Nuclear Medicine and Biology 40 (2013) 896–900

Contents lists available at ScienceDirect

Nuclear Medicine and Biology

journal homepage: www.elsevier.com/locate/nucmedbio

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

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ARTICLE INFO

Article history:
Received 21 March 2013
Received in revised form 11 June 2013
Accepted 20 June 2013

Keywords:
Radioligand receptor binding assay
[^{3}H]-flumazenil
Liquid scintillation
Filtration
Centrifugation

ABSTRACT

Radioligand receptor binding assays are a common method to evaluate the affinity of newly synthesized benzodiazepine ligands for the receptor. [^{3}H]-flumazenil is an antagonist of benzodiazepine receptors and is generally used as a radioligand. In this study, the binding parameters of [^{3}H]-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 [^{3}H]-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 [^{3}H]-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.

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 [^{3}H]-flumazenil was noticeably less than expected. Because of partial elution of bound [^{3}H]-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 [^{3}H]-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 [^{3}H]-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 CO_2 and then sacrificed.

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

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http://dx.doi.org/10.1016/j.nucmedbio.2013.06.010

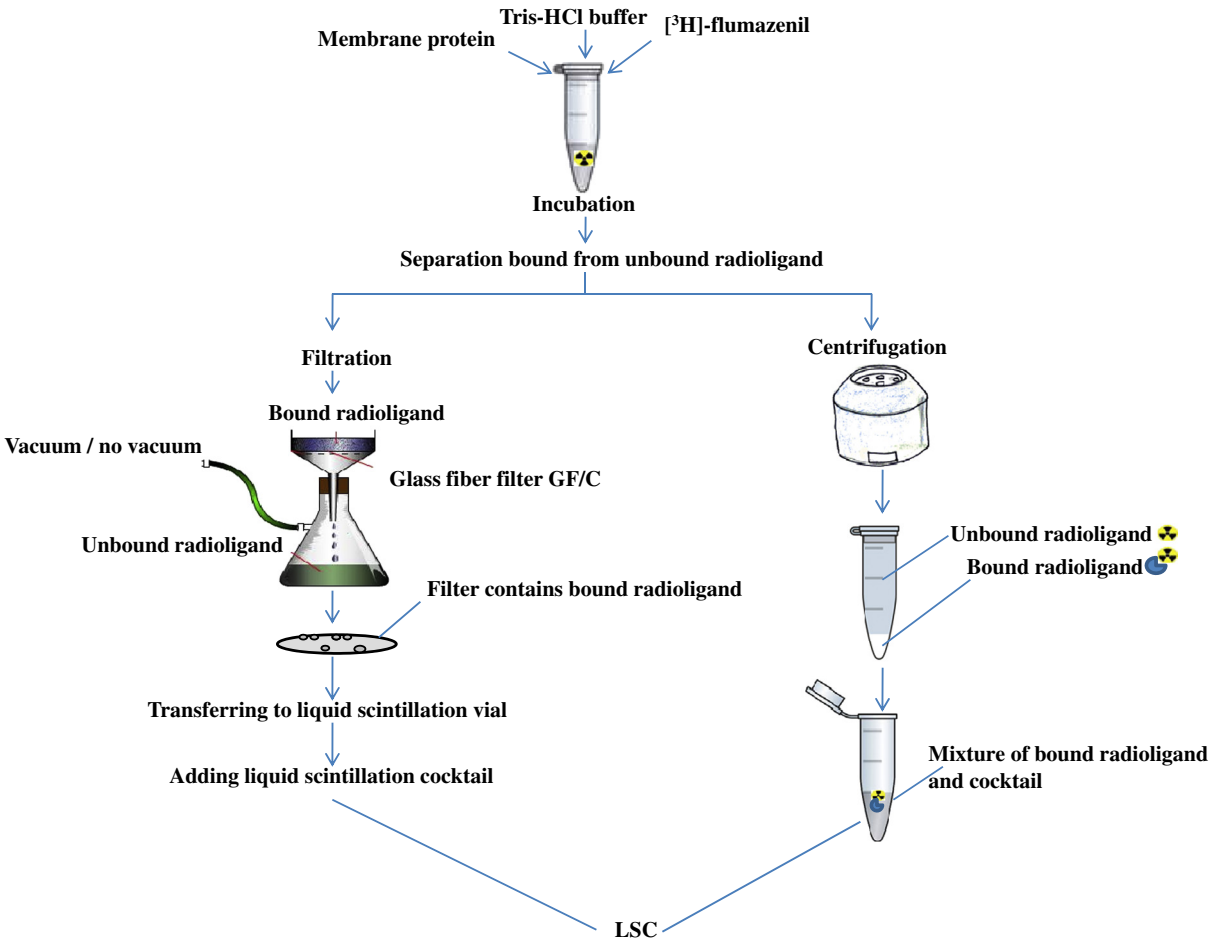

Fig. 1. Separation methods, filtration vs centrifugation.

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) [^{3}H]-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 [^{3}H]-flumazenil, seven different concentrations of [^{3}H]-flumazenil (ranging from 0.05 nM to