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Inclusion complexation of diazepam with different cyclodextrins in formulations for parenteral use

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A parenteral formulation for the water-insoluble benzodiazepine diazepam was developed. Different cyclodextrins (CDs) suitable for parenteral injection: hydroxypropyl-β-cyclodextrin (HP-β-CD), hydroxypropyl-γ-cyclodextrin (HP-γ-CD), sulfobutylether-7-β-cyclodextrin (SBE-7-β-CD) and maltosyl-β-cyclodextrin (malt-β-CD) were used as alternatives to cosolvents to increase solubility. The increase in solubility displayed a concentration dependency for the four CDs used. Diazepam's solubility is enhanced linearly as a function of each CD concentration. The highest improvements in solubility (dissolved concentration circa 3.5 mg/ml in 40% CD) were found by adding HP-β-CD or SBE-7-β-CD. The additional use of polyvinylpyrrolidone (PVP) did not further increase the solubility of diazepam with HP-β-CD. A parenteral aqueous diazepam solution was prepared containing 10 mg diazepam/5 ml 30% HP-β-CD or SBE-7-β-CD solution. The preparations are in agreement with the requirements for parenteralia. Sterilisation by filtration is required since autoclaving degrades the active compound. The stability of the preparations, with and without pH adjustment to pH 5, was investigated during 18 months and during this period no noticeable degradation was observed.

1. Introduction

Diazepam or 1,7-chloro-1,3-dihydro-1-methyl-5-phenyl-2*H*-1,4-benzodiazepine-2-one is a long-acting benzodiazepine (BZD) with anticonvulsant, anxiolytic, sedative, muscle relaxant and amnesic properties. Its actions are mediated by enhancement of the activity of gamma aminobutyric acid (GABA), a major inhibitory neurotransmitter in the brain (Martindale 1999). Diazepam is generally accepted as an intravenous (i.v.) sedative or anesthetic with applications in dentistry and medicine (Prancan et al. 1980). It is one of the more stable substituted 1,4-benzodiazepines, but it undergoes hydrolysis in aqueous solution (Connors et al. 1986; DAB 1999). Maximum stability towards hydrolysis occurs around pH 5 (Connors et al. 1986; Han et al. 1977).

Diazepam's solubility in water is low (European Pharmacopoeia 1997) and thus its parenteral administration is problematic. The composition of the currently marketed formulation Valium® (Roche) is diazepam 10 mg, propylene glycol 828.8 mg, ethanol 161.2 mg, sodium benzoate 97.6 mg, benzoic acid 2.4 mg, benzylic alcohol 31.4 mg and aqua ad injectabilia ad 2 ml (AVGI 2001; Van Sorge et al. 1986). Pharmaceuticals having poor water solubility are often formulated with the aid of cosolvents and/or by pH adjustment. These products tend to precipitate when diluted in blood or other aqueous fluids (Li et al. 1998). Diazepam injection is known to precipitate in the blood stream, thereby forming crystals which can produce severe pain and thrombophlebitis. Altered bioavailability also might be observed (Li et al. 1998; Prancan et al. 1980; Van Sorge et al. 1986; Yalkowsky et al. 1983, 1998). A frequent side effect upon intramuscular (i.m.) injection of diazepam is pain and skeletal muscle damage. The resorption after i.m. injection is irregular and unpredictable (Brazeau and Fung 1990; Prancan et al. 1980; Van Sorge et al. 1986).

Because of these shortcomings, the development of a safer diazepam formulation as an alternative to the one currently used should be considered of notable interest. A possibility in the development of an acceptable aqueous parenteral formulation is the use of cyclodextrins (CDs) (Brewster et al. 1995; Trapani et al. 1998). CDs are a group of cyclic oligosaccharides that have been shown to improve pharmaceutical properties of drugs, such as solubility and stability, by forming inclusion complexes (Loftsson and Brewster 1996; Rajewski and Stella 1996). The circular arrangement of the glucose units produces a torus-shaped molecule, with a hydrophobic interior cavity and a polar exterior. The interior cavity of the β-CDs is appropiate in size to accommodate a benzene ring. When a compound with appropriate geometry and a CD are in the same solution, the nonpolar aromatic part of the compound tends to enter the interior of the β-CD molecule. This complexation excludes the aromatic part of the drug molecule from the water, thereby increasing its aqueous solubility (Martin et al. 1983; McCandless and Yalkowsky 1998). Native CDs (α-, β- and γ-CDs), how-

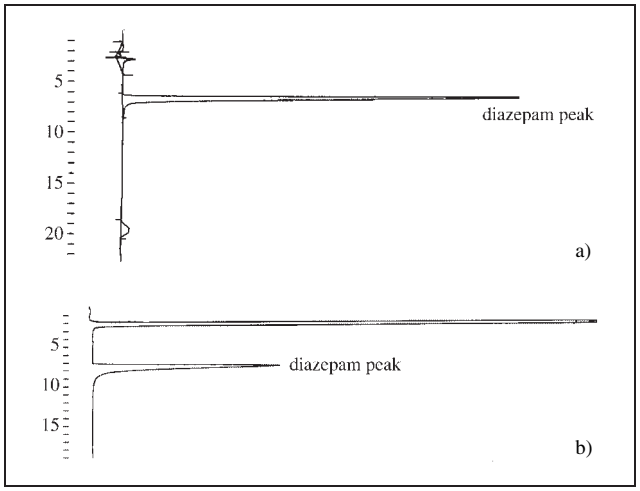

Fig. 1: a) Chromatogram of 2.01 mg diazepam/ml 30% HP-β-CD after autoclaving (10 times diluted before injection on HPLC). b) Chromatogram of about 0.2 mg diazepam/ml 2 N HCl, stored at 45 °C during 1 h

ever, cannot be used in parenteral formulations due to their low solubility. They also exhibit nephrotoxic and hemolytic activities (Duchêne and Wouessidjewe 1990; Torres-Labandeira et al. 1990; Zia et al. 1997). In addition, various modified CDs have been developed to improve the physical, chemical and safety properties of the native CDs (Ma et al. 2000; Narisawa and Stella 1998). Specific interest in HP-β-CD and SBE-7-β-CD has arisen from their better intrinsic solubility and safety compared to the parent material (Okimoto et al. 1996). Malt-β-CD also seems to be safe in most circumstances (Loftsson and Brewster 1997) was therefore also examined.
The aim of this paper was to compare the solubility enhancement of diazepam by complexation with different CDs. Parenteral formulations without cosolvents containing the required 10 mg diazepam were developed and tested.

2. Investigations, results and discussion

2.1. Evaluation of the HPLC method

The analytical HPLC assay of diazepam employing an isocratic elution system with a mobile phase of methanol-water (70 : 30, v/v), a flow rate of 1.0 ml/min and a detection wavelength of 314 nm, described by Rasool et al. (1991), was used here. The retention time for diazepam on the column applied was about 7.5 min (Fig. 1a). Standard curves were linear ($r = 0.9999$) over the concentration range 0.01–0.50 mg/ml diazepam. Before constructing diazepam solubility curves with the four CDs, the interference of 4% of each CD, the highest concentration occurring in the analytical samples, in the chromatogram was evaluated. No interference with the diazepam peak was observed. Only a solvent peak was observed due to the UV-transparancy of the CDs. An overview of the injection repeatability of 2 diazepam standards and of 4 diazepam-CD samples is given in Table 1. The data showed all RSD below 0.85%, which is a frequently used limit in the European Pharmacopoeia (2002). The results of the recovery studies conducted on the finally selected formulations are shown in Table 2. Each formulation was spiked at the 100% level, adding the known amount of 10 mg diazepam to the CD-solution and assaying the concentration of diazepam in the resulting solution by the HPLC method.

Table 1: Injection repeatability of the HPLC assay for diazepam

Sample	% RSD (n = 6)
Diazepam standard: 0.1074 mg/ml	0.67
Diazepam standard: 0.2104 mg/ml	0.26
2.46 mg diazepam/ml 30% HP-β-CD	0.79
1.02 mg diazepam/ml 15% SBE-7-β-CD	0.43
1.48 mg diazepam/ml 30% malt-β-CD	0.24
0.41 mg diazepam/ml 10% HP-γ-CD	0.39

Table 2: Accuracy/recovery of diazepam from the selected formulations

Formulation	Amount of diazepam (mg)		% Recovery
	Added	Recovered	
10 mg diazepam/5 ml 30% HP-β-CD, without pH adjustment	9.943	9.917	99.74
	10.016	10.029	100.13
with pH adjustment	9.961	10.036	100.75
	10.003	9.999	99.96
10 mg diazepam/5 ml 30% SBE-7-β-CD, without pH adjustment	10.019	10.109	100.90
	10.009	10.001	99.92
with pH adjustment	9.983	9.970	99.87
	9.956	9.963	100.07

The data show that quantitative recovery of diazepam is obtained and that the analysis method is accurate.
To ensure that the HPLC method was also stability-indicating and that the decomposition products of diazepam were not interfering with the diazepam peak, an attempt was made to degrade diazepam by heating. The diazepam peak then decreased and a small peak with a retention time of about 20 min appeared (Fig. 1a). The degradation of diazepam in 2M NaOH and in 2M HCl (Fig. 1b) was also investigated and no interference was observed in the neigbourhood of the mean peak. The applied HPLC system thus can be considered stability-indicating for diazepam as no degradation products interfered with the diazepam peak.

2.2. Solubility studies

Solubility curves of diazepam with each CD were drawn to define the amount of a CD needed to dissolve the desired 10 mg of diazepam. Solubility diagrams were constructed by plotting the experimentally determined dissolved concentrations of diazepam in water, expressed in mg/ml, as a function of the % (g/100 ml) CD concentration. This representation gives direct insight in incapsulation efficiency. Fig. 2 shows the diazepam solubility diagrams with HP-β-CD, HP-γ-CD, SBE-7-β-CD and malt-β-CD both at 25 °C and 4 °C ($n = 2$). Diazepam was found to interact with all tested CD derivatives. The interactions can be ranked in descreasing order: HP-β-CD ≈ SBE-7-β-CD > malt-β-CD ≫ HP-γ-CD. In the absence of CD, the solubility of diazepam is found around 0.05 mg/ml, i.e. 0.055 mg/ml at 4 °C ($n = 8$, RSD = 1.4%) and 0.063 mg/ml ($n = 8$, RSD = 1.2%) at 25 °C, which is in agreement with literature values: 0.069 mg/ml (Loftsson et al. 1994), 0.05 mg/ml (Connors et al. 1986) and 0.037 mg/ml (Rasool et al. 1991). The solubility of diazepam is notably affected by the presence of CDs. The solubility enhancement of diazepam for the different CDs is always somewhat lower at 4 °C than at 25 °C. Up to 3.5 mg/ml can be

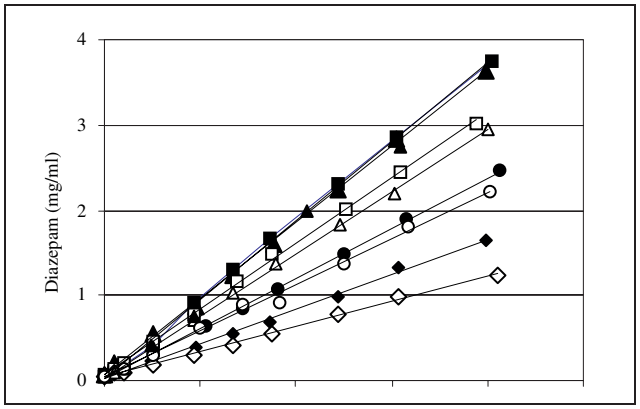

Fig. 2: Concentration of diazepam (mg/ml) in solution as a function of cyclodextrin concentration (g/100 ml) at 25 °C and 4 °C. ■: HP-β-CD at 25 °C, ▲: SBE-7-β-CD at 25 °C, ●: malt-β-CD at 25 °C, ◆: HP-γ-CD at 25 °C, □: HP-β-CD at 4 °C, △: SBE-7-β-CD at 4 °C, ○: malt-β-CD at 4 °C, ◇: HP-γ-CD at 4 °C

dissolved with either 40% (w/v) HP-β-CD or SBE-7-β-CD. About 2.2 mg/ml and 1.5 mg/ml diazepam can be dissolved with 40% malt-β-CD and 40% HP-γ-CD, respectively. Thus, a 40% HP-β-CD solution provided an about 70-fold increase in solubility of diazepam compared to water.

In summary, we achieved the targeted dissolution of 10 mg diazepam by preparing complexes of diazepam with 5 ml of a 30% or with 4 ml of a 40% HP-β-CD or SBE-7-β-CD solution.

The solubility diagrams can also be presented by plotting the molar concentrations of diazepam as a function of the molar CD concentration in order to determine the type of complexation (figure not shown since similar to Fig. 2). The solubility profiles correspond to the A_L type diagrams since there was a linear increase of diazepam in a wide range of CD concentrations (Higuchi and Connors 1965). Because such profiles are characterised by a slope below one, it was suggested that the increase in solubility is due to the formation of a 1 : 1 drug : CD complex (Higuchi and Connors 1965). The formation of a 1 : 1 complex is important concerning precipitation (see 2.3. clarity). Apparent stability constants ($K_{1:1}$ or K_c) are estimated from the solubility data [CD] in M versus solubilized diazepam in M, according to the equation:

$$K_{1:1} = \text{slope}/[S_0(1 - \text{slope})] \qquad (1)$$

where S_0 is the intrinsic solubility of the drug. The estimated $K_{1:1}$ values varied from 116 M^{-1} for HP-γ-CD up to 348 M^{-1} for SBE-7-β-CD at 25 °C and from 95 M^{-1} up to 313 M^{-1} at 4 °C. The differences in $K_{1:1}$ between the two temperatures are small. For HP-β-CD, the $K_{1:1}$ are situated around 200 M^{-1} and for malt-β-CD around 180 M^{-1}. The stability constant values with the β-CDs were higher than with the γ-CD derivative. Similar conclusions were drawn by Trapani et al. (2000) for the complexation of zolpidem with HP-β-CD and HP-γ-CD. The stability constant found for diazepam with malt-β-CD is similar to what is reported in the literature (Yamamoto et al. 1989). The most stable complexes are formed with SBE-7-β-CD. In general, drugs are more strongly bound to SBE-7-β-CD compared to HP-β-CD (Okimoto et al. 1996). The higher the K_c value, the more diazepam is bound and the slower the release of diazepam for the i.m. and, in general, for the peritoneal administration (Holvoet et al. 2003). However, for i.v. administration, the magnitude of the affinity constant is not important, as the preparation is diluted in the blood. HP-γ-CD and malt-β-CD are excluded from further investigations as they both encapsulate less diazepam than HP-β-CD and SBE-7-β-CD.

An approach to enhance drug solubilisation still more in CD solutions that has been suggested in the literature is the use of small quantities of pharmaceutical polymers, such as water-soluble cellulose derivates and other macromolecular agents (e.g. PVP) (Brewster et al. 1995; Loftsson and Brewster 1997). These compounds are thought to interact with CDs and their guests to form thermodynamically stable ternary complexes (Brewster et al. 1995; Loftsson et al. 1994). Hydroxypropylmethylcellulose (HPMC) and PVP, for instance, improved the solubility of pregnenolone with HP-β-CD (Brewster et al. 1995). The preparation of these CD systems includes heating the drug, polymer and CD mixture to 120–140 °C for 20–40 min (Brewster et al. 1995; Loftsson and Brewster 1997). However, autoclaving is not feasible, as diazepam then degrades. Therefore, in our study, the mixtures were shaken in a thermostatically controlled water bath shaker at 4 °C for 5 days. The hydrophilic polymers did almost not improve the solubilisation of diazepam (Fig. 3). No difference is noticed between the use of different PVPs (PVP 12 and PVP 17) nor of different concentrations (0.10 and 0.25%) (n = 1). Also Brewster et al. (1995) found no improvement by adding PVP to pregnanolone with HP-β-CD. Therefore, PVP will not be added to the selected parenteral solution.

2.3. Preparation and evaluation of the parenteral formulations

A parenteral solution can be prepared with 10 mg diazepam in 5 ml of 30% or in 4 ml of 40% HP-β-CD or SBE-7-β-CD solution, as mentioned above. The quality requirements for parenteralia, such as clarity, pH and sterility are checked for the four formulations, as well as compatibility with the packing material and stability during storage.

Isotonicity: A parenteral preparation is preferably isotonic (osmolality between 280 and 310 mosmol/kg) (DeLuca and Boylan 1992). The 10 mg diazepam solution in 5 ml 30% aqueous HP-β-CD is slightly hypertonic (330 mosmol/kg),

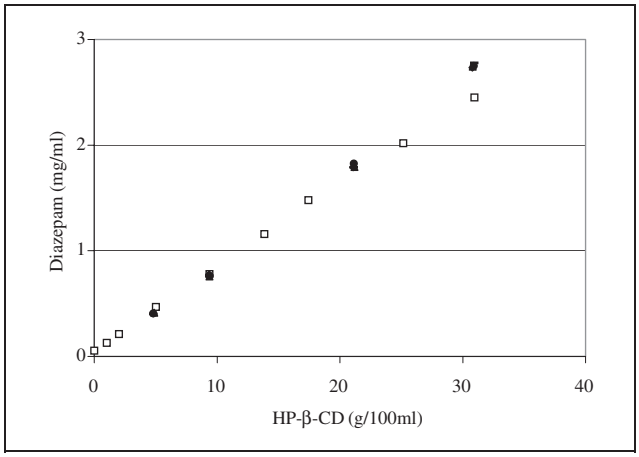

Fig. 3: Effect of different concentrations of water-soluble polymer PVP 12000 and 17000 on the solubilisation of diazepam with HP-β-CD at 4 °C. □: without PVP, ●: with 0.1% PVP 17000, ◆: with 0.25% PVP 17000, —: with 0.1% PVP 12000, ▲: with 0.25% PVP 12000

the one in 4 ml 40% HP-β-CD is hypertonic (520 mosmol/kg) and the one in 5 ml 30% SBE-7-β-CD is even more hypertonic (not measurable, out of scale). The tonicity of a preparation in HP-β-CD is lower than a similar one in SBE-7-β-CD. Therefore, the diazepam solution in 30% HP-β-CD is considered most suitable for parenteral use since it is less hypertonic than the other formulations.

pH: For intravenous application, a pH range of 3.0–10.5 is tolerated, while for parenterals administered by other routes the range narrows to 4–9. In both cases, the physiological pH is preferred. Below pH 3 extreme pain and phlebitis and above pH 9 tissue necrosis can occur (DeLuca and Boylan 1992). The pH of 10 mg diazepam in 30% HP-β-CD and in 30% SBE-7-β-CD, prepared in unbuffered water, is around 6.5.

Sterility: The formulations must be sterile (DeLuca and Boylan 1992). The formulation with 30% HP-β-CD was submitted to autoclaving (121 °C during 15 min): only 88% diazepam ($n = 2$, $RSD = 1.21\%$) is recovered. Therefore, sterilisation by autoclaving is not acceptable and sterile filtration was applied (recovery = 100.1%, $n = 3$).

Pyrogenicity was not checked as the volume of our formulations is less than 15 ml (DeLuca and Boylan 1992; European Pharmacopoeia 2002).

Clarity: Solutions for injection should be clear and free from particles (European Pharmacopoeia 2002). However, 1 : 1 complexes do not precipitate after dilution (Rajewski and Stella 1996) and this statement was verified by an experimental and a theoretical dilution method. Calculation of the theoretical possibility of precipitation of a solution can be done. When a 1 : 1 complex is formed, the amounts of free and bound diazepam in the selected formulations can be calculated for different dilutions using the K_c value and the total CD and diazepam concentrations (Fig. 4). Since the free diazepam concentration, in both the undiluted and diluted samples, was lower than the solubility in water, 2.1×10^{-4} M, it can be expected that the diluted solution will not precipitate.

In the experimental approach, both selected formulations were diluted applying the static serial dilution method of Li et al. (1998). In none of the dilutions (up to 1/1024), diazepam precipitated, which indicates the suitability to administer the parenteral formulations in infusions.

Compatibility with packing material: Loss of diazepam from solution by adsorption to components and containers used in the parenteral administration has been reported in a number of publications (AHFS 2002; AVGI 2001; Martindale 1999). More than 50% of diazepam in solution may be adsorbed onto the walls of polyvinylchloride infusion bags and their use should, therefore, be avoided. Suitable materials for infusion containers, syringes and administration sets when administering diazepam include glass, polyolefin, polypropylene and polyethylene (AVGI 2001; Martindale 1999). Our preparations were stored in small glass bottles during the stability study.

Stability: The chemical stability of the selected formulations containing 10 mg diazepam in 5 ml 30% HP-β-CD or SBE-7-β-CD, with and without pH adjustment to pH 5, was followed as a function of time. The pH of 5 is also evaluated as it is the pH value of maximum diazepam stability (Connors et al. 1986; Han et al. 1977). Degradation was monitored during 18 months. No degradation was observed in the four samples monitored. No difference in stability of diazepam was noted in the solutions with or without initial pH adjustment, nor between SBE-7-β-CD or HP-β-CD containing formulations.

In conclusion, this study evaluates the effects of various CD derivatives (HP-β-CD, HP-γ-CD, SBE-7-β-CD and malt-β-CD) on the solubilisation of diazepam. The interactions can be ranked as: HP-β-CD ≈ SBE-7-β-CD > malt-β-CD ≫ HP-γ-CD. We are able to achieve the target concentration of 10 mg diazepam by preparing an inclusion complex of diazepam in 5 ml of a 30% HP-β-CD or SBE-7-β-CD.

These formulations fulfilled the requirements for parenteral formulations. No precipitation is observed after dilution with water, with 0.9% NaCl-solution and with 5% dextrose, which indicates their suitability for administration with infusions. Sterilisation by sterile filtration is necessary. Concerning the tonicity, the preparation in 30% HP-β-CD is more suitable than in 30% SBE-7-β-CD. The stability of the selected formulations in solutions, without and with pH adjustment to pH 5, the pH of maximum stability of diazepam, is equal. No degradation was noted in either type of preparation. The parenteral formulations remained stable during the tested period of 18 months.

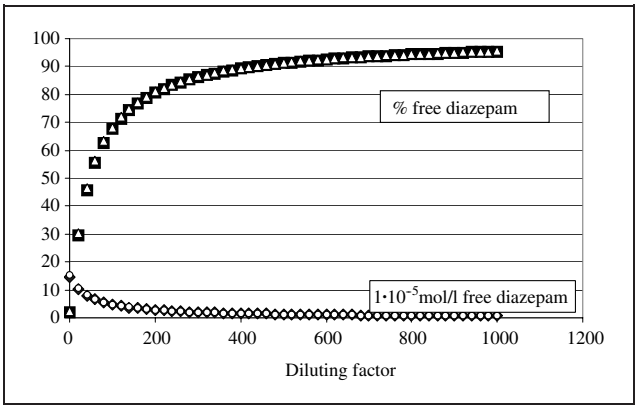

Fig. 4: Concentration of free diazepam ([AP]) ($\times 1.10^{-5}$ mol/l), and % free diazepam (% [AP]) as a function of dilution of the parenteral formulations. ■: % [AP] free from 10 mg diazepam in 5 ml of 30% HP-β-CD, △: % [AP] free from 10 mg diazepam in 5 ml of 30% SBE-7-β-CD, ◆: [AP] from 10 mg diazepam in 5 ml of 30% HP-β-CD, ○: [AP] from 10 mg diazepam in 5 ml of 30% SBE-7-β-CD

3. Experimental

3.1. Chemicals

Diazepam (Mw = 284.74) was obtained from Alpha Pharma (Zwevegem, Belgium). HP-β-CD (Mw = 1380) was a gift from Roquette (Lestrem, France). SBE-7-β-CD (Mw = 2200) was supplied by Cydex (Overland Park, Kansas). HP-γ-CD (Mw = 1703) and malt-β-CD (Mw = 1797) were purchased from Cyclodextrin Technology Development (High Springs, Florida). HPLC mobile phase was prepared with HPLC-grade methanol (Me) (BDH Laboratory Supplies, Poole, England, UK). Polyvinylpyrrolidone (PVP) 17000 and 12000 were obtained from BASF (Ludwigshafen, Germany). A PVDF (polyvinylidene difluoride) membrane filter from Macherey-Nagel (Düren, Germany) was used for filtration and an Acrodisc 13 NV filter from Gelman Sciences (Ann Arbor, MI) for sterile filtration. All membranes had a diameter pore of 0.2 μm. Sodium hydroxide (NaOH) pellets, hydrochloric acid (HCl) 37% and sodium chloride (NaCl) were obtained from Merck (Darmstadt, Germany) and dextrose from UCB (Brussels, Belgium). Milli-Q water, obtained from a milli-Q water purification system (Millipore, Molsheim, France) was used throughout the study.

3.2. Apparatus

The HPLC equipment consisted of a Merck-Hitachi L-6000 pump (Tokio, Japan), a Rheodyne injector (Cotati, California) fitted with a 20 μl loop, and a variable wavelength ultraviolet Perkin Elmer LC 90 UV Spectrophotometric detector (Shelton, Connecticut). The stationary phase was a RP C_{18}, Lichrospher®, 250 × 4 mm; 5 μm column (Merck) in conjunction with a guard column C_{18} Lichrospher® 100, 4 × 4 mm; 5 μm (Merck). For the pH measurements a Radiometer Copenhagen PHM 26 pH meter (Copenhagen, Denmark) was used, calibrated daily with pH 4.00, 7.00 and 10.00

standard buffers (Merck), while for the isotonicity measurements, a Knauer osmometer (Berlin, Germany) was used. The centrifuge was an IEC Centra (International Equipment Company, Bedfordshire, England). The analytical balance is from Sartorius (Göttingen, Germany), the microbalance from Mettler-Toledo (Zürich, Switzerland), the water bath shaker from Branson (Danbury, Connecticut) and the microscope from Carl Zeiss (Oberkochen, Germany), enlargement 10 × 100. A Memmert oven (Schwabach, Germany) was used to study the degradation of diazepam.

3.3. HPLC

As mobile phase, a 70/30 (v/v) Me/water mixture was used; the flowrate was 1 ml/min and the detection wavelength 314 nm. Analyses were performed at ambient room temperature (approximately 20 °C).

Standard preparation: An accurately weighed quantity of diazepam was dissolved in the mobile phase (by ultrasonification during 1 min) using a volumetric flask of 100.0 ml to obtain a stock solution of about 1.00 mg/ml. This solution was diluted 5 times in mobile phase.

Linearity: Linearity was tested in a range of 0.01–0.50 mg/ml. Solutions with a concentration of about 0.01, 0.02, 0.05, 0.10, 0.20, 0.30, 0.40 and 0.50 mg/ml were prepared by proper dilution of the stock solution.

Precision: The repeatability of injection was determined by making six replicate injections of two diazepam standard solutions containing 0.1074 mg/ml and 0.2104 mg/ml diazepam and of 2.46 mg diazepam/ml 30% HP-β-CD, 1.02 mg diazepam/ml 15% SBE-7-β-CD, 1.48 mg diazepam/ml 30% malt-β-CD and 0.41 mg diazepam/ml 10% HP-γ-CD. The procent relative standard deviation (% RSD) of the peak area was determined.

Accuracy: The finally selected formulations of diazepam were prepared, i.e. 10 mg diazepam was dissolved in 5 ml 30% HP-β-CD or in 5 ml 30% SBE-7-β-CD (see 2.). Each preparation was prepared twice and injected 3 times on the HPLC system after a 10 times dilution.

Forced degradation of diazepam: Degradation at increased temperature: a diazepam solution of 2.01 mg/ml 30% HP-β-CD in water was autoclaved (121 °C, 15 min) and analysed by the above mentioned HPLC procedure after a 10 times dilution. Degradation of diazepam under basic or acidic conditions: about 50 mg diazepam in 25 ml of 2N NaOH or of 2N HCl was stored at 45 °C for 1 h, protected from light. Before injection, the supernatant of the samples was diluted 10 times with mobile phase and eluted on the HPLC system. An acidic and basic blank were also injected.

3.4. Solubility

Solubility measurements of diazepam in aqueous solutions were carried out at constant temperatures (4 °C and 25 °C), in the dark, using different CD derivatives at different concentrations (0, 1, 2, 5, 10, 15, 20, 25, 30 and 40% w/v). An excess of diazepam was added to 10.0 ml of each CD solution in screw-capped test tubes. The mixtures were shaken in a thermostatically controlled water bath shaker up to equilibrium (5 days). To avoid changes in concentration due to evaporation, the tubes were wrapped in parafilm. The resulting suspensions were centrifuged for 20 min at 1787 × g and the supernatant filtered through a PVDF filter. The filtrate was diluted with mobile phase to a concentration situated in the linear calibration range.

In a second set of experiments, the effect of a polymeric additive was assessed. In these trials, an excess of diazepam was added to a 5, 10, 20 and 30% w/v solution of HP-β-CD in the presence of 0.1 and 0.25% of either PVP 12000 or PVP 17000.

3.5. Preparation and evaluation of the parenteral formulations

A mass of 10 mg diazepam was equilibrated with 5 ml 30% HP-β-CD or 30% SBE-7-β-CD in water. The mixture was vortexed for about 10 min and stirred at 25 °C for 3 h (until all diazepam was dissolved). The pH adjustment to pH 5.0 was done by adding 0.1 N HCl. The solution was sterilised through the Gelman Science 0.2 μm membrane filter.

Clarity: The selected formulations are sequentially diluted in a one-to-one ratio with solvent such as water, 0.9% NaCl in water and 5% dextrose in water. The occurrence of precipitation was microscopically observed.

Stability: The chemical stability of the selected formulations (10 mg diazepam/5 ml 30% HP-β-CD with and without pH adjustment to pH 5.0, and 10 mg diazepam/5 ml 30% SBE-7-β-CD with and without pH adjustment) was followed during a period of 18 months. For this study, 400 mg diazepam was dissolved in 200.0 ml of 30% aqueous HP-β-CD solution, without pH adjustment. An analogous solution was prepared with pH adjustment to pH 5.0 by adding 0.1 N HCl. The same was done using SBE-7-β-CD. Each 200 ml solution was divided in 40 screw-capped test tubes, wrapped with parafilm. All samples were stored in a thermostatically controlled room of 25 °C. At determined time intervals, a sample of the four different solutions was analysed. Before HPLC-analysis, the samples were diluted 10 times with mobile phase. The concentration in the sample was estimated by a one-point calibration with a 0.20 mg/ml diazepam standard.

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