^3H]EB for nAChRs. The percentage of specific [^3H]EB binding was plotted as the mean \pm S.E.M. from three individual experiments. Data were fit with a nonlinear regression one-site versus two-site competition curve fit. The K_i and Hill slope (n_H) values calculated from the fitted curves are shown. **B**, unwashed cortical homogenates were incubated in the absence or presence of saz-A at the indicated concentrations for 4 h, and then the homogenate was aliquoted in a [^3H]EB saturation binding assay without prior washing or centrifugation. The data are plotted as the mean \pm S.E.M. from three to six individual experiments and fit to a nonlinear regression saturation curve. Inset, saturation data were also plotted as a Scatchard plot and fit by linear regression. The K_d and K_{dA} values (pM) were calculated from the curves and are as follows: control, 61 \pm 6; 0.75 nM Saz-A, 136 \pm 17; 2.0 nM Saz-A, 323 \pm 20; and 3.5 nM Saz-A, 540 \pm 46.

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, eq. 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 predetermined 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 Fig. 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 yielded a K_i value of 182 pM.

We derived a transformed version of the Cheng-Prusoff equation to be used for competitive-saturation binding. In a standard competition binding assay, increasing concentrations of a nonradiolabeled ligand compete for binding against a fixed concentration of a radioligand; whereas in a competitive-saturation binding assay, increasing concentrations of a radioligand compete for binding against a fixed concentration of a nonradioligand, designated "I" (i.e., saturation binding assays are carried out in the absence and presence of a nonradioactive ligand that competes for the receptor, as shown in Fig. 1B). Thus, in the original Cheng-Prusoff equation, we substituted values that represent the radioligand with values that represent the nonradioligand and vice versa. This transformed Cheng-Prusoff equation is designated eq. 2a in Fig. 2A and is then rearranged to solve for the concentration of [I] in the assay (Fig. 2A, eq. 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 ([I]_{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 (Fig. 2A, eq. 3).

Figure 2B shows a sample calculation with this transformed Cheng-Prusoff equation, using the data from Fig. 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, eq. 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, because 200 μl of the tissue homogenate was diluted in a total volume of 500 μl 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 nine different known concentrations of saz-A. [^3H]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. Because the plotted

A

Equation 1: Cheng-Prusoff

$$K_i = \frac{IC_{50}}{1 + \frac{[L]}{K_d}}$$

Equation 2a: Modified Cheng-Prusoff

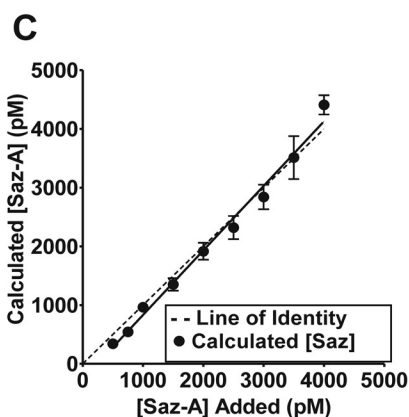
$$K_d = \frac{K_{dA}}{1 + \frac{[I]}{K_i}}$$

Equation 2b: Modified Cheng-Prusoff (Rearranged)

$$[I] = \frac{K_i (K_{dA} - K_d)}{K_d}$$

Equation 3: Accounting for Dilutions

$$[I]_{\text{Brain}} = ([I])(DF)$$



data fall close to the line of identity, Fig. 2C demonstrates that competitive-saturation binding provides an accurate, precise, and sensitive method ($< 1\text{nM}$) 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 after a single subcutaneous injection of saline or saz-A (2 mg/kg), and dissected cortex was prepared as an unwashed homogenate in 20 volumes of buffer. These homogenates were immediately aliquoted in $[^3\text{H}]\text{EB}$ saturation binding assays. The fitted saturation curves in these unwashed cortical homogenates (Fig. 3A) clearly show that the binding affinity of $[^3\text{H}]\text{EB}$ was shifted in the tissues from saz-A-treated rats. Specifically, $[^3\text{H}]\text{EB}$ 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-Prusoff equation to calculate the concentration of saz-A in the binding assay ($[I]$; Fig. 2A, eq. 2b). The concentration of saz-A in the brain, $[I]_{\text{brain}}$, was then calculated by multiplying $[I]$ by the dilution factors (Fig. 2A, eq. 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.

B

Example Calculation: 3.5 nM Saz-A Added

$$[I] = \frac{K_i (K_{dA} - K_d)}{K_d}$$

$$[I] = \frac{182\text{pM} (540\text{pM} - 61\text{pM})}{61\text{pM}}$$

$$[I] = 1.43 \text{ nM}$$

$$[I]_{\text{Homogenate}} = ([I])(DF)$$

$$[I]_{\text{Homogenate}} = (1.43 \text{ nM})(2.5)$$

$$[I]_{\text{Homogenate}} = 3.57 \text{ nM}$$

Fig. 2. Derivation and test of the equations used in competitive-saturation binding. A, eq. 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 nonradiolabeled ligand and vice versa. Equation 2b is a rearrangement of eq. 2a that solves for $[I]$. Equation 3 is used to account for DF resulting from dilution of the nonradiolabeled 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 Fig. 1. C, test of the method. Unwashed cortical homogenates were incubated in the absence or presence of nine 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 K_d and K_{dA} 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 \pm S.E.M. from three to six individual experiments against the actual concentrations of saz-A that were added. The data were fit by linear regression (solid line) and compared with the line of identity (dashed line).

Serum was also prepared from the same rats that received saline or saz-A (2 mg/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 $[^3\text{H}]\text{EB}$ were added to unwashed cortical homogenates (source of receptors), much like a standard competition binding assay. The percentage of specific $[^3\text{H}]\text{EB}$ binding in the presence of serum was then measured. To factor out any displacement of $[^3\text{H}]\text{EB}$ binding by serum alone, all results were normalized to the percentage of specific $[^3\text{H}]\text{EB}$ binding in the presence of serum from saline-treated rats. Figure 3B shows the normalized percentage of $[^3\text{H}]\text{EB}$ binding with 10 μl of serum from saline-treated rats and 1.0 μl of serum from saz-A treated rats. Serum from saz-A-treated rats displaced more than 50% of the $[^3\text{H}]\text{EB}$ binding, which clearly indicated that saz-A was present in the serum after injection. To calculate the actual concentration of saz-A, the percentage of $[^3\text{H}]\text{EB}$ binding was compared with competition binding curves with known concentrations of saz-A (Fig. 1A). The results indicate that 10 min after a single subcutaneous 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–

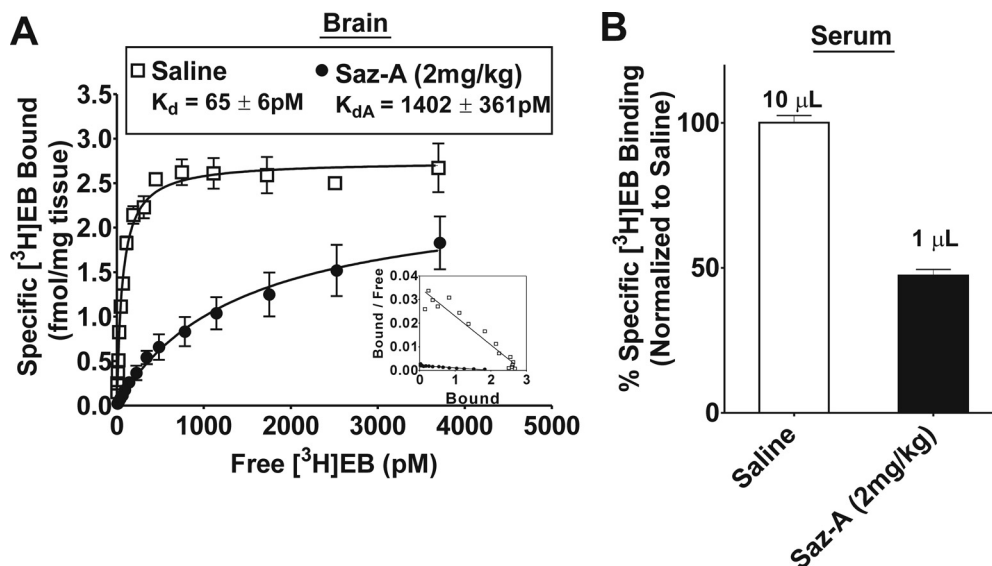


Fig. 3. Utilization of the competitive-saturation assay to measure saz-A in brain after acute injection. A, rats were sacrificed 10 min after a single subcutaneous injection of saline or 2 mg/kg saz-A. An unwashed homogenate was then prepared from dissected cortex and aliquoted in a [³H]EB competitive-saturation binding assay. Specific [³H]EB binding was plotted as the mean \pm S.E.M. from four 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 Inset. The K_d and K_{dA} values were calculated from the fitted curves and are displayed. 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 percentage of 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 \pm S.E.M. from four individual animals in each treatment group. The concentration of saz-A in the serum was calculated to be 1.3 ± 0.1 μM .

120 min) after a single subcutaneous 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) indicated that saz-A reached a peak concentration of ~ 150 nM in the brain and ~ 1.6 μM in serum. After the peak concentration, saz-A was eliminated from both the brain and serum with a calculated half-life ($t_{1/2}$) 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 subcutaneous injection of 2 mg/kg is ~ 1000 times its K_i value for $\alpha 4\beta 2^*$ 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. We 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 picomolar 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 re-

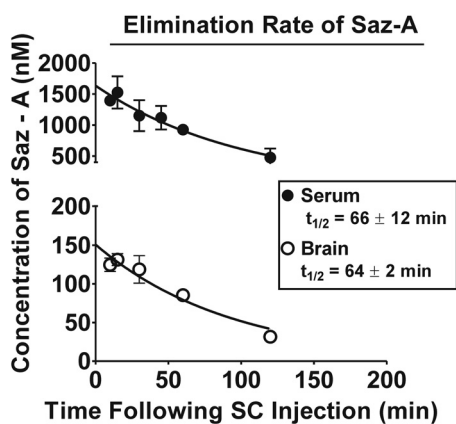


Fig. 4. Use of the competitive-saturation assay to measure saz-A elimination rate from brain after subcutaneous injection. Rats were given a single subcutaneous injection of saline or 2 mg/kg saz-A and sacrificed after increasing times after the injection. Cerebral cortex and serum were collected and 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 \pm S.E.M. from three individual animals for each time point. A nonlinear regression, exponential decay curve was fit to the data. The half-lives ($t_{1/2}$) of saz-A in the brain and serum were calculated from these curves and are displayed as the mean \pm S.E.M.

duced 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 subnanomolar range (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 subcutaneous injection and comparing it with the concentration of saz-A in rat serum, measured by a competitive radioligand binding assay (Fig. 3). Our results indicate that 10 min after a single subcutaneous injection (2 mg/kg), saz-A reached brain and serum concentrations of ~190 nM and ~1.3 μ M, 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 $\alpha 4\beta 2^*$ 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 after a single intraperitoneal injection of 3 mg/kg saz-A reached brain and plasma concentrations of ~38 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 intraperitoneal injections compared with subcutaneous 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 study (Hussmann et al., 2012), we used this competitive saturation method to measure the brain concentrations of saz-A, nicotine, and varenicline 10 min after a series of chronic subcutaneous 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 subcutaneous injection (2 mg/kg) saz-A displayed a half-life of ~65 min in brain and serum. Caldaroni et al. (2011) measured a somewhat faster elimination rate of saz-A from mouse brain and plasma after intraperitoneal administration, which again might be attributable to species differences. It is noteworthy that in mice, despite the rapid elimination of saz-A from the brain as a whole, receptor occupancy seemed 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 receptors

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 does not 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: Hussmann and Kellar.

Conducted experiments: Hussmann.

Performed data analysis: Hussmann and Kellar.

Wrote or contributed to the writing of the manuscript: Hussmann and Kellar.

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