Optimum Conditions of Radioligand Receptor Binding Assay of Ligands of Benzodiazepine Receptors

Fatemeh Ahmadi, Sara Dabirian, Mehrdad Faizi, Sayyed Abbas Tabatabai, Davood Beiki and Soraya Shahhosseini*

*a Department of Radiopharmacy, School of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran. b Department of Pharmaceutical Chemistry, School of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran. c Department of Pharmacology and Toxicology, School of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran. d Research center for Nuclear Medicine, Tehran University of Medical Sciences, Tehran, Iran.

Abstract

To obtain drugs which are more selective at benzodiazepine (BZD) receptors, design and synthesis of functionally selective ligands for BZD receptors is the current strategy of our pharmaceutical chemistry department. The affinity of newly synthesized ligands is assessed by radioligand receptor binding assays. Based on our previous studies, 2-phenyl-5-oxo-7-methyl-1,3,4-oxadiazolo[a,2,3]-pyrimidine (compound A) was chosen for design and synthesis of new triazole derivatives as GABA_BZD receptor agonist. The cortical membrane of male Sprague-Dawley rats was prepared as the source of the BZD receptors. Different concentrations of membrane protein and $[3H]$-flumazenil were incubated at room temperature at different time periods to reach the steady-state. To saturate the receptors, increased amounts of radioligand were incubated with membrane protein. The bound and un-bound ligands were separated by centrifugation. The affinity of compound A was measured in competition studies at optimum conditions by displacement of $[3H]$-flumazenil from rat cortical membrane. Based on results, the optimum conditions of radioligand receptor binding assay of benzodiazepines were 35 min incubation of ligands with 100 µg cortical membrane protein and $8.6 \times 10^{-5}$ nmole $[3H]$-flumazenil in a final volume of 0.5 mL Tris-HCl buffer (50 mM, pH 7.4) at 30 °C. The binding parameters of $[3H]$-flumazenil, $B_{\text{max}}$ and $K_d$ were determined through saturation studies as $0.638 \pm 0.099$ pmol/mg and $1.35 \pm 0.316$ nM respectively. The affinity of compound A was 1.9 nM comparable with diazepam (1.53nM). This finding makes the compound an interesting lead for further optimization. Starting from this compound, new ligands were synthesized and screened in-vitro by competitive binding assays.

Keywords: Radioligand receptor binding assay; $[3H]$-flumazenil; liquid scintillation; Benzodiazepine.

Introduction

Radioligand binging assays are used to determine the affinity of various ligands for a receptor, the binding site density of receptor families and their subtypes in different tissues or samples, the distribution of receptors, and effects of physiological and pathological conditions on the expression of the receptors. There are two basic types of receptor binding experiments: saturation and competition. Saturation studies
removed and homogenized for 30 s in 20 mL ice-cold Tris-HCl buffer (30 mM, pH 7.4) using a Silent S homogenizer (Heidolph, Germany) at medium speed. The homogenates were centrifuged at 600 g for 10 min using a Beckman Coulter L90K centrifuge. The resulting supernatant was centrifuged at 27000 g for 15 min. The pellet was washed 3 times with ice-cold buffer by re-suspension and re-centrifugation. The washed pellet was suspended in 20 mL buffer, incubated at 37 °C for 30 min and then centrifuged for 10 min at 27000 g. The pellet was washed once, and the final pellet was re-suspended in 30 mL Tris-HCl buffer (50 mM, pH 7.4). All of the centrifugation was performed at 4 °C (10-12). The amount of protein was estimated in the membrane preparation by the Bradford method (1976) using bovine serum albumin (BSA) as a standard (13). The membrane preparation was stored at -20 °C until it was used 1-15 days later.

Assay conditions

Incubation time

100 µg of membrane protein was incubated with $8.6 \times 10^{-5}$ nmole $[^3H]$-flumazenil (87Ci/mmol, Perkin-Elmer, USA Life and Analytical Science) in a final volume of 0.5 mL Tris-HCl buffer (50 mM, pH 7.4) at 30 °C. After incubation in different time periods (10, 20, 25, 30, 40 min), the contents of the tube were centrifuged at 1500 g for 4 min at 4 °C using Tomy MX-305 refrigerated centrifugation (Tomy, Japan). The supernatant was gently aspirated from the pellet. Pellet was washed by ice-cold Tris-HCl buffer, transferred to liquid scintillation vials, covered with 1mL of liquid scintillation cocktail (Maxilight, Hidex, Finland) and the activity was measured by liquid scintillation counter (Triathler multilabel tester, Hidex, Finland). Nonspecific binding (NSB) was determined in parallel assays performed in the presence of 100 µM diazepam. Total binding (TB) (receptor + radioligand) and NSB (receptor + radioligand + excess diazepam) were measured at various times of incubation (1, 14-16).

Receptor concentration (Zone A)

Different concentrations of membrane proteins (50, 100, 150, 200, 250, 300 µg) in Tris-HCl buffer
Optimum conditions of radioligand receptor binding assay of ligands

(50 mM, pH 7.4) were incubated with $8.6 \times 10^{-5}$ nmole $[^3H]$-Flumazenil in total volume of 0.5 mL at 30 °C. After a 35 minute incubation period, the homogenate was centrifuged and the activity of pellet was measured as previously mentioned. TB (receptor + radioligand) and NSB (receptor + radioligand + excess diazepam) were measured at various levels of added membrane protein. Total added (TA) is the count of radioligand in absence of receptor and diazepam (16).

Saturation binding studies

100 μg of membrane protein was added to Tris-HCl buffer (50 mM, pH 7.4) and incubated with seven different concentrations of $[^3H]$-Flumazenil at 30 °C for 35 min. The incubation was terminated by the centrifugation of reaction mixture at 1500 g for 4 min at 4 °C. The activity of pellet was measured as previously mentioned. TB (receptor + radioligand), NSB (receptor + radioligand + excess diazepam), and specific binding (SB) (SB = TB - NSB) were measured at various radioligand concentrations (1, 9).

Competition binding studies

100 μg of membrane protein in Tris-HCl buffer (50 mM, pH 7.4) was incubated with $8.6 \times 10^{-5}$ nmole $[^3H]$-Flumazenil and increasing amount of 2-phenyl-5-oxo-7-methyl-1,3,4-oxadiazolo[a,2,3]-pyrimidine, (compound A, Figure 1) in a final volume 0.5mL at 30 °C for 35 min (17). After incubation, the assay was terminated by centrifugation (1500 g, 4°C, 4 min). The activity of pellet was measured as previously mentioned. Binding (receptor + radioligand) and NSB (receptor + radioligand + excess diazepam) were measured at various concentrations of unlabeled ligand. TB is determined in the absence of any added competitor (non-radioactive ligand) (1, 9).

Data analysis

All of experiments were done in triplicates. The steady state curve was generated by plotting SB versus time in order to determine the proper time of incubation. Receptor concentration curve was generated by plotting [TB/TA] expressed as a percent versus membrane receptor protein concentration. Zone A, the level of membrane protein that yields <10% [TB/TA] was assessed from curve. The saturation curves were generated by plotting the SB versus the radioligand concentration. The binding parameters ($K_d$ and $B_{max}$) of $[^3H]$-Flumazenil were calculated from non-linear regression analysis of the saturation curve data by using the activity base software package (Program Prism, Graph Pad, San Diego, CA). The amount of SB was calculated by subtracting NSB from total binding (TB). TB is the amount of binding of the radioligand in the absence of diazepam. A large excess of diazepam was used in the control experiments to saturate the receptor sites to determine NSB of the radioligand (16-18).

Results and Discussion

To assess the affinity of newly synthesized ligands against benzodiazepine (BZD) receptors, radioligand receptor binding assays are frequently used. The assays are relatively simple but extremely powerful tool for studying receptors and identify a lead compound for future investigations (9). The assay includes incubation of radioligand with the receptor preparation, separation bound ligand from free ligand, quantify the amount of bound radioligand, and finally analyze the data. The optimum conditions are necessary for experiments in order to get

Figure 1. The structure of 2-phenyl-5-oxo-7-methyl-1,3,4-oxadiazolo[a,2,3]-pyrimidine (compound A).
at room temperature, which was measured to be 30 °C. The temperature was kept at 30 °C in all of the experiments. To minimize NSB and prevent ligand depletion, a low concentration of radioligand and membrane preparation is required in assay. Based on literature review, the range of radioligand concentrations should be from \((0.1-10) \text{k}_{d}\) if possible (1, 14-16). We obtained the appropriate radioligand concentration in preliminary experiments regarding NSB and reliable measurement of radioactivity (cpm). NSB was determined by excess concentrations of diazepam to occupy all of the available receptors in the presence of radioligand. For most receptor assays a tissue concentration in the range of 100-500 µg of membrane protein is used (1). In preliminary studies, we used 100 µg of membrane protein, which was later proved (in zone A determination) to be the right amount. The appropriate incubation time was determined with incubation of 100 µg of rat cortical membrane contains BZD receptors with the 8.6×10^{-5} \text{n mole}\(^{3}\)H-flumazenil at 30 °C until steady state conditions were reached. The amount of radioligand, which was bound to the receptors at various times after the start of the incubation, was measured. The results were plotted with bound on the Y-axis and time on the X-axis. The appropriate incubation time was determined from the curve where the binding is shown to be constant (1, 14-18). The results of our studies are shown in Table 1 and Figure 2.

Table 1. The [\(^{3}\)H]-flumazenil binding to rat cortical membrane in various time periods.

| Incubation time (min) | TB (cpm) | NSB (cpm) | SB (cpm) |
|-----------------------|----------|-----------|----------|
| 0                     | 0        | 0         | 0        |
| 10                    | 456.6±60.2 | 90.3±2.8 | 367±59.3 |
| 20                    | 516±9.8  | 104±1     | 412±10.58|
| 25                    | 537±32.5 | 104.3±2.08| 433.6±20.6|
| 30                    | 577.6±79.8 | 102.3±9.86 | 455.3±70.4|
| 40                    | 577.6±47.3 | 99.6±9.29 | 458±50.06|

TB: total binding, NSB: non specific binding, SB: specific binding.

The values shown are the Mean ± SEM of three independent determinations.
Optimum Conditions of Radioligand Receptor Binding Assay of Ligands

The amount of radioligand required to saturate the receptors was used to determine the receptor binding affinity of \([^3H]\)-Flumazenil (K$_d$) and the benzodiazepine receptor density (B$_{max}$) based on non-linear regression analysis of the saturation curve data (18). As the concentration of radioligand increases the amount of bound increases until a point is reached where no matter how much more radioligand is added, the amount bound does not increase further. As shown in Table 3 and Figure 4, the binding parameters (B$_{max}$ and K$_d$) of[^3H]-Flumazenil were calculated from the saturation binding experiments. B$_{max}$ and K$_d$ were calculated as 0.638 ± 0.099 pmol/mg and 1.35 ± 0.316 nM respectively.

**Table 2.** The[^3H]-flumazenil binding at various levels of membrane protein in optimum incubation time.

| Protein concentration (µg) | TB (cpm)     | TA (cpm) | (TB×100)/TA |
|---------------------------|--------------|----------|--------------|
| 0                         | 0            | 0        | 0            |
| 50                        | 194.3 ± 12.5 | 3974     | 4.85 ± 0.27  |
| 100                       | 316 ± 61.7   | 3974     | 7.9 ± 1.56   |
| 150                       | 457.6 ± 46.7 | 3974     | 11.48 ± 1.14 |
| 200                       | 517.6 ± 14.04| 3974     | 12.98 ± 0.35 |
| 250                       | 551.3 ± 105.02| 3974 | 13.83 ± 2.6  |
| 300                       | 580 ± 12.28  | 3974     | 14.55 ± 0.3  |

The values shown are the Mean±SEM of three independent determinations.

<10% TB/TA) must be evaluated from receptor concentration curve by plotting % TB/TA versus receptor concentration. To avoid ligand depletion, the TB of radioligand should be less than 10% of TA radioligand (9, 14-18). Saturation studies must be performed at <10% total ligand binding at all radioligand concentrations. In this study, we measured the amount of TB at various concentrations of receptors in optimum incubation time (35 min). Table 2 summarized the results of receptor concentration. The membrane receptor protein concentration that yields <10% TB/TA (Zone A) was 100 µg (Figure 3).

Saturation binding study: For the saturation binding studies of[^3H]-flumazenil, seven different concentrations of[^3H]-flumazenil (ranging from 0.05 nM to 0.97 nM) were used.

The amount of radioligand required to saturate the receptors was used to determine the receptor binding affinity of[^3H]-Flumazenil (K$_d$) and the benzodiazepine receptor density (B$_{max}$) based on non-linear regression analysis of the saturation curve data (18). As the concentration of radioligand increases the amount of bound increases until a point is reached where no matter how much more radioligand is added, the amount bound does not increase further. As shown in Table 3 and Figure 4, the binding parameters (B$_{max}$ and K$_d$) of[^3H]-Flumazenil were calculated from the saturation binding experiments. B$_{max}$ and K$_d$ were calculated as 0.638 ± 0.099 pmol/mg and 1.35 ± 0.316 nM respectively.

**Competition binding assay**
The affinity of the non-radioactive ligands for the receptor is determined indirectly by measuring their ability to compete and inhibit the binding of radioligand to its receptor. In a competition experiment, various concentrations of a non-radioactive ligand compete with a fixed concentration of radioligand for binding to the receptor. As the concentration of non-radioactive ligand increases, the amount of radioligand bound to the receptor decreases. The concentration of non-radioactive ligand that inhibits the binding of \([3H]\)-flumazenil by 50% is IC\(_{50}\) value (18).

In this study, the affinity of compound A was measured in competition studies at optimum conditions by displacement of \([3H]\)-Flumazenil from rat cortical membrane (Table 4 and Figure 5). The affinity was 1.9 nM comparable with diazepam (1.53 nM), a known benzodiazepine agonist. This finding makes the compound an interesting lead for further optimization. Starting from this compound, new ligands were designed and synthesized. The design was based on a pharmacophore model of the benzodiazepine binding site of GABA\(_A\). The affinity and IC\(_{50}\) of new compounds were measured in competition studies. The \textit{in-vivo} biological evaluation of compounds with good affinity is on the way. The results (design, synthesis, and biological evaluation) would be published soon.

In conclusion, radioligand receptor binding assays at optimum conditions provide \textit{in-vitro} screening of compounds quickly and precisely. Compounds with high affinity would go through biological evaluation. Based on receptor structure, essential pharmacophore groups, and affinity of ligands, a lead compound would be identified.

Table 3. The results of saturation binding at steady state conditions in the presence of seven different concentrations of \([3H]\)-flumazenil.

| \(3H\)-flumazenil (nM) | TB (cpm) | NSB (cpm) | SB (cpm) |
|------------------------|----------|-----------|----------|
| 0.97                   | 2129.67±11.2 | 153.5±5.3 | 1976.7±7.7 |
| 0.86                   | 2056.6±37.1  | 149.67±3.53 | 1907±33.65 |
| 0.63                   | 1836±107.3   | 141.33±3.48 | 1695±110.6 |
| 0.4                    | 1358±33±6.39 | 118.33±9.83 | 1240±3.93   |
| 0.28                   | 776.3±61.9    | 113.3±8.46  | 663±69.6   |
| 0.17                   | 429±67.1      | 101.67±5.5  | 327.36±4.9 |
| 0.05                   | 240±16.09     | 86.67±5.79  | 153.3±21.4 |

The values shown are the Mean ± SEM of three independent determinations.
Table 4. The results of competition experiment of 2-phenyl-5-oxo-7-methyl-1,3-4-oxadiazolo[a,2,3]-pyrimidine in the presence of increasing concentration of [3H]Flumazenil.

| log[L*] | %Specific binding |
|---------|-------------------|
| -11.0   | 66 ± 1            |
| -10.0   | 60 ± 1.5          |
| -9.5    | 56 ± 1.7          |
| -9.0    | 53 ± 1.5          |
| -8.5    | 46 ± 2.5          |
| -8.0    | 31 ± 3.7          |
| -7.0    | 25 ± 0.5          |

The values shown are the Mean±SEM of three independent determinations.

Acknowledgment

This study was supported by fund from Iranian National Science Foundation (INSF) and School of Pharmacy, Shahid Beheshti University of Medical Sciences.

References

(1) Bylund DB and Toews ML. Radioligand binding methods: practical guide and tips. American J. Physiol. (1993) 265: 421-429.
(2) Marangos PJ and Martino AM. Studies on the relationship of GABA stimulated diazepam binding and the GABA receptor. Molec. Pharmacol. (1981) 20: 16-21.
(3) Zarghi A, Faizi M, Shafaghi B, Ahadian A, Khojastepoor HR, Zanganer V, Tabatabai SA and Shafiee A. Design and synthesis of new 2-substituted-5-(2-benzylthiophenyl)-1,3,4-oxadiazoles as benzodiazepine receptor agonists. Bioorg. Med. Chem. Lett. (2005) 15: 3126-3129.
(4) Nilsson J, Nielsen EQ, Liljefors T, Nielsen M and Sterner O. 3-Alkyl- and 3-amido-isothiazoloquinolin-4-ones as ligands for the benzodiazepine site of GABAx receptors. Bioorg. Chem. (2012) 40: 125-130.
(5) Akbarzadeh T, Tabatabai SA, Khoshnoud MJ, Shafaghi B and Shafiee A. Design and Synthesis of 4H-3-(2-Phenoxy) phenyl-1,2,4-triazole Derivatives as Benzodiazepine Receptor Agonists. Bioorg. Medicin. Chem. (2003) 11: 769-773.
(6) Lager E, Nilsson J, Nielsen EQ, Nielsen M, Liljefors T and Sterne O. Affinity of 3-acetyl substituted quinolones at the benzodiazepine site of GABA receptors. Bioorg. Medicin. Chem. (2008) 11: 6936-6948.
(7) Carling RW, Moore KW, Street LJ, Wild D, Isted C, Leeson PD, Thomas Steven, Desmond O’Connor, McKernan Ruth M, Quirk Katherine, MCook Susan, Attack John R, Wafford Keith A, Thompson Sally A, Dawson Gerard R, Ferris Pushpinder and Castro Josel L. 3-Phenyl-6-(2-pyridyl) methoxy-1,2,3-triazolo[3,4-a]phthalazines and Analogues: High-Affinity t-Aminobutyric Acid-A Benzodiazepine Receptor Ligands with r2, r3, and r5-Subtype Binding Selectivity over r1. J. Med. Chem. (2004) 47: 1807-1822.
(8) Faizi M, SheikhhM, Ahangar A, Tabatabaie Ghomi H, Shafaghi B, Shafiee A and Tabatabai SA. Design, Synthesis and Pharmacological Evaluation of Novel 2-[2-(2-Chlorophenox) phenyl]-1,3-4-oxadiazole Derivatives as Benzodiazepine Receptor Agonists. Iran. J. Pharm. Res. (2012) 11: 83.
(9) Bigott-Hennkens HM, Dannoon S, Lewis MR and Jurisson SS. In-vitro receptor binding assays: general methods and considerations. The quarterly journal of nuclear medicine and molecular imaging (2008) 52: 245-253.
(10) Guerrini G, Ciciani G, Cambi G, Bruni F, Selleri S, Melani F, Montali M, Martini C, Ghelardini C, Norcini M and Costanzo A. Novel 3-arylopyrazolo [5,1-c] [1,2,4]benzotriazine 5-oxides 8-substituted, ligands at GABA, /benzodiazepine receptor complex: synthesis, pharmacological and molecular modeling studies. Bioorg. Medicin. Chem. (2008) 16: 4471-4489.
(11) Kahnberg P, Lager E, Rosenberg C, Schougaard J, Cane L, Nielsen M and Liljefors T. Refinement and evaluation of a pharmacophore model for flavones derivatives binding to the benzodiazepine site of the GABA Receptor. J. Med. Chem. (2002) 45: 4188-4210.
(12) Marini C, Lucacchini A, Ronca G, Hrelia S and Rossi CA. Isolation of putative benzodiazepine receptors from rat brain membrane by affinity chromatography. J. neurochem. (1982) 38: 15-19.
(13) Bradford M. M. A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein dye binding. Analytical. Biochem. (1976) 72: 248-254.
(14) Qume M. Overview of ligand receptor binding techniques. In: Methods in molecular biology. Receptor binding techniques. Keen M. (ed.) Humana Press Inc., Totowa, NJ (1995) 106: 3-23.
(15) Keen M. The problems and pitfalls of radioligand binding. In: Methods in molecular biology. Signal transduction protocols. Kendall DA and Hill SJ. (eds.) Humana Press Inc., Totowa, NJ (1995) 41: 1-16.
(16) McKinney M and Raddatz R. Practical aspects of radioligand binding. In: Enna Sj, Williams M, Frechette R, Kenakin T, McGonigle P, Ruggeri B and Wickenden AD (eds.) Current protocols in Pharmacology. Buffalo, New York (2006) 33: 1.3.1-1.3.42.
(17) Gehlen H, Simon B. Zur Kenntnis der 2-Amino-1,3,4-oxadiazole 35. Mitt.: Bildung und Reaktionen von 1,3,4-Oxadiazolo-[3,2-a]-pyrimidinen. Archiv der Pharmazie. (1970) 303: 501-510.
(18) Motulsky H. The GraphPad guide to analysing radioligand binding data. San Diego, California
Ahmadi F, Faizi M, Tabatabai SA, Beiki D and Shahhosseini S. Comparison \[^3\mathrm{H}\]-flumazenil binding parameters in rat cortical membrane using different separation methods, filtration and centrifugation. Nuclear Medicine and Biology (2013) 40: 896-900.

This article is available online at http://www.ijpr.ir