

Improved benzodiazepine radioreceptor assay using the MultiScreen[®] Assay System

M.J. Janssen, K. Ensing, R.A. de Zeeuw *

*Department of Analytical Chemistry and Toxicology, Groningen Institute for Drug Studies (GIDS),
University Centre for Pharmacy, A. Deusinglaan 1, 9713 AV Groningen, The Netherlands*

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Abstract

In this article, an improved benzodiazepine radioreceptor assay is described, which allows substantial reduction in assay time. The filtration in this method was performed by using the MultiScreen[®] Assay System. The latter consists of a 96-well plate with glass fibre filters sealed at the bottom, which allows both the incubation and the filtration of the specimen in the same plate. After the filtration, the filters were punched out for quantitation of the bound labeled ligand [³H]flunitrazepam. The results obtained with the MultiScreen Assay System did not differ significantly from the data obtained with the conventional filtration manifold (48S): The K_i 's of lorazepam were 2.4 ± 0.30 and 1.9 ± 0.15 nM, respectively. In case a radioactive label is replaced by a fluorescent label, the bound labeled-ligand usually cannot be determined in the presence of the receptor material. Here, the bound labeled-ligand has to be dissociated after the filtration step. To dissociate the ligand-receptor complex, Tris-HCl buffer, containing 10 μ M flumazenil, was added to the filters and the second filtrates were collected containing the previously bound fractions in the absence of receptor material. This approach showed the same K_i for lorazepam, 2.5 ± 0.04 nM as without dissociation, when using the radio-labeled benzodiazepine [³H]flunitrazepam. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

The use of radioreceptor assays (RRA) as an analytical method has several advantages, such as sensitivity and selectivity, ease of operation and speed. The principle is based on a competition between a radioactive labeled ligand and an analyte for binding to a particular receptor. After

equilibration, the bound and unbound fractions of the labeled ligand have to be separated to enable the quantification of one of these two fractions. In RRA the bound labeled ligand is most often quantitated after separation by filtration [1,2]. The filtration manifold (48S) used in our laboratory allows the incubation of maximally 48 samples in separate tubes, after which the samples have to be transferred one by one onto the filters. This total process is time consuming.

Millipore has developed an incubation and filtration system, the MultiScreen[®] Assay System,

* Corresponding author. Tel.: +31-50-3633336; fax: +31-50-3637582.

E-mail address: r.a.de.zeeuw@farm.rug.nl (R.A. de Zeeuw)

to rapidly and reproducibly perform the total process [3]. This system consists of a 96-well plate in which the well bottoms are made of glass fibre filters, the MultiScreen-FB or -FC plates. The specifications of the filter materials used are identical to Whatman GF/B and GF/C glass fibre filters, respectively. The well plate is mounted on top of an underdrain, as is shown in Fig. 1. The well plate and underdrain fit tightly together and vacuum can be applied at the underside of the underdrain. This allows maintenance of liquid volumes within the wells for prolonged and repeated incubations, even after vacuum filtration has been performed. It also enables both the incubation and the filtration of 96 individual samples in one on-line run, which means a reduction in sample handlings, in assay time and in variance. After the filtration, the filters can be punched out and the radioactivity retained on the filters can be counted.

Packard Instruments has also developed an instrument for the automation of radioreceptor assays, the TopCount [4]. This system provides a filtration harvester, the Filtermate, with 24- or 96-well microtiter plates containing GF/B or GF/C filters sealed at the bottom. The Filtermate enables the filtration of all samples at once. However, since the filtration plates of the TopCount do not contain an underdrain, the incubations have to be performed off-line in another microtiter plate.

In this paper, we compare a filtration method for the benzodiazepine radioreceptor assay employing a traditional filtration manifold (48S) with the MultiScreen Assay System, using [³H]flunitrazepam as radioactive labeled ligand.

Since radioactive methods have several disadvantages, attempts have been made to develop suitable non-radioactive receptor assays, such as by using a fluorescent label [5–10]. Since the bound fluorescent label usually cannot be determined directly on the filter, we have developed a modification of the assay in which the fluorescent label is dissociated from the benzodiazepine receptor on the filter and subsequently collected by filtration. This modification (FRA) has also been evaluated with the MultiScreen Assay System.

2. Materials and methods

2.1. Chemicals

[*N*-methyl-³H]flunitrazepam (82 Ci/mmol) was obtained from DuPont NEN (Wilmington, DE). [¹⁴C]Tributylmethylammonium iodide (21 Ci/mmol) was a gift from J.W. Smit (University Centre for Pharmacy, Groningen, The Netherlands). Lorazepam was a gift from Wyeth Laboratoria (Hoofddorp, The Netherlands) and flumazenil was a gift from Roche Nederland (Mijdrecht, The Netherlands). All other chemicals were of analytical grade and were purchased from Merck (Darmstadt, Germany).

The GF/B glass fibre filter discs (Ø25 mm) were obtained from Whatman (Maidstone, UK). The MultiScreen[®] Assay System and the MultiScreen-FB filtration plates were kindly donated by Millipore (Etten-Leur, The Netherlands). 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.

2.2. Preparation of membrane-bound receptors

We modified the method for the preparation of membrane-bound benzodiazepine receptors, described by Möhler and Okada [11]. 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 glass-teflon Potter-Elvehjem homogenizer (RW 20 DZW, Janke & Kunkel KG, Staufen i.Br., Germany) and centrifuged at

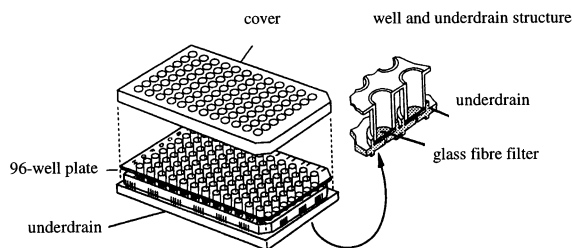


Fig. 1. MultiScreen Assay System 96-well filtration plate.

1000 × *g* for 10 min in a Beckman L8-55 Ultracentrifuge (Beckman Instruments, Mijdrecht, The Netherlands). The supernatant was collected and centrifuged at 100 000 × *g* for 60 min. The resulting pellet (P₂-pellet) was resuspended in sodium phosphate buffer (pH 7.4; 50 mM) and centrifuged at 10 000 × *g* for 30 min. This washing step was repeated four times. All operations were performed at 4°C. The washed P₂-pellet was resuspended in 5 volumes (w/v) phosphate buffer, frozen with liquid nitrogen and lyophilized (Hetosicc CD 52-1, Heto, Birkerød, Denmark). The lyophilized P₂-pellet was stored at –20°C. For the working receptor suspension, lyophilized P₂-pellet was resuspended in Tris–HCl buffer (pH 7.4; 50 mM) with the glass-teflon Potter–Elvehjem homogenizer.

2.3. Determination of the receptor loading of the MultiScreen filtration plates

The filters of a MultiScreen-FB filtration plate were pre-wetted by pipetting 200 µl ice-cold Tris–HCl buffer (pH 7.4; 50 mM) into each well. After waiting for at least 5 s, vacuum was applied by the MultiScreen vacuum manifold (Millipore, Etten-Leur, The Netherlands). Two hundred microliters receptor suspension with increasing concentrations of P₂-pellet in Tris–HCl buffer (0.25–8 mg/ml) were pipetted into the wells of six successive columns of the filtration plate. Vacuum was applied and the time to filter the receptor suspension was measured. During the filtration, the pressure was kept on 400 mbar.

2.4. Optimization of the wash procedure of the MultiScreen filtration plates

After pre-wetting the filters according to the procedure described above, 25 µl Tris–HCl buffer (pH 7.4; 50 mM), containing [³H]flunitrazepam and [¹⁴C]tributylmethylammonium iodide (both 1200 Bq per assay), and 25 µl Tris–HCl buffer for the maximal binding or 25 µl lorazepam solution (10 µM final concentration) for the non-specific binding were pipetted in duplicate into different wells of a filtration plate. To this mixture 200 µl receptor suspension (4 mg/ml P₂-pellet) were

added and the plate was shaken for one minute. After incubation at 4°C for 45 min, vacuum was applied (400 mbar) and the wells were washed 0, 1, 2 or 3 times with 200 µl ice-cold Tris–HCl buffer. After removing the underdrain, the bottom of the filtration plate was blotted with filtration paper and the filters were punched out. The filters were transferred into 6 ml 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 (Canberra Packard, Groningen, The Netherlands), using double-label counting.

2.5. Dissociation of the bound [³H]flunitrazepam after filtration

To select the best solvent for the dissociation of the bound [³H]flunitrazepam, the P₂-pellet was incubated with [³H]flunitrazepam in 6-fold in the presence or absence of lorazepam, as described in the previous procedure. After a single washing with 200 µl ice-cold Tris–HCl buffer (pH 7.4; 50 mM), the underdrain of the filtration plate was blotted and the bound [³H]flunitrazepam was dissociated after the addition of either 200 µl of Tris–HCl buffer, Tris–HCl buffer containing 10 µM flumazenil, methanol or acetate buffer (pH 4; 100 mM). After incubation at room temperature for 1 h under gentle shaking, vacuum was applied and the filtrates were collected in a microtiter plate placed under the MultiScreen-FB filtration plate. One hundred microliters of the filtrates were transferred into 6 ml counting vials, 3.5 ml Rialuma was added and the radioactivity was counted after vortexing the vials.

For the determination of the time necessary for dissociation of the bound fraction, the bound [³H]flunitrazepam was dissociated with Tris–HCl buffer containing 10 µM flumazenil. Incubation times were 5, 10, 20, 45 and 60 s and 2, 5, 10, 20 and 40 min, respectively. This was done in triplicate for each time point.

2.6. Inhibition curve of lorazepam with the MultiScreen assay system

After pre-wetting the filters of a MultiScreen-

FB filtration plate according to the procedure described earlier, two inhibition experiments were made by pipetting 25 μl [^3H]flunitrazepam solution (1.5 nM final concentration) in Tris–HCl buffer (pH 7.4; 50 mM) and 25 μl Tris–HCl buffer, containing lorazepam (100 nM–30 pM final concentration), in duplicate into the wells of the plate. To this mixture 200 μl receptor suspension (4 mg/ml P_2 -pellet, corresponding with 250 μg protein/assay) were added and the plate was shaken for 1 min. After the incubation at 4°C for 45 min, vacuum was applied (400 mbar) and the filters were rinsed once with 200 μl ice-cold buffer. In the first experiment 200 μl Tris–HCl buffer, containing 10 μM flumazenil, were pipetted in each well and incubated for 20 min at room temperature. The dissociated [^3H]flunitrazepam was collected in a microtiter plate by filtration. One hundred microliters of the filtrates were transferred into 6 ml counting vials and the radioactivity was counted as above.

In the second experiment the filters were punched out, transferred into 6 ml counting vials and dispersed in 3.5 ml Rialuma. The radioactivity was counted after shaking the vials for 2 h. Both experiments were done in duplicate.

2.7. Inhibition curve of lorazepam with the filtration manifold (48S)

For the inhibition curve with the filtration manifold (48S) [13], 25 μl [^3H]flunitrazepam solution (1.5 nM final concentration) in Tris–HCl buffer (pH 7.4; 50 mM) were mixed in duplicate with 25 μl Tris–HCl buffer, containing lorazepam (100 nM–30 pM final concentration), in polyethylene tubes. To this mixture 200 μl receptor suspension (4 mg/ml P_2 -pellet, corresponding with 250 μg protein/assay) were added and vortexed. 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 with the filtration manifold (48S) (University Centre for Pharmacy, Groningen, The Netherlands). The tubes were rinsed twice with 4 ml ice-cold

Table 1

Time required to filter 200 μl receptor suspensions containing different amounts of P_2 -pellet with the MultiScreen assay system at a pressure of 400 mbar ($n = 6$)

Concentration P_2 -pellet (mg/ml)	Filtration time (s)
0.25	<5
0.5	<5
1	<10
2	<10
4	<15
8	>60

buffer, which was also filtered. This filtration and washing procedure was completed in 15 s intervals for each sample. The filters were transferred into 6 ml counting vials and dispersed in 3.5 ml Rialuma. The vials were shaken for 2 h and counted as above. All inhibition experiments were performed in duplicate.

3. Results and discussion

The MultiScreen Assay System was tested for the automation of the filtration of the benzodiazepine radioreceptor assay. An inhibition curve of the benzodiazepine agonist lorazepam was obtained with this filtration system and the curve was compared with a curve obtained with the traditional filtration manifold (48S). Yet, before an inhibition curve of lorazepam with the MultiScreen Assay System could be obtained, the working procedures had to be optimized in view of the filtration properties of the MultiScreen filtration plates, the number of washings, the best solvent for dissociation and the dissociation time.

3.1. Receptor loading and wash procedure of the MultiScreen assay system

Table 1 depicts the time necessary to filter different concentrations of receptor suspension. The filtration time increased with higher concentrations of P_2 -pellet. The manufacturer of the MultiScreen Assay System advises a filtration

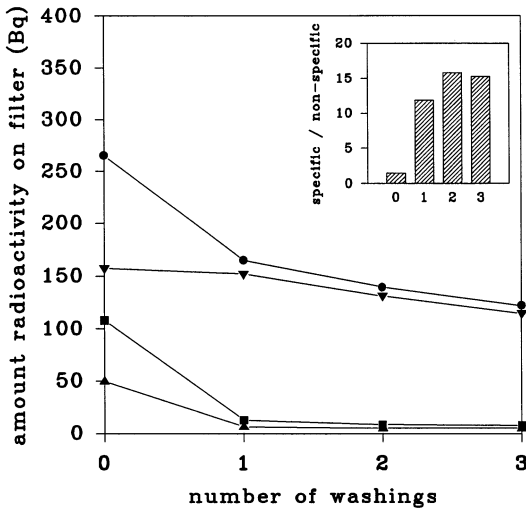


Fig. 2. Effect of the number of washings on the total binding (●), specific binding (▼), non-specific binding (■) and entrapment of [¹⁴C]tetrabutylammonium iodide (▲). The insert shows the effect of the number of washings on the ratio specific/non-specific binding. The amount of [¹⁴C]tributylmethylammonium iodide added was equal in radioactivity to the amount of [³H]flunitrazepam, namely 1200 Bq per assay.

time of less than 20 s. Therefore, we set the concentration P₂-pellet in the assay at 4 mg/ml, which corresponds with 0.8 mg P₂-pellet per filter.

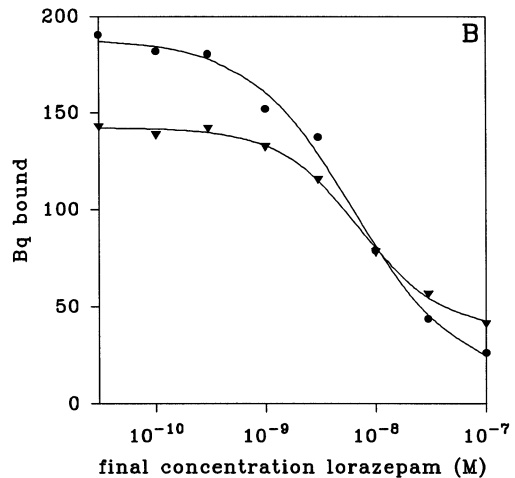
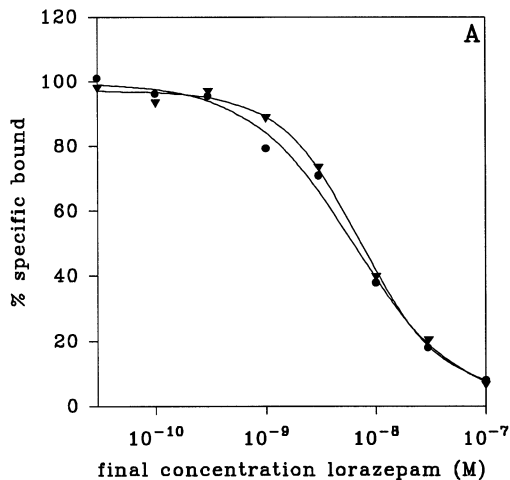


Fig. 3. RRA inhibition curves of lorazepam, obtained with the filter manifold (48S) (●) and the MultiScreen Assay System (▼). (A) represents the results corrected for non-specific binding and plotted as percentage of the maximal amount of bound [³H]flunitrazepam and (B) represents the amount of Bq bound to the receptors.

The number of washings of the filtration plates affects the apparent specific/non-specific binding ratio, as can be seen in Fig. 2. After one washing this ratio was higher than 10 which is currently considered as adequate. [¹⁴C]Tributylmethylammonium iodide was added to get an impression of the entrapment of the free labeled ligand during the filtration procedure, since this substance was expected to have no affinity for the benzodiazepine receptor and a negligible non-specific binding to membranes and filter material. Hence, the profile of the amount of [¹⁴C]tributylmethylammonium iodide on the filter is conceivably comparable with the non-specific binding of [³H]flunitrazepam to the filter. After one washing both non-specific bindings were diminished to very low values, and more washings had only negligible effects.

Washing of the filters also slightly reduced the specific binding after each washing step. The reason for the decrease of the specific binding is a partial dissociation of the bound [³H]flunitrazepam from the benzodiazepine receptors. Therefore, we reduced the number of washings from two, as necessary with the filtration manifold (48S), to one with the MultiScreen Assay System to minimize the loss of specific binding.

3.2. Comparison of the two filtration procedures

Fig. 3 compares the RRA inhibition curves obtained with the two different filtration methods. The curves are virtually identical when they are corrected for non-specific binding and plotted as percentage of the maximal amount of receptor bound [^3H]flunitrazepam (Fig. 3A). This indicates that the benzodiazepine radioreceptor assay can be carried out on the MultiScreen Assay System.

In Fig. 3B one can see that the total amount of bound [^3H]flunitrazepam with the filtration manifold (48S) is higher than with the MultiScreen Assay System. The assay with the filtration manifold (48S) had a non-specific binding of 5.3%, whereas the assay with the MultiScreen Assay System had a higher non-specific binding, 14%. A higher non-specific binding means a lower sensitivity of the RRA [12]. However, the automation of the assay has several advantages. With the MultiScreen assay system, 96 samples were filtered in 2 min, whereas it took 12 min to filter 48 samples with the filtration manifold (48S). Besides, the automation of the assay allows a reduction of 96% in liquid radioactive waste. The MultiScreen Assay System also provides lower variances than the filtration manifold (48S). The variation coefficient of twelve identical samples (maximal binding) was 5% with the MultiScreen Assay System and 10% with the filtration manifold (48S). The higher variation coefficient for the filtration manifold (48S) was probably caused by the fact that the time of vacuum on the filters with the receptor material on it differed from 15 s for the last filtered sample to 12 min for the first filtered sample. The radioactivity retained on a filter which was filtered first, and kept under vacuum for 12 min, was only 121 Bq ($n=6$, S.D. = 7.4), while the radioactivity on a filter which was filtered last was 185 Bq ($n=6$, S.D. = 5.0). This difference is significant when compared with Student's t -test ($P < 0.001$). This loss in radioactivity appears to be caused by dissociation of bound [^3H]flunitrazepam from the receptors during the time the filter is under vacuum.

Table 2

Capability of four solutions to dissociate bound [^3H]flunitrazepam ($n=6$)^a

	% [^3H]flunitrazepam dissociated (\pm S.D.)
50 mM Tris-HCl buffer, pH 7.4	71.6 (\pm 1.19)
10 μM flumazenil in Tris-HCl buffer	90.8 (\pm 0.39)
Methanol	^b
100 mM acetate buffer, pH 4	87.2 (\pm 1.16)

^a The receptors on the filters were incubated for 1 h at room-temperature.

^b Methanol was difficult to filter.

3.3. Dissociation of the bound [^3H]flunitrazepam

In RRA, the bound fraction can easily be determined by counting the radioactivity retained on the filter. The presence of the filters and the receptor material on it do not create an inaccurate measurement if the radioactivity is completely dispersed in the scintillation cocktail.

However, when a non-radioactive label, such as a fluorescent label or enzyme-bound label, is used as ligand in receptor assays, the presence of filters and/or receptor material makes the measurement of the bound labels impossible. Therefore, to measure the bound fraction in a non-radioactive assay, the bound label needs to be present in a clear solution. This can be achieved by dissociating the bound label from the receptor after the initial filtration and by collecting the dissociation solution in a second filtration step. Four different solutions were tested for their capability to dissociate the bound [^3H]flunitrazepam. The results are shown in Table 2. Methanol was not a good choice since it was found difficult to filter, due to the low surface tension of methanol. Parts of the filtrate remained at the underside of the under-drain of the MultiScreen filtration plate, so the volume of the collected filtrates varied from well to well. Besides, the filtrates were turbid.

Evaluation of the dissociation results with one-way ANOVA showed a significant difference between the three remaining dissociation solutions ($P < 0.05$). The dissociation in Tris-HCl buffer

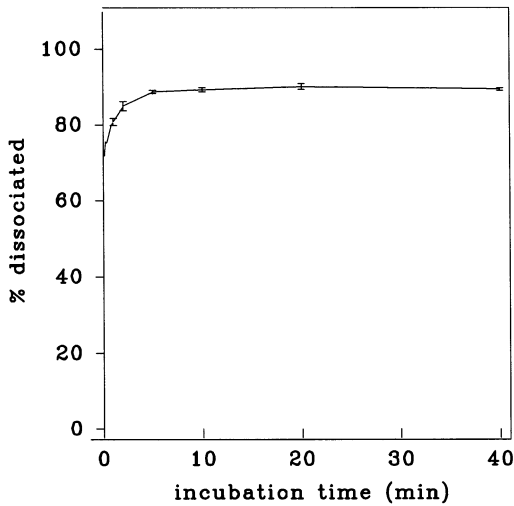


Fig. 4. Influence of the incubation time on the dissociation of [^3H]flunitrazepam. [^3H]flunitrazepam was dissociated in the presence of 10 μM flumazenil in 50 mM Tris–HCl buffer, pH 7.4, at room-temperature.

was incomplete after 1 h at room-temperature, due to reassociation of the label and receptor. However, with the addition of flumazenil in the Tris–HCl buffer, the dissociation of the bound [^3H]flunitrazepam was almost complete at about 91%. Apparently, flumazenil is capable of com-

pletely displacing the bound analyte from the receptor sites. Although the dissociation with acetate buffer (87.2%) was significantly lower, it is still a good alternative as dissociation solution.

In further experiments, we used 10 μM flumazenil in Tris–HCl buffer as dissociation solution. In Fig. 4 it can be seen that the dissociation of bound [^3H]flunitrazepam proceeds very quickly. The percentages dissociated were evaluated with one-way ANOVA and there was no significant difference ($P = 0.054$) between the results from 5 min till 40 min. To eliminate the impact of small changes in the dissociation rate, the dissociation time was set at 20 min in further experiments.

The procedure to dissociate and collect the bound fraction [^3H]flunitrazepam, was tested by the determination of two calibration series of lorazepam with the MultiScreen Assay System. In one series, the bound [^3H]flunitrazepam was dissociated after filtration and in the other series the bound fraction was determined by counting the radioactivity on the filters. The results of the two methods, corrected for the non-specific binding and plotted as percentage of the maximal amount of [^3H]flunitrazepam, are shown in Fig. 5A.

The inhibition curves are virtually identical, which implies that it is possible to dissociate the

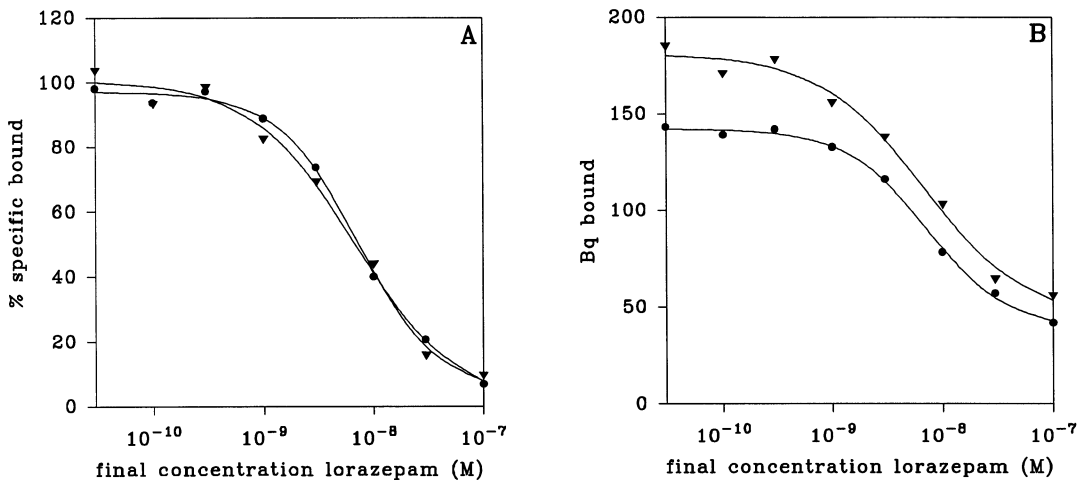


Fig. 5. RRA inhibition curves of lorazepam, obtained with the MultiScreen Assay System. (●) represents the curve without dissociation and (▼) the curve after dissociation of the bound [^3H]flunitrazepam. (A) represents the results corrected for non-specific binding and plotted as percentage of the maximal amount of bound [^3H]flunitrazepam and (B) represents the amount of radioactivity bound to the receptors.

Table 3
 K_i -values obtained with the three different inhibition procedures ($n = 2$)

	K_i -value (nM) (\pm S.D.)
Filtration manifold (48S)	1.9 (\pm 0.15)
MultiScreen assay system	
Without dissociation	2.4 (\pm 0.30)
With dissociation	2.5 (\pm 0.04)

bound label and to quantitate it in an environment free from receptors and filters. However, the non-specific binding after dissociation was higher than without dissociation, namely 22 and 14%, respectively, as can be seen in Fig. 5B (the amount of Bq bound at the receptor at a lorazepam concentration of 10^{-7} M was higher for the inhibition curve with dissociation). In the assay without dissociation, the underside of the filters were blotted after removing of the underdrain. Blotting the underside of the filters before the addition of the dissociation solution was not possible: The underdrain could not be removed after the filtration, since it was not possible to fix the plate back in the underdrain. The amount of non-specific binding could be reduced by more washings, but this also reduced the total amount of bound [3 H]flunitrazepam. Perhaps it may be possible in the future to adapt the MultiScreen Assay System, so that the underdrain can be removed and reinstalled after filtration and blotting the underside of the filtration plate.

The three inhibition curves, with the filtration manifold (48S), and the two with the MultiScreen Assay System, were also fitted with the program EBDA-Ligand, V4 (Biosoft, Cambridge, UK) [14] using the one-binding site model. The calculated K_i -values are presented in Table 3. Evaluation with one-way ANOVA shows that there is no significant difference between the K_i -values ($P = 0.104$), which implies that the three methods give comparable results.

3.4. Comparison of the MultiScreen assay system with the TopCount

With the TopCount, the radioactivity retained

on the filters can be measured directly in the filtration plate. After filtration, scintillation cocktail has to be dispersed in the filtration plate and after sealing the plate, the radioactivity can be measured by the TopCount, which can count up to 12 samples at once. With this system there is no need to punch out the filters after the filtration, which saves time. Direct counting of the radioactivity also reduces the amount of scintillation cocktail needed. Instead of 3.5 ml of scintillation cocktail per vial, 20–30 μ l cocktail is sufficient for each well of a 96-well plate. With special adaptors it is even possible to count the MultiScreen filtration plates directly with the TopCount.

The advantage of the MultiScreen Assay System over the TopCount is that the incubation, and an eventual second incubation, can be done in the filtration plates, in contrast to the TopCount. Also, with the TopCount it is not possible to collect the filtrates. When the bound label has to be dissociated and collected after filtration, this can only be done with the MultiScreen Assay System, not with the TopCount.

4. Conclusions

The benzodiazepine radioreceptor assay can be automated successfully with the MultiScreen Assay System. When using the latter, the bound fraction can be determined by counting the punched-out filters, as well as by dissociating the label followed by counting the second filtrate. The MultiScreen Assay System allows a substantial reduction in filtration time in comparison with the filtration manifold (48S), namely from 12 to 2 min. In addition, the variation coefficient of twelve identical samples was 5% with the MultiScreen Assay System and 10% with the filtration manifold (48S).

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