Chapter 5

Solubilized benzodiazepine receptors for use in receptor assays

5.1 Introduction

The receptor material frequently used in radioreceptor assays (RRA) is a crude homogenate of calf brain. Besides the membrane-bound receptor, this homogenate contains mitochondria, myelin, and other membrane fragments. For RRA, the purity of the receptor material has little influence, since the receptor material will not interfere with the counting of the radioactive label. However, in fluorescent receptor assays (FRA), the turbid solution of the membrane-bound receptors may cause a significant background signal, due to scattering and autofluorescence, which disturbs the determination of the non-radioactive label. Therefore, the crude benzodiazepine receptor homogenate has to be purified to minimize the background signal. A first step in purification is the solubilization of membrane proteins, including the benzodiazepine receptor.

Up till now solubilized receptors have been used primarily for pharmacodynamic studies of the benzodiazepine and the GABA receptor. In our experiments, the benzodiazepine receptor was solubilized with sodium deoxycholate, since the latter solubilizes the maximum number of GABA- and benzodiazepine binding sites from brain membrane preparations [1]. After the solubilization, the binding characteristics of the solubilized receptor were compared to those of the membrane-bound receptor by saturation experiments and by inhibition experiments of the benzodiazepine antagonist flumazenil and the agonist lorazepam. These experiments were done with [3H]flunitrazepam in order to be able to determine the binding properties of the membrane-bound receptors which could not be achieved with fluorescent ligands.

In RRA performed with membrane-bound receptors, the bound and free labeled ligand were separated by filtration through GF/B glass fibre filters. Solubilized receptors, however, will pass these filters, so an alternative separation method is required. Three separation methods, precipitation of the receptor with polyethylene glycol followed by filtration, filtration through ion exchange filters and charcoal adsorption, were compared to select the method which gives the highest yield for the bound fraction and the best reproducibility.

Slightly modified version of:
5.2 Materials and Methods

Chemicals

\([N\text{-}methyl^3\text{H}]\text{flunitrazepam (82.0 Ci/mmol}) was obtained from DuPont NEN (Wilmington, DE, USA). Lorazepam was a gift from Wyeth Laboratoria (Hoofddorp, The Netherlands) and flumazenil was a gift from Roche Nederland (Mijdrecht, The Netherlands). Sodium deoxycholate (>95%), bovine serum albumin (Fraction V, BSA), bovine globulins (Cohn Fraction II, III) and the protease inhibitors were supplied by Sigma Chemical Co. (St. Louis, MO, USA). Polyethylene glycol (PEG) 6000 was obtained from Genfarma (Maarssen, The Netherlands), and charcoal (Carbo activus, Ph.Eur) was obtained from OPG Farma (Utrecht, The Netherlands). All other chemicals were of analytical grade and were purchased from Merck (Darmstadt, Germany).

The GF/B glass fibre filters and the ion exchange filters (DE81) were obtained from Whatman (Maidstone, UK). Rialuma, used as scintillation cocktail, was obtained from Lumac (Olen, Belgium).

Demineralized water was further purified by an Elgastat Maxima instrument (Elga, High Wycombe, UK) before use in the buffers.

Preparation of membrane-bound receptors

We modified the method for the preparation of membrane-bound receptors, described by Möhler and Okada [2]. Calf brains, obtained from the slaughterhouse and stored at -80°C after discarding the cerebella, were homogenized in 6 volumes (w/v) of ice-cold 0.32 M sucrose in a Potter-Elvehjem homogenizer (RW 20 DZW, Janke & Kunkel KG, Staufen i.Br., Germany) fitted with a teflon pestle and centrifuged at 1,000 \( \times \) g for 10 min in a Beckman L8-55 Ultracentrifuge (Beckman Instruments, Mijdrecht, The Netherlands). The supernatant was centrifuged at 100,000 \( \times \) g for 60 min. The resulting pellet (P\(_2\)) was resuspended in sodium phosphate buffer (pH 7.4; 50 mM) and centrifuged at 100,000 \( \times \) g for 30 min. This washing step was repeated once. All operations were performed at 4°C. The washed P\(_2\)-pellet was resuspended in 5 volumes (w/v) phosphate buffer, frozen with liquid nitrogen and lyophilized (Hetrosicc CD 52-1, Heto, Birkerød, Denmark). The lyophilized P\(_2\)-pellet was stored at -20°C.

Preparation of solubilized receptors

The procedure to solubilize the benzodiazepine receptors was a compromise between several protocols [3-5]. The lyophilized P\(_2\)-pellet was resuspended with a glass/Teflon homogenizer in Tris-HCl buffer (pH 7.4; 50 mM), containing 150 mM KCl and the protease inhibitors EDTA (1 mM), benzamidine HCl (1 mM), bacitracin (200 µg/ml) and fresh phenylmethylsulfonyl fluoride (0.3 mM). The concentration of the P\(_2\)-pellet was 8 mg/ml. A 5% (w/v) solution of sodium deoxycholate in water was added dropwise to the magnetically stirred suspension till a
final concentration of 0.5% (w/v). The suspension was stirred for 30 min at 4°C and the solubilized receptors were recovered by centrifugating at 15,000 x g for 15 min in a Heraeus Biofuge A microcentrifuge (Heraeus-Sepatech GmbH, Osterode am Harz, Germany) and collecting the supernatant. The solubilized receptors were directly used in the binding assays.

**Membrane-bound receptor binding assays**

For the saturation experiments 20 µl [³H]flunitrazepam solution (0.2 - 20 nM final concentration) in Tris-HCl buffer (pH 7.4; 50 mM) were mixed in duplicate with either 20 µl Tris-HCl buffer for the maximal binding or 20 µl lorazepam solution (10 µM final concentration) for the non-specific binding in polyethylene tubes. 160 µl receptor suspension (4 mg/ml P₂-pellet, this corresponds with 150 µg protein/assay) were added, mixed on a Vortex mixer and the mixture was incubated at 4°C for 45 min. The incubation was terminated by adding 4 ml ice-cold buffer and the mixture was filtered through pre-wetted GF/B filters. The tubes were rinsed twice with 4 ml ice-cold buffer, which was also filtered. The filters were transferred into 6 ml polyethylene tubes and dispersed in 3.5 ml Rialuma. The vials were shaken for 2 hours and counted for 5 min in a Tri-Carb 4000 Packard scintillation counter (Canberra Packard, Groningen, The Netherlands).

For the inhibition experiments 20 µl [³H]flunitrazepam solution (4 nM final concentration) were mixed in duplicate with either 20 µl Tris-buffer, containing the benzodiazepine antagonist flumazenil (300 nM - 10 pM final concentration), or the agonist lorazepam (300 nM - 30 pM final concentration). The further procedure was the same as for the saturation experiments. The saturation experiments and the inhibition experiments were performed in duplicate.

**Solubilized receptor binding assays**

The binding assays for the solubilized receptors were performed as described for membrane receptors, but now with the amount of protein being 250 µg/assay. Flumazenil and lorazepam solutions were made in Tris-HCl buffer, containing 0.5% sodium deoxycholate and the protease inhibitors. However, the separation of bound and free [³H]flunitrazepam was executed differently, since the solubilized receptors will pass the glass fibre filters.

The incubation was terminated by adding 100 µl Tris-HCl buffer, containing 0.5% (w/v) γ-globulin and 30% (w/v) PEG 6000 to precipitate the solubilized receptors, followed by incubation at 4°C for 13 min [5]. Three ml ice-cold Tris-HCl buffer, containing 7.5% PEG, was added and the mixture was filtered through pre-wetted GF/B filters. The tubes were rinsed twice with 3 ml ice-cold buffer, which was also filtered, and the filters were dispersed in 3.5 ml Rialuma and counted as above.
Fluorescence spectra of the receptor materials
The fluorescence background of the two receptor materials was recorded by registration of the emission spectra at several excitation wavelengths with a Kontron SFM 25 spectrofluorometer (Kontron Instruments, Basle, Switzerland). The membrane-bound receptors were diluted to a protein concentration of 60 µg/ml, the solubilized receptors had a protein concentration of 360 µg/ml.

Comparison of the separation techniques for bound and free labeled ligand
For the comparison of the three separation methods, the [³H]flunitrazepam, and the lorazepam for the non-specific binding, were added to the bulk receptor preparation. This is done to minimize the variation due to pipetting.

For each separation method 280 µl [³H]flunitrazepam in Tris-HCl buffer (pH 7.4; 50 mM) (4 nM final concentration) were mixed with either 280 µl lorazepam in Tris-buffer with inhibitors (10 µM final concentration), or 280 µl buffer for the non-specific binding or maximal binding respectively. To this mixture 2.24 ml solubilized receptor were added with a final concentration of 1 mg/ml protein. From these mixtures twelve 200 µl aliquots were pipetted into 12 ml polyethylene tubes (for the filtration methods) or into 1.5 ml Eppendorf vials (for the charcoal adsorption method). After incubation for 45 min at 4°C, the bound and free fractions were separated according to the following three methods.

Polyethylene glycol precipitation/filtration: This method was performed as described under "Solubilized receptor binding assays".

Ion exchange filtration: The incubation was ended by adding 4 ml ice-cold Tris-HCl buffer (pH 7.4; 50 mM), and this mixture was applied to pre-wetted Whatman DE81 filters [6]. The tubes were rinsed twice with 4 ml ice-cold buffer which was also filtered and the filters were transferred into 6 ml polyethylene counting vials and dispersed in 3.5 ml Rialuma. The vials were shaken for 2 hours and counted for 5 min in a Tri-Carb 4000 Packard scintillation counter.

Charcoal adsorption: The incubation was terminated by the addition of 200 µl of an ice-cold charcoal solution (10% w/v charcoal, 2% w/v BSA) in Tris-HCl buffer (pH 7.4; 50 mM) [7]. After mixing and centrifugation for 5 min at 15.000 x g, 250 µl aliquots of the supernatant were pipetted in scintillation counting vials and the radioactivity was measured after mixing with 3.5 ml Rialuma.

The variance of the filtration method for membrane-bound receptors was also determined by performing the total binding and the non-specific binding in twelvefold. The pipetting scheme was the same as for the solubilized receptors, only 2.24 ml membrane-bound receptors (4 mg/ml P₂-pellet) were used instead of the solubilized receptors.
Protein determination
The amount of protein used in the saturation experiments was assayed by a modified version of the method developed by Lowry [8], using bovine serum albumin as the standard. Before assaying, the protein was precipitated with trichloroacetic acid, to avoid interference of the Tris-HCl buffer and formation of precipitates caused by the detergent [9]. The precipitates, both from membrane-bound receptors as well as from solubilized receptors, were directly dissolved in the so called Lowry reagent "C".

5.3 Results and discussion

Solubilization of the benzodiazepine receptor
The saturation and inhibition curves were fitted with the program EBDA-Ligand, V4 (Biosoft, Cambridge, UK) [10] using the one-binding site model. The results are presented in Table 5.1. Solubilization with 0.5% sodium deoxycholate extracted about 80-85% of the protein amount and about 50-55% of the benzodiazepine binding sites ($B_{\text{max}}$) present in the membrane-bound receptor preparation. This corresponds with the results from Sigel and Barnard [4], who found a recovery of receptor binding sites of about 55%. Figure 5.1 shows representative saturation curves for the membrane-bound and solubilized benzodiazepine receptors.

![Saturation curves of membrane-bound receptors (●) and solubilized receptors (○). The closed symbols represent the specific binding and the open symbols the non-specific binding.](image)

Figure 5.1  Saturation curves of membrane-bound receptors (●) and solubilized receptors (○). The closed symbols represent the specific binding and the open symbols the non-specific binding.
The binding affinity of \[^3\text{H}\text{]flunitrazepam}\} for the solubilized receptor (K\text{d}) decreased from 1.20 ± 0.11 nM to 4.1 ± 0.7 nM. The \(B_{\text{max}}\) decreased from 1.01 ± 0.01 pM/mg protein to 0.54 ± 0.13 pM/mg protein after solubilization. The changes in K\text{d} and \(B_{\text{max}}\) were significant when compared with Student's \(t\)-test (\(p<0.05\)). The non-specific binding increased after solubilization.

The inhibition experiments were performed with a \[^3\text{H}\text{]flunitrazepam}\} concentration which was equal to the K\text{d} of \[^3\text{H}\text{]flunitrazepam}\} for the solubilized receptor. Representative inhibition curves for the benzodiazepine antagonist flumazenil and the agonist lorazepam are shown in Figures 5.2A and 5.2B respectively. For both benzodiazepines, the use of solubilized receptors caused a shift in the inhibition curves to the right, as can be seen from the IC\text{50}-values in Table 5.1. From the inhibition curves the inhibition constants (K\text{i}) were calculated (Table 5.1).

**Table 5.1** Comparison of binding properties of membrane-bound and solubilized benzodiazepine receptors (n=2).

<table>
<thead>
<tr>
<th></th>
<th>membrane-bound receptors (±SD)</th>
<th>solubilized receptors (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K\text{d} ([(^3\text{H}\text{]flunitrazepam}})</td>
<td>1.20 (± 0.11)</td>
<td>4.1 (± 0.7)</td>
</tr>
<tr>
<td>(B_{\text{max}}) (pM/mg protein)</td>
<td>1.01 (± 0.01)</td>
<td>0.54 (± 0.13)</td>
</tr>
<tr>
<td>flumazenil: IC\text{50} (nM)</td>
<td>2.7 (± 0.4)</td>
<td>6.3 (± 0.4)</td>
</tr>
<tr>
<td>: K\text{i} (nM)</td>
<td>0.67 (± 0.10)</td>
<td>3.2 (± 0.3)</td>
</tr>
<tr>
<td>: detection limit (nM)</td>
<td>0.46 (± 0.14)</td>
<td>0.72 (± 0.01)</td>
</tr>
<tr>
<td>lorazepam: IC\text{50} (nM)</td>
<td>5.51 (± 0.13)</td>
<td>15.3 (± 0.2)</td>
</tr>
<tr>
<td>: K\text{i} (nM)</td>
<td>1.49 (± 0.13)</td>
<td>8.4 (± 1.0)</td>
</tr>
<tr>
<td>: detection limit (nM)</td>
<td>0.98 (± 0.05)</td>
<td>1.5 (± 0.4)</td>
</tr>
</tbody>
</table>
A significant difference ($p<0.05$) in the affinity for the two receptor preparations was observed for both flumazenil and lorazepam. This means that solubilization of the benzodiazepine receptor causes a loss of affinity of about a factor 5 for both benzodiazepines.

**Fluorescence spectra of the receptor materials**

In Figure 5.3 fluorescence spectra of the membrane-bound and solubilized receptors are shown at 2 different excitation wavelengths for some common fluorescent labels ($\lambda_{ex}$ 350 nm corresponds to several coumarins and $\lambda_{ex}$ 500 nm corresponds to fluorescein). The membrane-bound receptors gave a high fluorescence signal, which would prevent the detection of low label concentrations. As can be seen from the curves labeled "II", the solubilized receptors produced a much lower background signal, so that the latter appear to be a promising receptor material for developing fluorescent receptor assays, despite the observed loss in affinity as compared to the membrane-bound material.

**Comparison of the different separation methods for bound and free labeled ligand**

The principles of the three methods used are as followed: In the PEG precipitation/filtration method, the PEG precipitates the solubilized receptor. The $\gamma$-globulin is a carrier for the precipitation reaction, since the $\gamma$-globulin is being precipitated by PEG. The anionic filtration is based on the presence of negative charges of the benzodiazepine receptor, which is an acidic glycoprotein with a pI of 5.6 [11]. The filters bear positive charges, so the receptor complex may be retained on the filter by ionic forces during filtration [12].
Table 5.2

Comparison of the three different separation techniques for bound and free labeled ligand (n=12).
The receptor-bound amount $[^3H]$flunitrazepam is represented as percentage of the amount $[^3H]$flunitrazepam added. The results in the brackets are correlated to the polyethylene glycol precipitation/filtration method (= 100%).

<table>
<thead>
<tr>
<th></th>
<th>Polyethylene glycol precipitation/filtration (±SD)</th>
<th>Ion exchange filtration (±SD)</th>
<th>Charcoal adsorption (±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total binding</td>
<td>12.5 (± 0.31)</td>
<td>2.8 (± 0.27)</td>
<td>10.0 (± 0.35)</td>
</tr>
<tr>
<td></td>
<td>(100%)</td>
<td>(22.5%)</td>
<td>(79.6%)</td>
</tr>
<tr>
<td>Non-specific binding</td>
<td>0.83 (± 0.13)</td>
<td>0.52 (± 0.05)</td>
<td>0.91 (± 0.05)</td>
</tr>
<tr>
<td></td>
<td>(100%)</td>
<td>(63.3%)</td>
<td>(110%)</td>
</tr>
</tbody>
</table>

Charcoal adsorbs small organic molecules, such as the free $[^3H]$flunitrazepam, which then can be removed by centrifugation. The supernatant contains $[^3H]$flunitrazepam bound to the receptor.

The results of the three separation methods are presented in Table 5.2. The binding is expressed as percentage of the total amount of $[^3H]$flunitrazepam added, and compared with the value obtained with the PEG precipitation/filtration method. The results were evaluated with one-way ANOVA.

Both the total binding results as well as the non-specific binding results of the three methods differ significantly from each other ($p<0.05$). The PEG precipitation/filtration method gives the highest collected bound fraction for the total binding. The lower results for the charcoal adsorption method indicate that besides the free $[^3H]$flunitrazepam also a part of the receptor material is adsorbed to the charcoal, although the charcoal was presaturated with albumin. For the non-specific binding the charcoal adsorption method gives the highest binding. It seems that the charcoal does not adsorb the free $[^3H]$flunitrazepam properly.

The recovery of the filtration with the ionic exchange filters is only 22.5% of the PEG precipitation/filtration method. Apparently, the ionic forces between the receptors and the filters are not strong enough to retain the receptor material on the filters. An other explanation of the poor binding of the ionic exchange filters maybe that sodium deoxycholate molecules contain a negative charge. Therefore, the sodium deoxycholate molecules may also bind to the filters, thus reducing the capacity of the filters for benzodiazepine receptor binding.

There was no significant difference between the variance of the three methods, neither for the total binding, nor for the non-specific binding. Wang et al. [13], however, described that the reproducibility of the charcoal method is poor, when compared to the filtration method.

From these results, the conclusion can be made that the PEG precipitation/filtration method is the best separation technique for solubilized receptors, since it has the highest yield of bound
fraction and a lower non-specific binding than the charcoal method. Moreover, the PEG precipitation/filtration method is the easiest method to perform, especially with large numbers of samples.

**Calculation of the detection limit**

The detection limit was calculated by subtracting three times the variation coefficient of the maximal binding of a standard curve and to calculate the intersection of this value with the standard curve. The variation coefficients of the filtration method for membrane-bound receptors was 4.1% for the total binding and 10.3% for the non-specific binding. For the PEG precipitation/filtration method for solubilized receptors, the variation coefficient was 2.5% for the total binding and 15.5% for the non-specific binding. The detection limits were then calculated at 87.7% of the maximal binding for the membrane-bound receptors and at 92.5% of the maximal binding for the solubilized receptors. This resulted for flumazenil in detection limits of 0.46 nM and 0.72 nM for the membrane-bound and the solubilized benzodiazepine receptors respectively. For lorazepam the detection limits were 0.98 nM and 1.5 nM for the membrane-bound and the solubilized benzodiazepine receptors respectively. When compared with Student's t-test, these detection limits were not significantly different (flumazenil: \( p = 0.13 \); lorazepam: \( p = 0.20 \)) for the two receptor materials.

For the IC\(_{50}\)-values, however, there was a significant difference for the two receptor preparations. This was caused by the fact that the variation coefficients for the separation of the bound and free fractions were taken into account for the determination of the detection limit but not for the determination of the IC\(_{50}\).

The higher variation coefficient in the filtration method for membrane-bound receptors was somewhat surprising because the filtration method for solubilized receptors contains one extra step, the precipitation of the solubilized receptors. Possibly the solubilized receptors give a coarser precipitate than the membrane-bound receptors which may lead to small, yet variable losses of membrane-bound receptors through the glass fibre filters.

### 5.4 Conclusions

Solubilization of the benzodiazepine receptor appears to be a suitable alternative to circumvent background fluorescence which is rather prominent with membrane-bound receptors. Hence, solubilized receptors are to be preferred in the development of fluorescent receptor assays. On the other hand, solubilization of the benzodiazepine receptor caused a reduction in affinity of \( ^{3} \text{H} \)flunitrazepam, flumazenil and lorazepam for the receptor. However, the detection limits of the benzodiazepine antagonist flumazenil and the agonist lorazepam were not significantly affected by the solubilization.
Polyethylene/glycol precipitation followed by glass fibre filtration appeared to be the best method to separate free and bound ligand when working with solubilized receptors.

References