



Comparison [³H]-flumazenil binding parameters in rat cortical membrane using different separation methods, filtration and centrifugation

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ABSTRACT

Radioligand receptor binding assays are a common method to evaluate the affinity of newly synthesized benzodiazepine ligands for the receptor. [³H]-flumazenil is an antagonist of benzodiazepine receptors and is generally used as a radioligand. In this study, the binding parameters of [³H]-flumazenil to rat cortical membranes were evaluated using two separation methods: filtration with GF/C filters and centrifugation. Additionally, the effects of vacuum pressure, exposure time to the cocktail, and geometry on the filtration method were studied. The binding parameters of [³H]-flumazenil (K_d and B_{max}) were determined through saturation studies using two methods. The results from this study showed that the filtration method is time consuming and requires more steps to be completed. Because filtration causes partial elution of bound [³H]-flumazenil into the liquid scintillation cocktail, the results are not reproducible, which result in inaccurate estimation of the binding parameters. The centrifugation method in contrast to filtration is straightforward and produces reproducible as well as reliable results, all of the steps are performed in a single polypropylene tube, which eliminates the loss of tissue and avoids other systematic errors associated with transfer and handling.

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1. Introduction

The pharmacological effects of benzodiazepines (BZD) such as anxiolytic, anticonvulsant, muscle relaxant, and sedative-hypnotic, make them the most important GABA_A receptor modulating drugs currently in clinical use. It is suggested that the specific pharmacological effects of BZDs may be mediated by binding to the BZD binding site of the central GABA-A receptor [1].

New BZD receptor ligands with more selective effects such as anti-anxiety, anti-seizer and fewer adverse drug reactions were synthesized in the last two decades [2–7]. To assess the affinity of novel ligands for the binding site, radioligand receptor binding assays are frequently used. These assays are widely utilized by investigators to quickly and inexpensively screen the affinity of ligands for the receptors *in vitro* [8]. There are various methods to separate bound from unbound ligands in these studies, and the advantages and disadvantages of the different separation methods have been previously described [9]. In most of the radioligand receptor binding assays for

BZD receptors, the filtration method has been employed to separate bound from unbound ligand [10–17]. We also used filtration with a glass fiber GF/C filter in the initial studies. However, our preliminary results were variable and depended on the various parameters of filtration. In addition, the amount of specifically bound [³H]-flumazenil was noticeably less than expected. Because of partial elution of bound [³H]-flumazenil into the cocktail, reliable results could not be obtained through the filtration method. Therefore, evaluation of centrifugation as a proper separation method for separating the bound from unbound ligand in radioligand receptor binding assays for BZDs was considered (Fig. 1). The reproducibility and reliability of the method also need to be evaluated.

In this work, the effects of vacuum pressure, exposure time, and geometry on the filtration method to separate bound radioligand from unbound were also studied. The binding parameters of [³H]-flumazenil were determined from our saturation studies using the filtration and centrifugation methods. In this paper, we report the results of these studies using rat cortical membranes as the source of the BZD receptors and [³H]-flumazenil as the radioligand.

2. Materials and methods

2.1. Membrane preparation

Male Sprague–Dawley rats with weights of 300–350 g (Pasteur Institute, Tehran, Iran) were anesthetized with CO₂ and then sacrificed.

Abbreviations: TB, Total binding; NSB, Non-specific binding; SB, Specific binding.
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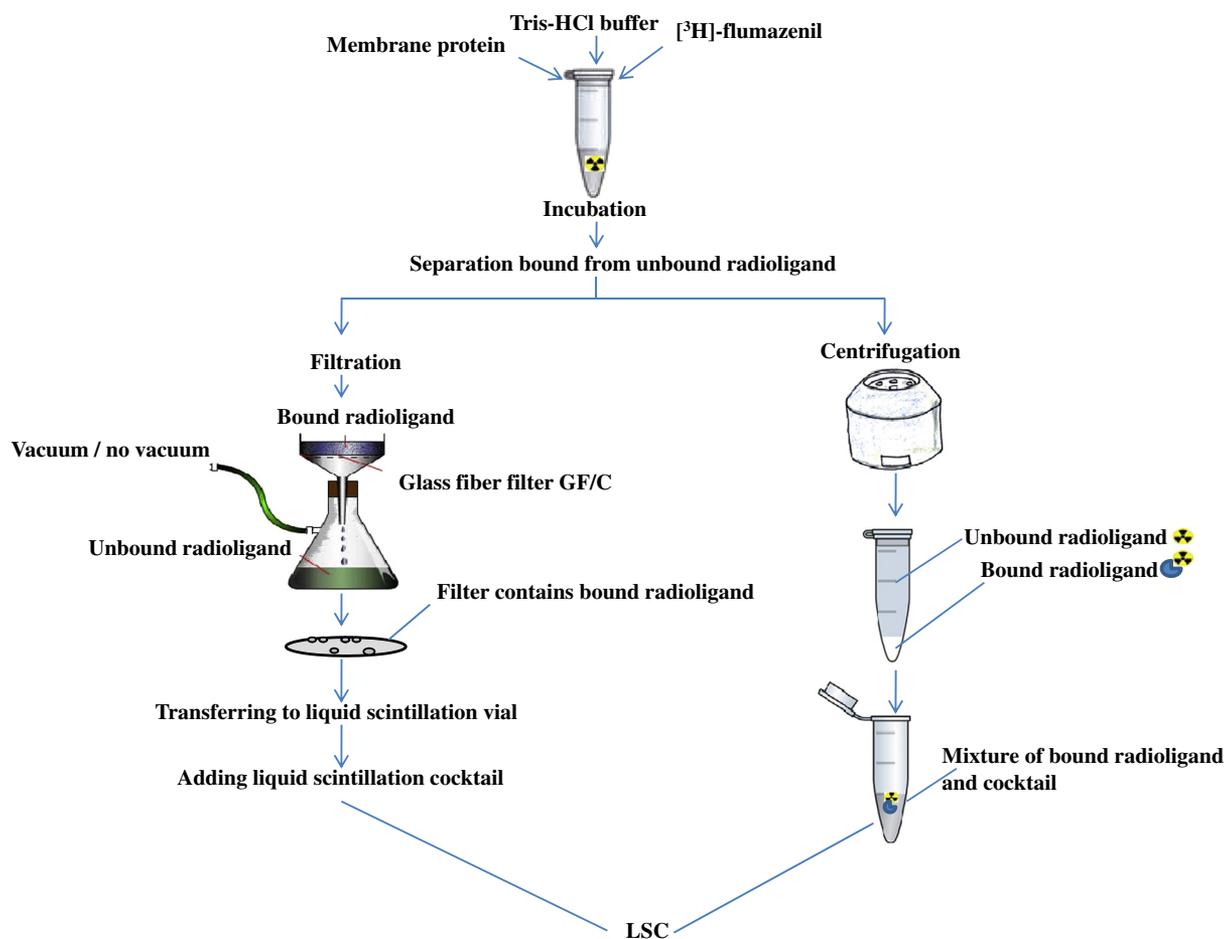


Fig. 1. Separation methods, filtration vs centrifugation.

The cortical membrane tissue was immediately 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 27,000 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 27,000 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 [16–18]. The amount of protein was estimated in the membrane preparation by the Bradford method (1976) using bovine serum albumin (BSA) as a standard [19]. The membrane preparation was stored at –20 °C until it was used 1–15 days later.

2.2. Filtration assay

The membrane protein (100 µg) in Tris-HCl buffer (50 mM, pH 7.4) was incubated with 8.6×10^{-5} nmol (7.482 nCi) [³H]-flumazenil (87 Ci/mmol, Perkin-Elmer, Life and Analytical Science, USA) at 30 °C for 35 min. After incubation, the contents of the tube were immediately filtered through glass fiber GF/C filters (Whatman, 25 mm circles, grade 1.2 µm), which had been presoaked in bovine serum albumin at 4 °C for 30 min. The filters were washed with 1.5 mL of ice-cold Tris-HCl buffer (50 mM, pH 7.4) and placed in scintillation vials (Hidex, Finland) covered with 4 mL of liquid scintillation cocktail (Maxilight, Hidex, Finland). The bound radioactivity was measured by liquid scintillation counting (Triathler multi-

label tester, Hidex, Finland). All of the procedures in the filtration assay were performed at 0–4 °C. The effects of the vacuum, the exposure time to the cocktail, and the geometry of the counting vials on the filtration method were studied. A peristaltic pump for making 8 bar vacuum pressure was used. Non-specific binding (NSB) was determined in parallel assays performed in the presence of 100 µM diazepam. All of the experiments were performed in triplicate. In all of the filtration samples, the filtrate was centrifuged, and the activity of the pellet and supernatant was measured.

2.3. Centrifugation assay

The membrane preparation and radioligand used in the centrifugation method were the same as in the filtration method. Triplicate reaction mixtures were prepared for each experiment. For the centrifugation assay, the samples were placed in 1.5 mL micro-centrifugation tubes. After a 35 min incubation at 30 °C, the tubes were centrifuged at 1500 g for 4 min at 4 °C using a Tomy MX-305 refrigerated centrifuge (Tomy, Japan). The supernatant was gently aspirated from the pellet. The pellet was washed with ice-cold Tris-HCl buffer, transferred to liquid scintillation vials, covered with 1 mL of liquid scintillation cocktail (Maxilight, Hidex, Finland) and the activity was measured by liquid scintillation counting. The NSB was determined in parallel assays performed in the presence of 100 µM diazepam.

2.4. Saturation studies

For the saturation binding studies of [³H]-flumazenil, seven different concentrations of [³H]-flumazenil (ranging from 0.05 nM to

Table 1
The effect of vacuum pressure during filtration on binding of [³H]-flumazenil in rat cortical membranes.

Method of analysis		TB (cpm)	NSB (cpm)	SB (cpm)
Vacuum filtration	Count of filter	808 ± 43.5***	136.67 ± 5.13**	671.33 ± 41.1***
	Count of pellet and filter	1194 ± 13.86	Not-applicable	Not-applicable
Simple filtration	Count of filter	1385 ± 24.63	216.67 ± 8.5	1168 ± 16.56

The values shown are the mean ± SEM of three independent determinations. The filter count in the vacuum method is significantly different compared to the corresponding filter count using simple filtration. (***P* < 0.01, ****P* < 0.001; Student's *t*-test).

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 [20]. The results of the filtration and centrifugation methods for separation of the bound radioligand from unbound were compared.

2.5. Data analysis

The saturation curves were generated by plotting the specific binding (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. In the centrifugation and filtration assays, NSB included [³H]-flumazenil physically entrapped in the pellet or filter, as well as what was bound to the membranes [20].

3. Results

3.1. Filtration

3.1.1. The effect of vacuum pressure on specific [³H]-flumazenil binding

The filtration method was performed either under vacuum pressure or with no vacuum. The results showed that the amount of SB was relatively higher with simple filtration (no vacuum) than with vacuum filtration. However, this decreased considerably under vacuum pressure. The effects of vacuum pressure on the counting process are shown in Table 1. The lack of vacuum pressure during filtration significantly increased the amount of measured SB. To determine the reason for this, the filtrate was centrifuged, and the activity of the pellet was measured. As shown in Table 1, the sum of the pellet activity and the filter is approximately equal to TB after simple filtration.

3.1.2. The effect of liquid scintillation exposure time and sample counting geometry on counting efficiency

To obtain the optimum conditions for the assays and to obtain proper counting efficiency, the effect of two parameters (exposure time and geometry) were evaluated. After separating the bound from unbound radioligand, the filters were transferred to liquid scintilla-

tion vials covered with a liquid scintillation cocktail and counted at different time points. The vials were counted immediately after adding the cocktail and after overnight exposure. The results of the counts immediately after adding the cocktail and after the overnight exposure are shown in Table 2. There is a significant improvement in the counts by increasing the scintillate exposure time.

To investigate the effect of geometry on the counting efficiency, further experiments were carried out using two sizes of liquid scintillation counting plastic vials, 10 mL (25 mm) and 4 mL (15 mm). The counting results showed that the amount of measured TB and NSB increased considerably when the filters were placed in 4 mL scintillation vials compared to the 10 mL vials (Table 2).

3.1.3. Centrifugation

To separate the bound from unbound radioligand, the centrifugation method was used. TB and NSB in the centrifugation procedure were significantly higher than the simple filtration when counting after an overnight exposure. Table 3 summarizes the results of the [³H]-flumazenil binding at 7 different concentrations in rat cortical membranes for each method. The total binding and non-specific binding were remarkably higher at all ligand concentrations when the centrifugation method was used. The saturation curves for the filtration and centrifugation methods are shown in Figs. 2 and 3, respectively.

3.1.4. Saturation studies

The radioligand equilibrium dissociation constant (*K_d*), receptor density (*B_{max}*), and correlation coefficient values from the non-linear regression derived from the centrifugation and filtration methods in the saturation studies are compared in Table 3.

4. Discussion

[³H]-flumazenil has been used as a radioligand by researchers in radioligand receptor binding studies of BZDs [21–24]. Filtration using glass fiber filters is the most commonly used method to study the binding properties of the radioligand [10–17]. We also used the filtration method to separate the bound from unbound radioligand in our initial studies. However, our results showed that the filtration method is not the proper method to determine the binding parameters of [³H]-flumazenil. In this study, we evaluated the impact of different factors on the filtration method and also compared the results of filtration with the centrifugation method.

In the first experiments, we studied the effects of vacuum pressure, exposure time to liquid scintillation, and geometry on the amount of bound radioligand using the filtration method. Our studies showed

Table 2
The effect of exposure time and geometry during filtration on binding of [³H]-flumazenil in rat cortical membranes.

	Exposure Time	Vial size	TB(cpm)	NSB(cpm)	SB(cpm)
Simple filtration	Overnight	10 mL	418.33 ± 15.82	79.67 ± 8.5	338.67 ± 18.14
	Overnight	4 mL	1385 ± 24.63***	216.67 ± 8.5***	1168 ± 16.56***
	Immediately	4 mL	417.80 ± 22.7###	79.20 ± 6.14###	338.60 ± 22.7###

*Results of overnight counts using the 4 mL vials are significantly different from 10 mL vials (****P* < 0.001 one-way ANOVA with Dunnett's post test).

#Results of overnight counts are significantly different from immediate filtration (###*P* < 0.001 one-way ANOVA with Dunnett's post test).

Table 3
Comparison of filtration and centrifugation methods of [³H]-flumazenil in rat cortical membranes.

³ H-flumazenil (nM)	Filtration method			Centrifugation method		
	TB (cpm)	NSB (cpm)	SB (cpm)	TB (cpm)	NSB (cpm)	SB (cpm)
0.97	423.3 ± 2.6	87.33 ± 2.9	336 ± 5.5	2129.67 ± 11.2***	153.53 ± 5.3***	1976.7 ± 7.7***
0.86	413.67 ± 13.6	79.67 ± 4.9	334 ± 15.1	2056.6 ± 37.1***	149.67 ± 3.53***	1907 ± 33.65***
0.63	390.33 ± 12.4	79.333 ± 3.8	311 ± 12.5	1836 ± 107.3***	141.33 ± 3.48***	1695 ± 110.6***
0.4	252.67 ± 3.2	61.33 ± 5.1	191.33 ± 5.2	1358.33 ± 6.39***	118.33 ± 9.83**	1240.3 ± 3.93***
0.28	231.6 ± 2.9	56 ± 2.0	175.6 ± 1.8	776.3 ± 61.9***	113.3 ± 8.46**	663 ± 69.6**
0.17	204 ± 4.3	63 ± 12.1	141 ± 13.0	429 ± 67.1*	101.67 ± 5.5*	327.36 ± 4.9***
0.05	165 ± 4.9	58.33 ± 10.05	107 ± 11.8	240 ± 16.09*	86.67 ± 5.79	153.33 ± 21.4

All values for total binding (TB), non-specific binding (NSB), and specific binding (SB) represent the mean ± SEM of three independent determinations. Centrifugation method values marked with (*) are significantly different from corresponding filtration method values (**P* < 0.05, ***P* < 0.01, ****P* < 0.001; Student's *t*-test).

that the amount of measured TB and NSB is significantly decreased when a vacuum is employed compared to simple filtration. It seems that some parts of the radioligand-bound receptors pass through the filter when the vacuum is applied. This was demonstrated by centrifuging the filtrate and measuring the activity of the pellet (Table 1). The sum of the pellet and filter activity is approximately equal to TB with simple filtration. The effect of geometry on the counting efficiency was evaluated using two types of plastic liquid scintillation counting vials, which are recommended for low activity counting applications [25]. Because of the better counting geometry of the filter in the 4 mL scintillation plastic vials, the amount of TB and NSB is considerably increased compared to the 10 mL scintillation plastic vials. Another factor was the exposure time to the liquid scintillation fluid. The filters covered with the cocktail were counted immediately and at different time points (data not shown). The maximum counts were obtained with an overnight exposure. This means that the bound radioligand is partially eluted from the filter into the cocktail, and some of the bound radioligand, which is embedded within the filter, requires more time to become accessible to the liquid scintillation for photon generation and signal detection. When the filter is covered with the cocktail, the bound radioligand is partially eluted from the filter. The soluble fraction is counted with 4π geometry, while the filter-bound portion is counted with 2π geometry. The presence of both 2π and 4π geometry within the counting mixture provides inaccurate and irreproducible results [26]. To avoid a partial elution and to make a complete elution situation where the samples were completely dissolved or eluted into the cocktail, two attempts were performed. Prior to adding the cocktail, different solvents and reagents were used to extract or elute the bound radioligand from the filter. In another attempt, three different cocktails were used to elute the bound radioligand into the cocktail. However, there was no significant change in count rate. We also tried to make a zero elution

situation (where the sample remains bound to the filter), which was also not successful (data not shown).

The centrifugation method resulted in a significantly greater TB and SB with reproducible results at all of the radioligand concentrations tested. In the centrifugation method, the pellet was dissolved in liquid scintillation fluid and counted immediately. When the sample is dissolved in the liquid scintillation cocktail, it makes for homogeneous counting, in which the photons of scintillations are free to radiate in any direction (4π). In heterogeneous counting, the presence of the filter or the membrane restricts the photons of scintillation to radiate in any direction (2π). NSB was higher using the centrifugation method compared to the filtration method because the unbound radioligand can be trapped in the pellet. NSB was reduced by washing and a second centrifugation step. One explanation for the decreased count after filtration compared to centrifugation might be rapid dissociation of the radioligand from the receptors on the filter. The sum of the counts in the filtrate and filter is consistent with this assumption. The centrifugation method avoids loss of binding due to this dissociation. This has also been mentioned by Barrett R.W. when assaying for ³H-DADLE enkephalin binding to mouse brain membranes [27].

The saturation binding studies with [³H]-flumazenil were performed using seven different concentrations of [³H]-flumazenil. The amount of radioligand required to saturate the receptors was used to determine both the receptor binding affinity of [³H]-flumazenil (*K_d*) and the benzodiazepine receptor density (*B_{max}*), which were based on non-linear regression analysis of the saturation curve data [20,28,29]. The binding parameters of [³H]-flumazenil using two separation methods, namely centrifugation (1500 g, 4 °C, 5 min) and simple filtration with overnight counting, were compared (Table 4, Figs. 2–3). We found that there is a significant difference in the *B_{max}* values measured by filtration and centrifugation. Our results clari-

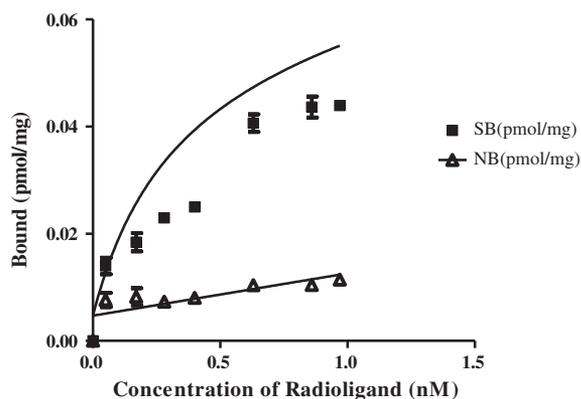


Fig. 2. Saturation curve of [³H]-flumazenil. The [³H]-flumazenil binding to rat cortical membranes using the filtration method to separate bound from free radioligand.

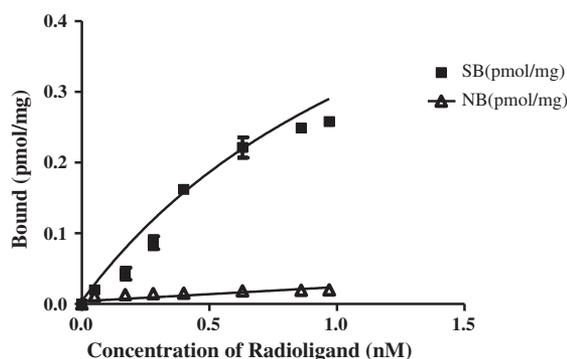


Fig. 3. Saturation curve of [³H]-flumazenil. The [³H]-flumazenil binding to rat cortical membranes using the centrifugation method to separate bound from free radioligand.

Table 4
Analysis of [³H]-flumazenil binding parameters on rat cortical membranes.

Parameter	Method of separation bound from free	
	Centrifugation	Filtration
K _d (nM)	1.35 ± 0.316**	0.32 ± 0.084
B _{max} (pmol/mg)	0.638 ± 0.099***	0.057 ± 0.006
Correlation coefficient	0.97	0.92

The values are shown as the mean ± SEM of three independent determinations. K_d and B_{max} values shown in centrifugation are significantly different from corresponding filtration method values (***P* < 0.01, ****P* < 0.001; Student's *t*-test).

find that the number of experimentally determined binding sites for [³H]-flumazenil is increased (90%) when centrifugation is used to assess binding.

In summary, this study showed that the filtration method is time consuming, requires several steps to be completed, is not reproducible, and results in inaccurate estimation of binding parameters. However, the centrifugation method is easy and produces reproducible, as well as reliable results. Another advantage of centrifugation is that all of the steps are performed in a single polypropylene tube, which eliminates loss of tissue and other errors associated with transfer and handling. Therefore, because of time spent, number of steps, reproducibility, and reliability, we recommend the centrifugation method for radioligand receptor binding studies of BZDs using [³H]-flumazenil as the radioligand and rat cortical membranes as tissue that contains BZD receptors.

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