Development and perspectives of fluorescent receptor assays

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Chapter 2

Synthesis and characterization of fluorescent-labeled 1,4-benzodiazepines

2.1 Introduction

Up till now, most receptor binding studies have been performed with radioactive labeled ligands. This allows measurements with high sensitivity and since the use of the radioisotopes $[^3]$H and $[^14]$C does not change the structures of the molecules, these ligands have the same binding affinities towards the receptor as the corresponding unlabeled analogues. However, the use of radioactivity has disadvantages, such as potential health hazards, high costs, generation of radioactive waste and the requirement for special laboratory facilities. Therefore, several groups have attempted to synthesize non-radioactive ligands. For the benzodiazepine receptor, fluorescent-labeled ligands [1-6] and biotin-labeled ligands [7-9] have been used as non-radioactive labels.

However, coupling of a fluorophore to a benzodiazepine molecule most often reduces the binding affinity towards the benzodiazepine receptor. To minimize this undesired effect, we coupled the fluorophore at different positions of 1,4-benzodiazepine molecules to gather insight in the positions of the benzodiazepine molecule which are essential for the binding towards the benzodiazepine receptor and in those positions can be used for labeling. We also examined the impact of the type of the fluorescent label by comparing different fluorophores.

In this paper we describe the synthesis of several fluorescent-labeled 1,4-benzodiazepines, their purification and the determination of their affinity towards the benzodiazepine receptor.

The fluorescent-labeled benzodiazepine most suitable as fluorescent ligand in receptor binding studies was selected by comparing the respective $K_i$-values and fluorescence characteristics of the fluorescent-labeled ligands, as described in Chapter 4.

2.2 Materials and Methods

Chemicals and apparatus

[N-methyl-H]flunitrazepam (82 Ci/mmol) was obtained from DuPont NEN (Wilmington, DE, USA). Lormetazepam was a gift from Wyeth Laboratoria (Hoofddorp, The Netherlands) and didesethylflurazepam-HCl (Ro7-1986) and 7-aminonitrazepam (Ro5-3072) were gifts from Roche Nederland (Mijdrecht, The Netherlands). Oxazepam was purchased from Genfarma (Maarssen, The Netherlands). The fluorescent-labeled benzodiazepines Bodipy®-Ro7-1986 and NBD-Ro7-1986 were obtained from Molecular Probes, Inc. (Eugene, OR, USA). Quinine sulphate dihydrate (99+%), succinic anhydride, 60% suspension of sodium hydride in mineral oil, 4-bromomethyl-7-methoxycoumarin, 18-crown-6 ether and dansyl chloride were supplied by Janssen Chimica (Beersel, Belgium). Methanol, hplc-grade, was supplied by Lab-Scan (Dublin, Ireland). All other chemicals were of analytical grade and were purchased from Merck (Darmstadt, Germany).
Empore® Extraction Disks (C18, Ø47 mm) were obtained from Varian (Harbor City, CA, USA) and GF/B glass fibre filter discs (Ø25 mm) were obtained from Whatman (Maidstone, UK). Rialuna, 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.

The HPLC-system used for the identification of the fluorescent-labeled 1,4-benzodiazepine consisted of a Spectraflow 400 HPLC pump (ABI Analytical Kratos Division, Manchester, UK), a Spectraflow 757 variable wavelength UV detector (Kratos) and a modified Nermag R 3010 triple quadrupole mass spectrometer (Delsi-Nermag, Argenteuil, France), equipped with a custom-built prototype atmospheric pressure ionization (API) source. The spectra were recorded in the positive-ion mode. Injections were made using a Rheodyne 7125 injection valve, fitted with a 20 µl sample loop (Rheodyne, Cotati, CA USA).

The HPLC-system used for the purification of the fluorescent-labeled 1,4-benzodiazepines consisted of a SP 8800 HPLC pump (Spectra Physics, San Jose, CA, USA), an autosampler model 460 fitted with a 20 µl loop (Kontron Instruments, Basle, Switzerland), a SPD-6A variable wavelength UV detector (Shimadzu, Tokyo, Japan) and a HeliFrac fraction collector (Pharmacia LKB Biotechnology, Uppsala, Sweden).

Synthesis

Synthesis of Mmc-O-CO-(CH₂)₂-CO-oxazepam
To a suspension of 0.5 g oxazepam in 35 ml dry tetrahydrofuran was added 90 mg of a 60% suspension of sodium hydride in mineral oil. After stirring for 0.5 hour at room temperature under a nitrogen atmosphere, 0.27 g succinic anhydride was added and stirring was continued at 40°C. After 2 hours, the tetrahydrofuran was evaporated under vacuum. The residue was suspended in 20 ml water, transferred into a separation funnel, acidified with acetic acid, and extracted twice with 30 ml dichloromethane. After drying the dichloromethane phase with anhydrous sodium sulphate, the dichloromethane was evaporated to half its volume and the addition of hexane afforded the desired product, 7-chloro-1,3-dihydro-3-hemisuccinyloxy-5-phenyl-2H-1,4-benzodiazepin-2-one (oxazepam hemisuccinate) [10].

For the labeling reaction, 10 mg of the latter product were suspended in 2 ml acetonitrile. To this suspension, 10 ml of a 4-bromomethyl-7-methoxycoumarin solution in acetonitrile (2 mg/ml), 0.25 ml of a 18-crown-6 ether solution in acetonitrile (10 mg/ml) and 10 mg potassium carbonate were added [11]. After derivatization for 1 hour at 60°C, the mixture was analyzed by HPLC-MS to identify the derivatization product (pag 30). The reminder was purified according the method described at pag 30.

Synthesis of Mmc-O-CO-(CH₂)₂-CO-lormetazepam
To a suspension of 0.2 g lormetazepam in 10 ml dry tetrahydrofuran, 30 mg of a 60% suspension of sodium hydride in mineral oil were added. After stirring for 0.5 hour at room temperature under a nitrogen atmosphere, 95 mg succinic anhydride were added and stirring was continued at 40°C. After 2 hours, the tetrahydrofuran was evaporated under vacuum. The residue was suspended in 20 ml water, transferred into a separation funnel, acidified with acetic acid, and extracted twice with 30 ml dichloromethane. After drying the dichloromethane phase with anhydrous sodium sulphate, the dichloromethane was evaporated to half its volume and the addition of hexane afforded the desired product, 7-chloro-1,3-dihydro-3-hemisuccinyloxy-1-methyl-2H-1,4-benzodiazepin-2-one (lormetazepam hemisuccinate) [10].

For the labeling reaction, 10 mg of the latter product were suspended in 2 ml acetonitrile. To this suspension, 10 ml of a 4-bromomethyl-7-methoxycoumarin solution in acetonitrile (2 mg/ml), 0.25 ml of a 18-crown-6 ether solution in acetonitrile (10 mg/ml) and 10 mg potassium carbonate were added [11]. After derivatization for 1 hour at 60°C, the mixture was analyzed by HPLC-MS to identify the derivatization product (pag 30). The reminder was purified according the method described at pag 30.

Synthesis of dansyl-Ro5-3072
To a glass test-tube with 100 mg dansyl chloride, 0.5 ml of 0.5 M sodium carbonate solution and 10 ml of a 7-aminotrazepam (Ro5-3072) solution (1 mg/ml in acetone) were added [12]. After incubation for 3 hours at 45°C, the clear solution was transferred into a second glass test-tube and the solvent was evaporated under a nitrogen atmosphere. The residue was dissolved in 10 ml methanol.
Synthesis of dansyl-Ro7-1986
To a glass test-tube with 100 mg dansyl chloride, 0.5 ml of 0.5 M sodium carbonate solution and 10 ml of a didesethylflurazepam (Ro7-1986) solution (1 mg/ml in acetone) were added [12]. After incubation for 3 hours at 45°C, the clear solution was transferred into a second glass test-tube and the solvent was evaporated under a nitrogen atmosphere. The residue was dissolved in 10 ml methanol.

Identification of the derivatization products by HPLC-MS
Since we synthesized only small amounts of the fluorescent-labeled benzodiazepines, we purified them by HPLC, using an analytical RP-C\textsubscript{18} column. After collecting the fractions containing the desired compounds, the fluorescent-labeled benzodiazepines were isolated from the mobile phase by extraction with C\textsubscript{18} Empore\textsuperscript{TM} Extraction Disks. After the derivatization reactions, 100 µl of the solutions were evaporated and the residues were dissolved in 1 ml methanol. From these solutions, 20 µl were injected onto a reversed phase column (LiChrospher\textsuperscript{®} 100 RP-18 (5 µm), 125 x 4 mm i.d., Merck, Darmstadt, Germany) and eluted with a linear gradient water/methanol mobile phase, starting with 70 % water and 30% methanol and finishing with 100% methanol after 10 min. The flow was 1 ml/min and the eluents were monitored by UV detection at 254 nm and mass spectrometric detection.

Purification with HPLC
After the derivatization, 20 µl aliquots of the derivatization solutions were injected onto a reversed phase column (LiChrospher\textsuperscript{®} 100 RP-18 (5 µm), 125 x 4 mm i.d., Merck, Darmstadt, Germany). The mobile phases used were different for each fluorescent-labeled 1,4-benzodiazepine and are listed in Table 2.1. The eluent was monitored by UV detection at 254 nm and the fractions containing the fluorescent-labeled 1,4-benzodiazepine were collected with the fraction collector. Water was added to reduce the methanol concentration to 10%. The fluorescent-labeled 1,4-benzodiazepine was isolated from the water/methanol solution with a C\textsubscript{18} Empore\textsuperscript{TM} Extraction Disk.

Table 2.1

<table>
<thead>
<tr>
<th>m/z ratios of the synthesized fluorescent-labeled 1,4-benzodiazepines and the mobile phase composition used for the purification of these compounds.</th>
<th>m/z</th>
<th>mobile phase composition H\textsubscript{2}O/MeOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mmc-O-CO-(CH\textsubscript{2})\textsubscript{2}-CO-oxazepam</td>
<td>575</td>
<td>70/30</td>
</tr>
<tr>
<td>Mmc-O-CO-(CH\textsubscript{2})\textsubscript{2}-CO-lormetazepam</td>
<td>623</td>
<td>70/30</td>
</tr>
<tr>
<td>dansyl-Ro5-3072</td>
<td>485</td>
<td>40/60</td>
</tr>
<tr>
<td>dansyl-Ro7-1986</td>
<td>565</td>
<td>30/70</td>
</tr>
</tbody>
</table>
The disk was kept under vacuum for 15 min after the solution passed the disk and the fluorescent-labeled 1,4-benzodiazepine was eluted by 5 ml methanol. This extraction procedure was repeated until the entire water/methanol solution had been treated. The methanol eluates were collected in a weighed glass test-tube and the methanol was evaporated under vacuum with an Univapo 150 H centrifuge (UniEquip, Martinsried, Germany). After the evaporation of the methanol, the weight of the glass test-tube was determined again. The difference in weight was considered as the yield of the purified fluorescent-labeled benzodiazepine. The fluorescent-labeled benzodiazepines were dissolved in 10.0 ml methanol and the purity was checked by HPLC (same conditions as were used for the purification). The solutions of the fluorescent-labeled benzodiazepines were stored at -20°C.

Determination of the fluorescence characteristics of the fluorescent-labeled 1,4-benzodiazepines

The stock solutions of the fluorescent-labeled 1,4-benzodiazepines were diluted one hundred fold with Tris-HCl buffer (50 mM, pH 7.4) and the fluorescence spectra were recorded on a Kontron SFM 25 spectrofluorometer (Zürich, Switzerland).

Since not all spectra were recorded on the same day, a calibrator, quinine sulfate, was also determined at an excitation wavelength of 351 nm and an emission wavelength of 448 nm. Quinine sulphate was dried at 100°C to constant weight and dissolved in 1 N sulphuric acid (5 mM) [13].

Preparation of membrane-bound receptors

Calf brains, obtained from the local slaughterhouse and stored at -80°C after discarding the cerebella, were homogenized in six volumes (w/v) of ice-cold 0.32 M sucrose in a Potter-Elvehjem homogenizer (RW 20 DZW, Janke & Kunkel KG, Staufen i. Breisgau, Germany) fitted with a Teflon pestle and centrifuged for 10 min at 10000 x g in a Beckman L8-55 Ultracentrifuge (Beckman Instruments, Mijdrecht, The Netherlands). The supernatant was centrifuged for 60 min at 100.000 x g. The resulting pellet (P<sub>2</sub>) was resuspended in sodium phosphate buffer (pH 7.4; 50 mM) and centrifuged for 30 min at 100.000 x g. This washing step was repeated once. All operations were performed at 4°C. The washed P<sub>2</sub>-pellet was resuspended in five volumes (w/v) of phosphate buffer, frozen with liquid nitrogen and lyophilized (Hetosisc CD 52-1, Heto, Birkerod, Denmark). The lyophilized P<sub>2</sub>-pellet was stored at -20°C. For the receptor binding assays, the lyophilized P<sub>2</sub>-pellet was resuspended in Tris-HCl buffer (pH 7.4; 50 mM) with a glass-teflon Potter-Elvehjem homogenizer (2.5 mg/ml).

Receptor binding assay

For the binding assay, 50 µl [H]flunitrazepam solution (0.5 nM final concentration) in Tris-HCl buffer (pH 7.4; 50 mM) was mixed in duplicate with 50 µl Tris-HCl buffer, containing the fluorescent-labeled benzodiazepines or their respective parent compounds (200 nM - 6 pM final concentration). To this mixture, 400 µl of the receptor suspension were added, vortexed and incubated for 45 min at 4°C. The incubation was ended by adding 4 ml ice-cold Tris-HCl buffer and this 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 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).

2.3 Results and discussion

Synthesis of the fluorescent-labeled benzodiazepines

The chemical structures of the fluorescent-labeled benzodiazepines are shown in the Appendix. After the synthesis of the fluorescent-labeled 1,4-benzodiazepines, the derivatization products were checked by reversed-phase HPLC-MS. The fluorescent-labeled 1,4-benzodiazepines were purified by reversed-phase HPLC with UV detection. The identities of the fluorescent-labeled 1,4-benzodiazepines were not further confirmed by NMR, since the labels could only be coupled to one position of the benzodiazepine molecule. The verification of the mass by MS was considered sufficient for identification. The m/z ratios of the different fluorescent-labeled 1,4-benzodiazepines are reported in Table 2.1.

For the synthesis of different fluorescent-labeled substances, 1,4-benzodiazepines with reactive groups, such as hydroxy- or amino-groups, were required. We selected the 3-hydroxybenzodiazepine oxazepam and lormetazepam, the only active 1,4-
Synthesis and characterization of fluorescent-labeled 1,4-benzodiazepines

benzodiazepines with a reactive group. We also used two metabolites, 7-aminonitrazepam, with no affinity for the benzodiazepine receptor, and didesethylflurazepam, Ro7-1986, for labeling.

The reactive groups of 7-aminonitrazepam, didesethylflurazepam and the 3-hydroxybenzodiazepines oxazepam and lormetazepam are located at three different positions of the basic structure of the 1,4-benzodiazepine molecule, which is shown in Figure 2.1.

By using 1,4-benzodiazepines with different labeling positions, we also wanted to examine which position of the benzodiazepine molecule can be labeled so that the resulting product still exhibits sufficiently high binding affinity to the benzodiazepine receptor. The influence of the fluorophores used in this study on the affinity for the benzodiazepine receptor can also be examined because others have labeled didesethylflurazepam with different labels [1-3].
Purification of the fluorescent-labeled 1,4-benzodiazepines

The synthesized fluorescent-labeled 1,4-benzodiazepines were purified by reversed-phase HPLC. This was done with an analytical C<sub>18</sub>-column, since a semi-preparative column did not provide adequate resolution. The use of an analytical column had the disadvantage that only small amounts of the fluorescent-labeled 1,4-benzodiazepines could be purified. In one run, about 30 µg of the derivatization product could be brought onto the column. Applying more onto the column caused peak broadening, resulting in insufficient resolution. The fluorescent-labeled 1,4-benzodiazepines were isolated from the mobile phase to obtain the product in a dry state. The amount of purified fluorescent-labeled 1,4-benzodiazepine was established by weighing the glass test-tubes with and without the purified product. To minimize the error in weighing, the minimum amount of purified fluorescent-labeled benzodiazepine had to be at least 2 mg. The recovery from this purification procedure was 50-70%. The residues were dissolved in 10 ml methanol and the purity was checked by reversed-phase HPLC, under the same conditions as the purifications were done, and was minimal 95%. Takeuchi and Rechnitz [1] purified their fluorescent-labeled benzodiazepine, AMCA-Ro7-1986, also by HPLC, but they did not isolate their product from the mobile phase. After the removal of the acetonitrile by evaporation, the concentration in their remaining eluent was determined from the molar extinction coefficient of the fluorophore, assuming that the molar extinction coefficient of the label had not been affected by coupling the benzodiazepine to the fluorophore. Because this assumption is questionable, we preferred to isolate the fluorescent-labeled benzodiazepine from the mobile phase.

Fluorescence characteristics of the fluorescent-labeled 1,4-benzodiazepines

The optimum excitation and emission wavelengths of the fluorescent-labeled 1,4-benzodiazepines in Tris-HCl buffer (pH 7.4; 50 mM) are reported in Table 2.2.

<table>
<thead>
<tr>
<th>Relative fluorescence</th>
<th>( \lambda_{\text{ex}} ) (nm)</th>
<th>( \lambda_{\text{em}} ) (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-aminonitrazepam</td>
<td>-NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>-H</td>
</tr>
<tr>
<td>Didesethylflurazepam</td>
<td>-Cl</td>
<td>-CH&lt;sub&gt;2&lt;/sub&gt;CH&lt;sub&gt;2&lt;/sub&gt;NH&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>Oxazepam</td>
<td>-Cl</td>
<td>-H</td>
</tr>
<tr>
<td>Lormetazepam</td>
<td>-Cl</td>
<td>-CH&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
</tbody>
</table>

Fluorescence signals were measured as relative fluorescence values with quinine sulphate as calibration sample. The molar fluorescence signals of the fluorescent-labeled 1,4-benzodiazepines were next expressed relative to quinine (5 mM) and are

The fluorescence characteristics of the fluorescent-labeled 1,4-benzodiazepines are summarized in Table 2.2.
reported in Table 2.2. In this way, the fluorescence sensitivity of the different fluorophores can be compared easily to see which fluorescent-labeled 1,4-benzodiazepine has the most suitable fluorescence characteristics for use in a fluorescence receptor assay. This comparison will be done in Chapter 4.

Table 2.3  

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_i$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>oxazepam</td>
<td>16.7</td>
</tr>
<tr>
<td>Mmc-O-CO-(CH$_2$)$_2$-CO-oxazepam</td>
<td>&gt;1 µM</td>
</tr>
<tr>
<td>lormetazepam</td>
<td>1.2</td>
</tr>
<tr>
<td>Mmc-O-CO-(CH$_2$)$_2$-CO-lormetazepam</td>
<td>114 nM</td>
</tr>
<tr>
<td>nitrazepam</td>
<td>7.4</td>
</tr>
<tr>
<td>7-aminonitrazepam (Ro5-3072)</td>
<td>470 nM</td>
</tr>
<tr>
<td>dansyl-Ro5-3072</td>
<td>&gt;1 µM</td>
</tr>
<tr>
<td>flurazepam</td>
<td>10.4</td>
</tr>
<tr>
<td>didesethylflurazepam (Ro7-1986)</td>
<td>4.9</td>
</tr>
<tr>
<td>dansyl-Ro7-1986</td>
<td>167 nM</td>
</tr>
<tr>
<td>Bodipy FL-Ro7-1986</td>
<td>67 nM</td>
</tr>
<tr>
<td>NBD-Ro7-1986</td>
<td>51 nM</td>
</tr>
<tr>
<td>NBD-(CH$_2$)$_2$CO-Ro7-1986</td>
<td>132 nM</td>
</tr>
<tr>
<td>NBD-(CH$_2$)$_5$CO-Ro7-1986</td>
<td>163 nM</td>
</tr>
<tr>
<td>fluorescein-Ro7-1986</td>
<td>74 nM</td>
</tr>
<tr>
<td>AMCA-Ro7-1986 [1]</td>
<td>8.6 nM</td>
</tr>
</tbody>
</table>

Binding affinities of the fluorescent-labeled 1,4-benzodiazepines
The affinities of the fluorescent-labeled 1,4-benzodiazepines were calculated from their inhibition curves. The inhibition curves were fitted with the program EDBA-Ligand, V4 (Biosoft, Cambridge, UK) [14] using a one-binding site model. The results are presented in Table 2.3 and the discussion about the most suitable position for labeling and best fluorophore to be used will be done in Chapter 4.

References

Synthesis and characterization of fluorescent-labeled 1,4-benzodiazepines