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Solubilized benzodiazepine receptors for use in receptor assays[1]

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Abstract

In the development of non-radioactive receptor assays for benzodiazepines, employing fluorescent ligands, it was observed that the fluorescence measurements were hampered by the background fluorescence of the receptor preparation. This receptor preparation is a brain tissue homogenate in which the benzodiazepine receptors are membrane-bound. To minimize the influence of the receptor material on the fluorescence detection, the benzodiazepine receptors were solubilized with 0.5% sodium deoxycholate. The binding characteristics of the receptors were examined after solubilization and compared with membrane-bound receptors. The K_d and B_{max} values for membrane-bound receptors were 1.20 nM and 1.01 pM mg^{-1} protein and for solubilized receptors they were 4.1 nM and 0.54 pM mg^{-1} protein respectively. Inhibition curves with the benzodiazepine antagonist flumazenil and the agonist lorazepam revealed that their affinities for the solubilized receptor as compared to the membrane-bound receptor were also reduced from 0.67 nM to 3.2 nM and from 1.49 nM to 8.4 nM respectively. The detection limits for the two benzodiazepines, however, were not affected by the solubilization. Furthermore, three different methods to separate the fraction of free labelled ligand and the fraction bound to the solubilized receptor were compared, namely polyethylene glycol precipitation/filtration, ion exchange filtration and charcoal adsorption. Polyethylene glycol precipitation/filtration gave the highest yield for the bound fraction and the best reproducibility.

Keywords: Receptor assay; Benzodiazepine; Receptor solubilization; Separation methods

1. Introduction

Radioreceptor assays (RRA) can be used to measure drug levels in biological matrices. RRA are simple and rapid to perform, selective and sensitive. Their sensitivity is proportional to the potency of the drug to be measured. However, the use of radioactive labels has several disadvantages, such as cost, health hazards, radioactive waste, and requirements of special licences. Therefore, several researchers have attempted to develop non-radioactive receptor assays for benzo-

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diazepines, using fluorescence detection [1–5] or enzymatic detection [6–8].

The receptor material commonly used in 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 non-radiactive receptor assays (RA), the turbid 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-bound proteins, including the benzodiazepine receptor.

Up until now solubilized receptors have been used primarily for pharmacodynamic studies of the benzodiazepine and the GABA receptor. In the experiments of this group 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 [9]. After the solubilization, the binding characteristics of the solubilized receptor were compared with 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 [^{3}H]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 labelled ligand were separated by filtration of the 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.

2. Materials and methods

2.1. Chemicals

[*N*-methyl-^{3}H]flunitrazepam (82.0 Ci mmol^{-1}) was obtained from DuPont NEN (Wilmington, DE). Lorazepam was a gift from Wyeth Laboratoria bv (Hoofddorp, The Netherlands) and flumazenil was a gift from Hoffmann–La Roche (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). 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).

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

2.2. Preparation of membrane-bound receptors

We modified the method for the preparation of membrane-bound receptors, described by Möhler and Okada [10]. Calf brains, obtained from the slaughterhouse and stored at −80°C after discarding the cerebellum, were homogenized in six volumes (w/v) of ice-cold 0.32 M sucrose in a Potter-Elvejehem homogenizer (RW 20 DZW, Janke & Kunkel KG, Staufen i.Br., Germany) fitted with a Teflon pestle and centrifuged at 1000 *g* for 10 min in a Beckman L8-55 Ultracentrifuge (Beckman Instrucments, Mijdrecht, The Netherlands). The supernatant was centrifuged at 100 000 *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 *g* for 30 min. This washing step was repeated once. All opera-

tions were performed at 4°C. The washed P_2-pellet was resuspended in five volumes (w/v) of phosphate buffer, frozen with liquid nitrogen and lyophilized (Hetosicc CD 52-1, Heto, Birkerød, Denmark). The lyophilized P_2-pellet was stored at −20°C.

2.3. Preparation of solubilized receptors

The procedure to solubilize the benzodiazepine receptors was a compromise between several protocols [11–13]. 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 $\mu g\ ml^{-1}$) and fresh phenylmethylsulfonyl fluoride (0.3 mM). The concentration of the P_2-pellet was 8 $mg\ ml^{-1}$. A 5% (w/v) solution of sodium deoxycholate in water was added dropwise to the magnetically stirred suspension until a final concentration of 0.5% (w/v) was reached. The suspension was stirred for 30 min at 4°C and the solubilized receptors were recovered by centrifuging at 15 000 *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.

2.4. Membrane-bound receptor binding assays

For the saturation experiments 20 µl [^{3}H]flunitrazepam solution (0.2–20 nM final concentration) in Tris–HCl buffer (pH 7.4; 50 mM) was 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 $mg\ ml^{-1}$ P_2-pellet, corresponding to 150 μg protein per assay) was 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 h and counted for 5 min in a Tri-Carb 4000 Packard scintillation counter (Canberra Packard, Groningen, The Netherlands).

For the inhibition experiments 20 μl [^{3}H]flunitrazepam solution (4 nM final concentration) was 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.

2.5. 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 per 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 [^{3}H]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 [13]. Ice-cold Tris–HCl buffer (3 ml), 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.

2.6. 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 Instrucments, Basle, Switzerland). The membrane-bound receptors were diluted to a protein concentration of 60 $\mu g\ ml^{-1}$; the solubi-

lized receptors had a protein concentration of 360 μg ml^{-1}.

2.7. Comparison of the separation techniques for bound and free labelled ligand

For the comparison of the three separation methods, the [^{3}H]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 [^{3}H]flunitrazepam in Tris–HCl buffer (pH 7.4; 50 mM) (4 nM final concentration) was 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 was added with a final concentration of 1 mg ml^{-1} protein. From these mixtures 12 200 μl aliquots were pipetted into 12 ml polyethylene tubes (for the filtration methods) or into 1.5 ml Eppendorf tubes (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.

2.7.1. Polyethylene glycol precipitation/filtration

This method was performed as described in Section 2.5.

2.7.2. 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 [14]. 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 h and counted for 5 min in a Tri-Carb 4000 Packard scintillation counter.

2.7.3. 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) [15]. After mixing and centrifugation for 5 min at 15 000 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 12 times. The pipetting scheme was the same as for the solubilized receptor except that 2.24 ml membrane-bound receptors (4 mg ml^{-1} P_2-pellet) was used instead of the solubilized receptors.

2.8. Protein determination

The amount of protein used in the saturation experiments was assayed by a modified version of the method developed by Lowry [16], 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 [17]. The precipitates, from both membrane-bound receptors as well as from solubilized receptors, were directly dissolved in the so-called Lowry reagent "C".

3. Results and discussion

3.1. Solubilization of the benzodiazepine receptor

The saturation and inhibition curves were fitted with the program EBDA-Ligand, V4 (Biosoft, Cambridge, UK) [18] using the one-binding site model. The results are presented in Table 1. Solubilization with 0.5% sodium deoxycholate extracted about 80–85% of the protein and about 50–55% of the benzodiazepine binding sites (B_{max}) with regard to the membrane-bound receptor preparation. This corresponds to the results of Sigel and Barnard [12], who found a recovery of receptor binding sites of about 55%. Fig. 1 shows representative saturation curves for the membrane-bound and solubilized benzodiazepine receptors. The binding affinity of [^{3}H]flunitrazepam for the solubilized receptor (K_d) decreased from 1.20 ± 0.11 nM to 4.1 ± 0.7 nM. The B_{max} value

Table 1
Comparison of binding properties of membrane-bound and solubilized benzodiazepine receptors

	Membrane-bound receptors	Solubilized receptors
K_d (nM) ([^{3}H]flunitrazepam)	1.20 ± 0.11	4.1 ± 0.7
B_{max} (pM mg^{-1} protein)	1.01 ± 0.01	0.54 ± 0.13
Flumazenil		
IC_{50} (nM)	2.7 ± 0.4	6.3 ± 0.4
K_i (nM)	0.67 ± 0.10	3.2 ± 0.3
Detection limit (nM)	0.46 ± 0.14	0.72 ± 0.01
Lorazepam		
IC_{50}	5.51 ± 0.13	15.3 ± 0.2
K_i (nM)	1.49 ± 0.13	8.4 ± 1.0
Detection limit (nM)	0.98 ± 0.05	1.5 ± 0.4

decreased from 1.01 ± 0.01 pM mg^{-1} protein to 0.54 ± 0.13 pM mg^{-1} protein after solubilization. The changes in K_d and B_{max} were significant when compared with Student's t-test ($p < 0.05$). The non-specific binding increased after solubilization.

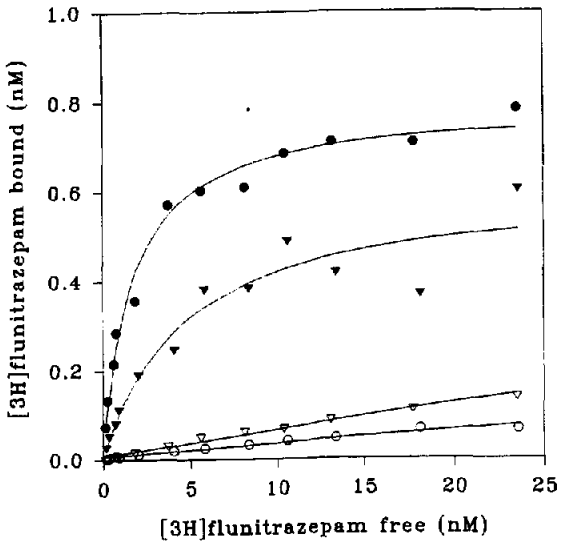

Fig. 1. Saturation curves of membrane-bound receptors (circles) and solubilized receptors (triangles). The closed symbols represent the specific binding and the open symbols the non-specific binding.

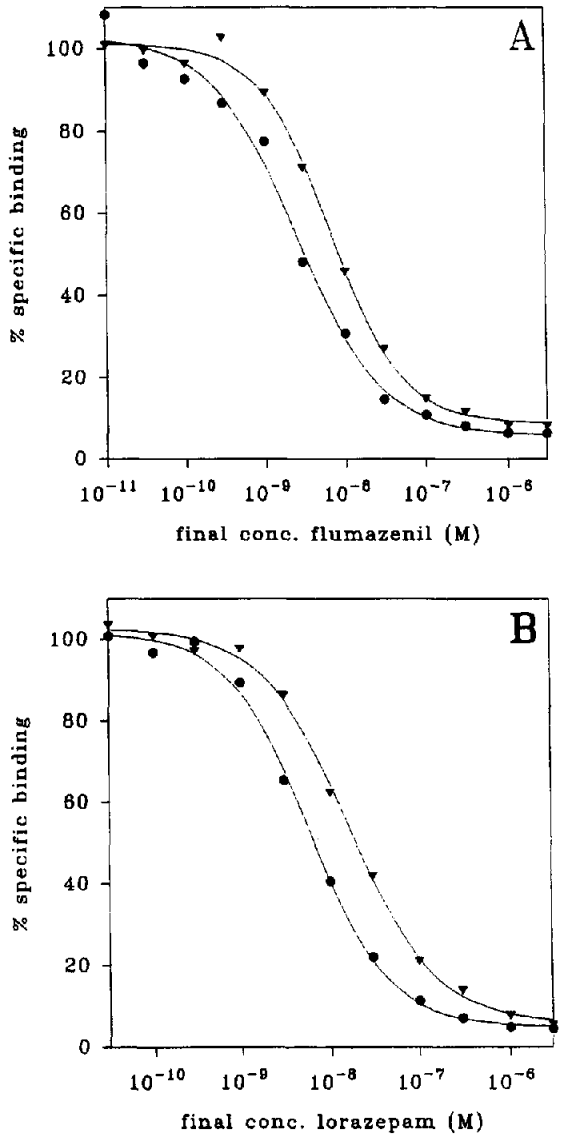

Fig. 2. Inhibition curves of membrane-bound receptors (●) and solubilized receptors (▼) for flumazenil (A) and lorazepam (B).

The inhibition experiments were performed with a [^{3}H]flunitrazepam concentration which was equal to the K_d value of [^{3}H]flunitrazepam for the solubilized receptor. Representative inhibition curves for the benzodiazepine antagonist flumazenil and the agonist lorazepam are shown in Fig. 2A and 2B respectively. For both benzodi-

azepines, the use of solubilized receptors caused a shift in the inhibition curves to the right, as can be seen from the IC_{50} values in Table 1. From the inhibition curves the inhibition constants (K_i) were calculated (Table 1). 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 sensitivity by about a factor of five for both benzodiazepines.

3.2. Fluorescence spectra of the receptor materials

In Fig. 3 fluorescence spectra of the membrane-bound and solubilized receptors are shown at two different excitation wavelengths for some common fluorescent labels ($\lambda = 350$ 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 labelled "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.

3.3. Comparison of the different separation methods for bound and free labelled ligand

The principles of the three methods used are as follows. In the PEG precipitation/filtration method, the PEG precipitates the solubilized receptor. The γ-globulin is a carrier for the precipitation reaction, since the γ-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 [19]. The filters bear positive charges, so the receptor complex may be retained on the filter by ionic forces during filtration [20]. Charcoal adsorbs small organic molecules, such as the free [^{3}H]flunitrazepam, which can then be removed by centrifugation. The supernatant contains [^{3}H]flunitrazepam bound to the receptor.

The results of the three separation methods are presented in Table 2. The binding is expressed as the percentage of the total amount of [^{3}H]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

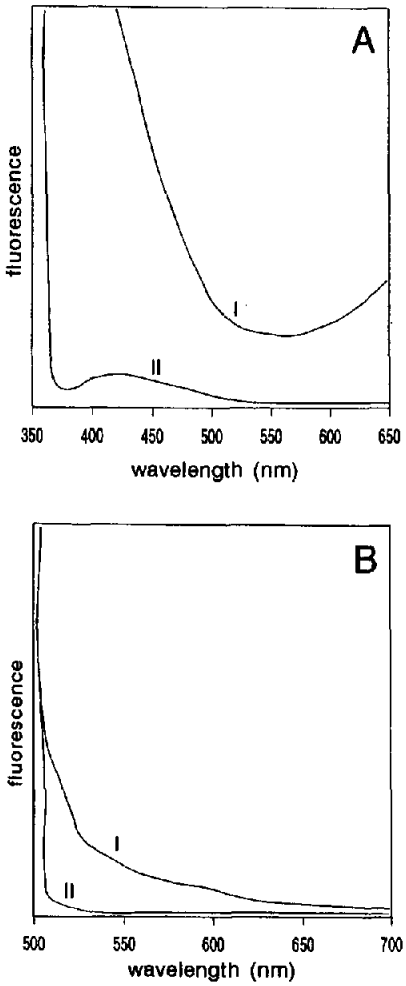

Fig. 3. Emission spectra of membrane-bound receptors (I) and solubilized receptors (II). The excitation wavelengths correspond to different fluorescence labels: $\lambda_{ex} = 350$ nm to several coumarins (A) and $\lambda_{ex} = 500$ nm to fluorescein (B).

Table 2
Comparison of the three different separation techniques for bound and free labelled ligand. (The receptor-bound amount of [^{3}H]flunitrazepam is represented as the percentage of the amount of [^{3}H]flunitrazepam added. The results in the parentheses are correlated to the PEG precipitation/filtration method (= 100%))

	PEG precipitation/ filtration	Ion exchange filtration	Charcoal adsorption
Total binding	12.5 ± 0.31 (100%)	2.8 ± 0.27 (22.5%)	10.0 ± 0.35 (79.6%)
Non-specific binding	0.83 ± 0.13 (100%)	0.52 ± 0.05 (63.3%)	0.91 ± 0.05 (110%)

binding. The lower results for the charcoal adsorption method indicate that besides the free [^{3}H]flunitrazepam a part of the receptor material is also 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 [^{3}H]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. Another explanation of the poor binding of the ionic exchange filters is that sodium deoxycholate molecules also 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 is no significant difference between the variance of the three methods, either for the total binding, or for the non-specific binding. Wang et al. [21], however, described that the reproducibility of the charcoal method is poor, when compared to the filtration method.

From these results, the conclusion can be drawn 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 use, especially with large amounts of samples.

3.4. Calculation of the detection limit

The detection limit was calculated by subtracting three times the relative standard deviation (RSD) of the maximal binding of a standard curve and calculating the intersection of this value with the standard curve. The RSD 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 RSD 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 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 to 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 RSDs 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} values.

The higher RSD 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.

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. In contrast, solubilization of the benzodiazepine receptor caused some loss in affinity of [^{3}H]flunitrazepam, flumazenil and lorazepam for the receptor. However, the detection limits of the benzodiazepine anatagonist flumazenil and the agonist lorazepam were not affected by the solubilization.

PEG precipitation followed by glass fibre filtration appeared to be the best method to separate free and bound ligand when working with solubilized receptors.

References

[1] T. Takeuchi and G.A. Rechnitz, Anal. Biochem., 194 (1991) 250–255.
[2] T. Takeuchi, M. Yoshida, Y. Kabasawa, R. Matsukawa, E. Tamiya and I. Karube, Anal. Lett., 26 (1993) 1535–1545.
[3] R.H. Havunjian, B.R. De Costa, K.C. Rice and P. Skolnick, J. Biol. Chem., 265 (1990) 22181–22186.
[4] J.L. Velazquez, C.L. Thompson, E.M. Barnes, Jr. and K.J. Angelides, J. Neurosci., 9 (1989) 2163–2169.
[5] R.T. McCabe, B.R. De Costa, R.L. Miller, R.H. Havunjian, K.C. Rice and P. Skolnick, FASEB J., 4 (1990) 2934–2940.
[6] T. Takeuchi, S. Tanaka and G.A. Rechnitz, Anal. Lett., 24 (1991) 2111–2121.
[7] T. Takeuchi, S. Tanaka and G.A. Rechnitz, Anal. Biochem., 203 (1992) 158–162.
[8] S. Tanaka, T. Takeuchi and G.A. Rechnitz, J. Chromatogr., 597 (1992) 443–448.
[9] F.A. Stephenson, A.E. Watkins and R.W. Olsen, Eur. J. Biochem., 123 (1982) 291–298.
[10] H. Möhler and T. Okada, Life Sci., 20 (1977) 2101–2110.
[11] E. Sigel, F.A. Stephenson, C. Mamalaki and E.A. Barnard, J. Biol. Chem., 258 (1983) 6965–6971.
[12] E. Sigel and E.A. Barnard, J. Biol. Chem., 259 (1984) 7219–7223.
[13] F.A. Stephenson, in E.C. Hulme (Ed.), Receptor Biochemistry, A Practical Approach, IRL Press, Oxford, 1990, pp. 177–201.
[14] B. Lang, E.A. Barnard, L-R. Chang and J.O. Dolly, FEBS Lett., 104 (1979) 149–153.
[15] H. Gorissen, G. Aerts, B. Ilien and P. Laduron, Anal. Biochem., 111 (1981) 33–41.
[16] S. Clark in J.C. Venter and L.C. Harrison (Eds.), Receptor Purification Procedures, Alan R. Liss, Inc., New York, 1984, pp. 149–161.
[17] A. Bensadoun and D. Weinstein, Anal. Biochem., 70 (1976) 241–250.
[18] P.J. Munson and D. Rodbard, Anal. Biochem., 107 (1980) 220–239.
[19] F.A. Stephenson, S.O. Casalotti, C. Mamalaki and E.A. Barnard, J. Neurochem., 46 (1986) 854–861.
[20] R.F. Burns, K. Lawson-Wendling and T.A. Pugsley, Anal. Biochem., 132 (1983) 74–81.
[21] J-X. Wang, H.I. Yamamura, W. Wang and W.R. Roeske in E.C. Hulme (Ed.) Receptor–Ligand Interactions, A Practical Approach, IRL Press, Oxford, 1992, pp. 213–234.