International Journal of Pharmaceutics 379 (2009) 244–250

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Development and formulation of a 0.2% oral solution of midazolam containing γ-cyclodextrin

Frédéric Marçon[a], David Mathiron[b], Serge Pilard[b], Anne-Sophie Lemaire-Hurtel[c], Jean-Marc Dubaele[a], Florence Djedaini-Pilard[d,*]

[a] Pharmacie Centrale, Centre Hospitalier Universitaire, place Victor Pauchet, F-80054 Amiens, France
[b] Plateforme-analytique, Université de Picardie Jules Verne, 33 rue Saint-Leu, F-80039 Amiens, France
[c] Laboratoire de pharmacologie & des gaz du sang, Centre Hospitalier Universitaire – Groupement hospitalier sud, avenue René Laënnec, F-80054 Amiens, France
[d] Laboratoire des Glucides UMR6219, Université de Picardie Jules Verne, 33 rue Saint-Leu, F-80039 Amiens, France

ARTICLE INFO

Article history:
Received 23 February 2009
Received in revised form 17 April 2009
Accepted 18 May 2009
Available online 23 May 2009

Keywords:
γ-Cyclodextrin/midazolam inclusion complex
Pre-anesthesia
Oral solution
NMR
LC–HRMS
HPLC quantification

ABSTRACT

In absence of dedicated children formulation, intravenous formulations of midazolam, which exhibit strong bitterness, are occasionally used for oral or sublingual administration. In order to improve the quality and the acceptance by children of a midazolam anesthesia premedication, a new 0.2% (w/v) aqueous solution for oral administration has been prepared. The final formulation was obtained by the adjunction of a sweetener (sucralose), an aroma (orange aroma) and γ-cyclodextrin to a citric acid solution of midazolam. The γ-cyclodextrin forms an inclusion complex with the hydrophobic midazolam as evidenced using nuclear magnetic resonance spectroscopy (stoichiometry 1:1, $K = 283\ M^{-1}$). A sterile filtration method was selected for the formulation microbial preservation using liquid chromatography coupled to high resolution mass spectrometry (LC–HRMS). Finally, a routine high performance liquid chromatography (HPLC) method is proposed for the quantitative determination of global midazolam amount in the pharmaceutical preparation.

1. Introduction

Midazolam is a preoperative anesthetic, it belongs to the class of 2,3-dihydro-1*H*-1,4-benzodiazepine and exhibits anticonvulsant, anxiolytic, muscle relaxant and sedative properties. Clinically the midazolam is quite appreciated because of its pharmacokinetics properties (short half-life and short delay of action), the decrease in post-surgical behavior's troubles (McGraw and Kendrick, 1998; Ko et al., 2001), its intrinsic anxiolytic properties and the anterograde amnesia it induces. Midazolam is commonly employed in pre-anesthesia of children due to its easiness of use when administered orally or by sublingual routes. It is generally used at 0.2 mg/kg when administered by sublingual route and 0.4 mg/kg when administered by the oral one (Davies and Waters, 1998). However its main drawback is its strong bitterness which may be disgusting for children. Moreover midazolam formulations are hampered by its low solubility in aqueous solution. The development of a new 0.2% (w/v) aqueous oral solution which hides the bitterness of midazolam will be more comfortable for the use of midazolam as pre-anesthetic agent in pediatrics.

Midazolam is a weak acid with a p*K*a of 6.2 in water. It is well documented that this acido-basic equilibrium involves the opening of the diazepin ring by cleavage of the imine function leading to the formation of an amino ketone, known as "ring-open" form (Loftsson et al., 2001). At neutral and physiological pH, midazolam is a hydrophobic drug with a partition coefficient log *P* (octanol/water) = 4.8, having a weak solubility in water (approximately 10.3 mg/mL pH 3.4, 25 °C) (Ali and Upadhyay, 2008). For pharmaceutical applications, acid buffer such as citric acid/sodium citrate can be added to improve midazolam solubility in aqueous solution. On the other hand, it is well-known that cyclodextrins (CDs) can form inclusion complexes with a variety of guest molecules and that inclusion in CD is a convenient alternative to solve the problem encountered in the administration of hydrophobic drugs in terms of stability, solubility and also bioavailability (Dodziuk, 2006; Loftsson et al., 2007). In some cases the CDs are also used to reduce several drawbacks such as local drug irritation or bad tastes (Loftsson and Duchêne, 2007). Several and recent investigations have attempted to form inclusion complexes between midazolam and CDs (Loftsson et al., 2001; Armijo et al., 2004; Ali and Upadhyay, 2008; Amorim et al., 2008). T. Loftsson has shown that solubilization of benzodiazepines (alprazolam, midazolam and triazolam) was enhanced in presence of β-CD or sulfobutylether-β-CD (17 mg/mL pH 4.3, SBEβ-CD 14% (w/v) for midazolam) leading to

* Corresponding author.
E-mail address: florence.pilard@u-picardie.fr (F. Djedaini-Pilard).

0378-5173/$ – see front matter.
doi:10.1016/j.ijpharm.2009.05.029

the formulation of a midazolam nasal spray (Loftsson et al., 2001). A complete characterization by NMR of midazolam hydrochloride/β-CD complex has been also reported pointing out the formation of 1:1 inclusion complex (Ali and Upadhyay, 2008).

In the present work, we wish to demonstrate that the use of γ-CD can offer solutions for midazolam bitterness masking and solubility improvement. At our knowledge, no inclusion complex has been previously described between γ-CD and midazolam. Furthermore, γ-CD is the less toxic (Bar and Ulitzur, 1994) and the more water soluble (Szejtli, 1992) of the natural CDs. In a first approach, the advantages of the γ-CD formulation, in terms of solubility and taste have been investigated. NMR characterization of the complex formed with midazolam used as guest and γ-CD used as host in citric acid aqueous solution is presented. ^{1}H NMR studies have confirmed the formation of inclusion complex and allowed the determination of stoichiometry and associate constant value. The 3D structure in solution of the complex was elucidated using T-Roesy experiment (Péan et al., 1999). The influence of the different components of the dedicated preparation upon the stability of the complex has been also evaluated. On the other hand, the midazolam stability according to the formulation sterilization process was monitored using LC–HRMS. Finally, a high performance liquid chromatography (HPLC) quantification method was validated for controlling the midazolam concentration in pharmaceutical batches.

2. Materials and methods

2.1. Materials

Midazolam, citric acid, sucralose and orange aroma were purchased from INRESA pharma (Bartenheim, France), CDs were purchased from Wacker Chimie (Lyon, France), sterile water for injection from Lavoisier (Paris, France). All components were pharma-grade and/or approved for human use. Analytical grade HPLC solvents were purchased from Fischer Scientific (Illkirch, France) or VWR (Pessac, France). D_2O for NMR experiments was purchased from CortecNet (Voisins le Bretonneux, France).

2.2. Solubility studies

Phase solubility studies were carried out as described by Higuchi and Connors (1965). Excess amounts of midazolam were added (300 mg) to a 10 mL citric acid solution (4.2 g/L) containing different concentrations (from 2.5 to 20 mM) of CDs. The suspensions were maintained at 25 °C for 24 h. Then, an aliquot was filtered through 0.2 μm cellulose filters from Grace (Templemars, France), 1:1000 diluted in the citric acid solution and analyzed by UV (λ_{max} = 223–227 nm) using a SECOMAM UviLight PC 2 spectrophotometer (Alès, France).

2.3. NMR studies

All NMR experiments were performed at 500.13 and 300.13 MHz using Bruker DRX500 and DMX300 spectrometers equipped with a *Z*-gradient unit for pulsed-field gradient spectroscopy. Me_4Si was used as an external standard and calibration was performed using the signal of the residual protons of the solvents as a secondary reference. Measurements were performed at 300 K with careful temperature regulation. The length of the 90° pulse was approximately 7 μs. 1D NMR data spectra were collected using 16K data points. 2D experiments were run using 1K data points and 512 time increments. The phase sensitive (TTPI) sequence was used and processing resulted in a 1K × 1K (real–real) matrix. Details concerning experimental conditions are given in the figure captions.

Table 1
Formulation components.

Components	Quantities
Midazolam	200 mg
γ-Cyclodextrin	8000 mg
Sucralose	150 mg
Citric acid	420 mg
Orange aroma	12 drops
Sterile water	QSP 100 mL

2.3.1. Determination of the stoichiometry

Job Plot procedure (Job, 1925) was applied. ^{1}H NMR spectra of twelve samples of mixture of γ-CD and midazolam were recorded in 40 mM of citric acid solution. The sum of both species was kept constant at 10 mM, the molar ratio r of each component was varied from 0 to 1. The observation of any chemical shift of the host or guest varying in a linear fashion with the concentration of bound species was obtained and affords the corresponding Job plots as described elsewhere (Djedaini-Pilard et al., 1990).

2.3.2. Determination of the associate constant value

The concentration of γ-CD was kept constant at 0.2 mM in presence of 40 mM of citric acid while the concentration of midazolam was varied from 20 to 35 mM. Measurements of γ-CD chemical shift were performed at 300 K with careful temperature regulation (Benesi and Hildebrand, 1949; Péan et al., 1999).

2.4. Formulation and study of the oral solution

2.4.1. Final formulation

Several formulations were prepared with different excipient concentrations. The final solution is described in Table 1. The midazolam concentration selected was 2 g/L for an usual dose estimated at 0.2 mg/kg. Solutions were prepared by dissolution at room temperature of components. The pH of the final formulation was 2.8.

2.4.2. Sterilization methods

Two sterilization processes were investigated using LC–HRMS: sterile filtration via Stericup filter (0.22 μm) from Millipore (Guyancourt, France) and steam sterilization with a Subtil Crepieux (Lyon, France) autoclave (120 °C, 35 min). Pharmaceutical solutions were prepared at University Hospital of Amiens following good manufacturing practice used in France (AFSSaPS, 2007).

2.4.3. LC–HRMS studies

LC–HRMS analyses were performed on a Prominence UFLC system (Shimadzu, Duisburg, Germany) coupled with a Q-TOF *Ultima Global* hybrid quadrupole time-of-flight instrument (Waters-Micromass, Manchester, UK), equipped with a pneumatically assisted electrospray ionization (ESI) source (Z-spray) and an additional sprayer for the reference compound (Lock Spray). The elution was performed using a 1 mL/min mobile phase gradient programmed from water containing 0.1% formic acid (A) to acetonitrile containing 0.1% formic acid (B) as follows (A:B): 95:5 (t = 0 min), 5:95 (t = 30 min), 5:95 (t = 40 min). The solutions were loaded on a Symmetry C_8 (3.5 μm, 100 mm × 4.6 mm) column (Waters, Saint Quentin en Yvelines, France) using a sample injection volume of 2 μL (dilution to 1/20 in water of the original formulations, corresponding to solutions at 0.1 mg/mL). The effluent was flow-split via a peek tee with 1:4 of the flow directed toward the electrospray (ESI) source of the Q-TOF instrument and the residual 3:4 directed toward a UV detector (Shimadzu SPD-20A) set to 220 nm. ESI-MS data were recorded in the positive ion mode. The source and desolvation temperatures were 120 and 250 °C, respectively. Nitrogen was used as a drying and nebulizing gas at flow rates of 450 and 100 L/h, respectively. Typically, the capillary voltage was 1 kV and the cone

voltage 180 V. Lock mass corrections, using appropriate cluster ions of an orthophosphoric acid solution (0.1% in H_2O/CH_3CN 50/50, v/v), were applied for accurate mass measurements (elemental composition determination). The mass range was 50–1000 Da and spectra were recorded at 1 s/scan in the profile mode at a resolution of 10,000 (FWHM). Data acquisition and processing were performed with MassLynx 4.0 software.

2.4.4. HPLC quantification

2.4.4.1. Chromatographic conditions. Quantification of midazolam solutions was designed on a Waters Alliance (Saint Quentin en Yvelines, France) chromatography system composed of a Waters 2695 injection module and a Waters 2996 diode array detector (DAD). Chromatography column used was a Waters symmetry C_8 (5 μm, 250 mm × 4.6 mm) protected by a guard column (Waters symmetry C_{18}; 5 μm, 20 mm × 3.9 mm). The flow rate was 1 mL/min and the optimized mobile phase was a 25/75 (v/v) mixture of phosphate buffer (pH 3.8) and acetonitrile. The column was conditioned during 35 min prior each run.

2.4.4.2. Midazolam peaks integration. At pH 3.8, the midazolam is present in open and closed ring forms inducing two chromatogram peaks and UV spectra. The retention time was 2.5 min for the ring-open form (λ_{max} 212 and 253 nm) and 3.6 min for the ring-closed form (λ_{max} 217 nm). The ratio between open and closed form is altered by pH (Orive et al., 1989), mobile phase and γ-CD. To avoid the multiplicity of equilibriums, the two chromatogram peaks were integrated and the corresponding areas were added for the determination of total midazolam content.

2.4.4.3. Validation of HPLC quantification method. Based on the data of the Société Française des Sciences et Techniques Pharmaceutiques (Hubert et al., 2007a,b), the validation method consisted in a four day trials. First day, the linearity of the calibration curve was assessed in a fixed concentration range. Six 5-points calibration curves were established using standard solutions. These solutions were prepared by dilution of midazolam in a phosphate buffer (pH 2.8). Final concentrations of standard solutions used for calibration were 0.05, 0.1, 0.2, 0.3 and 0.4 g/L. Injection volume was set to 10 μL. Then the quantification method was achieved for the three other days. Three control batches containing exactly the same ingredients than the pharmaceutical solution dosed at 1.60, 2.00 and 2.40 g/L (i.e. 2.00 g/L ± 20%) were prepared for this purpose. These control batches were diluted to 1:10 in phosphate buffer (pH 2.8) and the injection volume was set to 10 μL. Each control batch was analyzed six times (i.e. 6 samples × 3 levels × 3 days) for linearity, trueness and precision.

2.4.4.4. Assessment of matrix effect. Assay for matrix effect was evaluated by comparing result of dosages of the 2.00 g/L control batch with a control solution dosed at 2.00 g/L (3 samples × 3 days), made as mentioned before for calibration curves solutions.

2.4.4.5. Data processing. Linearity was controlled by successive linear regression, ANOVA and residuals plots analysis. All statistics were performed using R software (R Development Core Team, 2008) and p-values < 0.05 were regarded as statistically significant.

3. Results and discussion

3.1. Preliminary and solubility studies

The potentiality of the three main CDs (α-, β- and γ-CD) to improve the solubility in water and to mask the bitterness of midazolam was evaluated using a preliminary experiment. Aqueous solutions containing 10 mM of α-, β- or γ-CD and 5 mM of midazolam were prepared in presence of 40 mM of citric acid. The resulting solutions were tasted to appreciate the influence of each CD on midazolam bitterness. At this stage, the taste of aqueous solution was not modified by the presence of α-CD. Conversely, solutions containing β- or γ-CD exhibited a significantly decrease of the bitterness consistent with the formation of inclusion complexes. It should be pointed out that, in these conditions, the sample obtained in presence of γ-CD appeared completely clear indicating an increase of apparent solubility of midazolam while in presence of α- or β-CD a precipitate appeared rapidly.

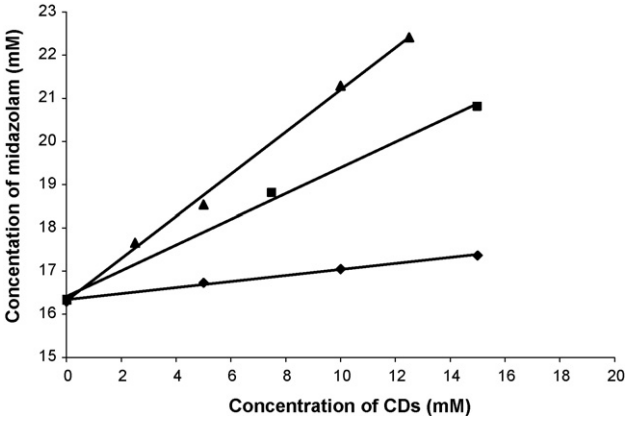

Fig. 1. Phase solubility diagram of midazolam in citric acid buffer (4.2 g/L) at 25 °C: α-CD (♦), β-CD (■) and γ-CD (▲).

Solubility of midazolam alone has been evaluated to 5.3 g/L (16.3 mM) in the formulation citric acid buffer (4.2 g/L, 25 °C). Phase solubility techniques were used to assess the formation of inclusion complexes between midazolam and α-, β- or γ-CD in the citric acid buffer. As shown in Fig. 1, a linear increase of midazolam solubility was verified as a function of CD concentration, suggesting the formation of a solution complex in all cases.

Moreover, it is well known that the value of the apparent stability constant is directly related to the slope of the linear part of the solubility curves (Higuchi and Connors, 1965). In these conditions, the inclusion complex obtained in presence of α-CD seemed to be very weak while β-CD and above all γ-CD formed stronger inclusion complexes.

3.2. NMR studies

First, we will highlight the formation of midazolam/γ-CD inclusion complex by ^{1}H NMR. Then, we will present its stoichiometry determined by NMR titration experiment and an estimation of the association constant will be given. Structural analysis using 2D ROESY will be described to explain the mode of penetration of midazolam into the γ-CD.

3.2.1. NMR evidence of the formation of midazolam/CD inclusion complex

In order to determine the best CD candidate to form inclusion complex, the α-, β- and γ-CD have been selected based on the preliminary and solubility results. To demonstrate whether or not the interaction between midazolam and CDs occurred via an inclusion complex, ^{1}H NMR experiments study has been performed based on the variations of chemical shifts of the protons H-3, H-5 from the CDs (10 mM in D_2O with 40 mM of citric acid).

In the presence of midazolam, the signals of protons from α-CD did not show significant chemical shifts changes in ^{1}H NMR spectra. Furthermore, it is noteworthy that a precipitate quickly appeared in the sample. In view of these results, α-CD failed to solubilize

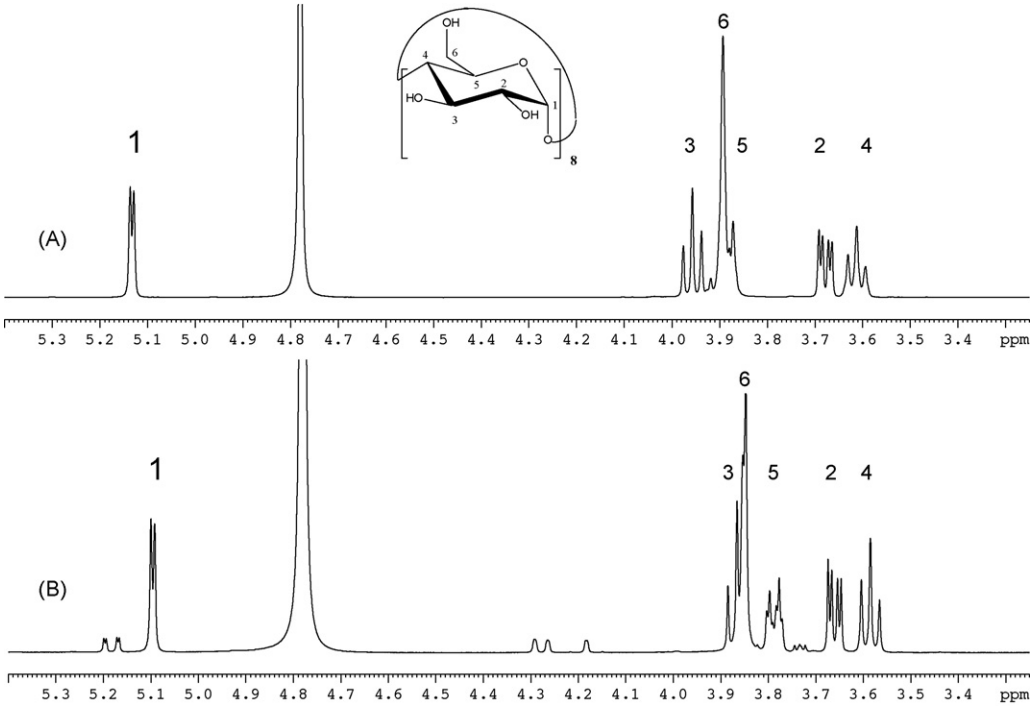

Fig. 2. Partial 500 MHz ^{1}H NMR spectra of γ-CD (10 mM in D_2O with 40 mM of citric acid) in the absence (A) and in the presence of 12 mM of midazolam (B) at 300 K, with assignment of the signals from the CD moiety.

efficiently midazolam through an inclusion complex; this could be explained by the too small size of the α-CD's cavity.

In the case of β-CD (with successive additions of aliquots of midalozam solution leading to final concentrations in range of 1–10 mM), the data obtained in ^{1}H NMR are in agreement with the literature (Ali and Upadhyay, 2008) since upfield shifts on H-3, H-5 were observed confirming the formation of a host-guest complex with midazolam.

By comparison of the ^{1}H NMR spectra obtained for the γ-CD in the absence as well as in the presence of midazolam, we observed large variations of chemical shifts for H-3 and H-5 protons (Fig. 2). The addition of midazolam led to a shielding of H-3, H-5, H-6 whereas H-1, H-2, H-4 seemed to be less affected by this addition. The shifts of the CD cavity protons surely due to the anisotropic current effect of the aromatic cycles from midazolam allowed to evidence interaction of the midazolam with the γ-CD via the formation of an inclusion complex.

As a conclusion to this section, it should be noted that β-CD as well as γ-CD gave inclusion complexes with midazolam evidenced by the induction of strong variations of the chemical shifts of protons of the cavity of CDs while this was not the case for the α-CD. In agreement to our preliminary studies (Section 3.1), γ-CD seems to be the more appropriate host to enhance the solubility and decrease the bitterness of midazolam.

3.2.2. NMR determination of the stoichiometry of midazolam/γ-CD inclusion complex

As described above, it has been shown the formation of a midazolam/γ-CD inclusion complex thanks to the variation of chemical shifts from the protons H-3, H-5 located inside the hydrophobic cavity of the γ-CD. The stoichiometry of the complex could not be determined directly, that is the reason why we used the continuous variation's method so called "Job's method" based on NMR titration experiments (Djedaini-Pilard et al., 1990). As a prerequisite to apply this method, the total concentration of γ-CD and midazolam was kept constant (10 mM), the ratio r being varied from 0 to 1. Plots of the observed $\Delta\delta\cdot[\gamma\text{-CD}]_t$ as a function of r led to Job plots depicted in Fig. 3. This procedure was performed for all protons of the γ-CD and yielded identical results. In all cases, the Job plots showed a maximum at $r = 0.5$ and symmetrical shapes indicating that the complex had 1:1 stoichiometry and that no other complex was present.

3.2.3. NMR determination of the associate constant of midazolam/γ-CD inclusion complex

The apparent association constant K was determined by the Benesi–Hildebrand method (Amato et al., 1992; Péan et al., 1999). The γ-CD concentration was set to 0.2 mM and that of the midazolam varied between 20 and 35 mM, the citric acid concentration was kept constant at 40 mM. The Benesi–Hildebrand procedure use a graphical approach, however, this one presents some limitations with systems exhibiting limited solubility, as in the present case.

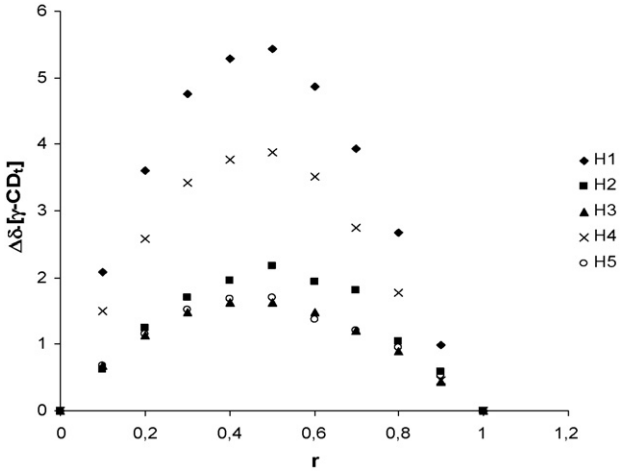

Fig. 3. Continuous variation plot (Job plot) for all protons of the γ-CD.

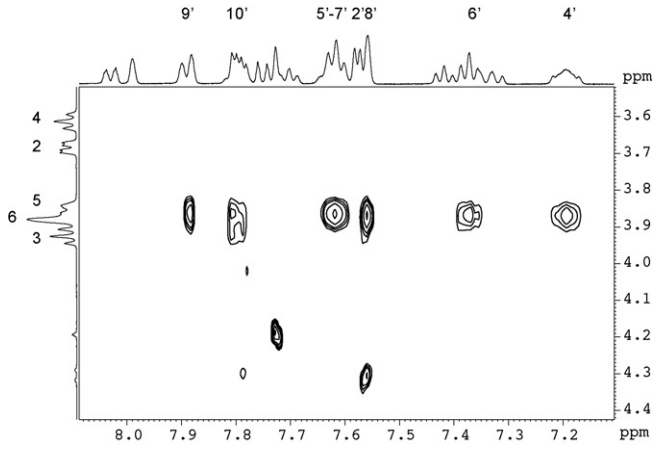

Fig. 4. Partial contour plot of a T-ROESY experiment (spin lock: 300 ms, 22 dB) performed at 500 MHz on γ-CD/midazolam inclusion complex (10 mM, D_2O, 40 mM citric acid). Vertical scale: CD area, horizontal scale: aromatic area of midazolam.

Nevertheless, the average association constant K has been evaluated from the protons H-3, H-5 of the γ-CD to yield a value of 283 M^{-1} at 300 K.

3.2.4. Structure of midazolam/γ-CD inclusion complex

Based on the T-Roesy spectral data, the structure of the midazolam/γ-CD inclusion complex was established (Péan et al., 1999). The presence of cross-peaks between aromatic protons of midazolam and those from γ-CD fully supports the formation of an inclusion complex. Strong interactions between protons H-5 and H-6 located on the upper rim of γ-CD and protons of fluoro-substituted aromatic ring (H-4′, H-5′, H-6′ and H-7′) of midazolam have been observed (Fig. 4). H-8′, H-9′ and H-10' protons of chloro-substitued aromatic ring exhibited also interactions with H-5 and H-6 of γ-CD. It should be pointed out that H-3 of γ-CD located inside the cavity did not show any cross-peak with proton of midazolam.

Based on these results and taking account the 1:1 stoichiometry of γ-CD/midazolam inclusion complex, it should be concluded that the midazolam moiety implicated is the fluoro-aromatic ring as also described with the β-CD/midazolam inclusion complex (Ali and Upadhyay, 2008). But in our case, the penetration of the guest inside the highest CD's cavity is deeper since H-8′, H-9′ and H-10′ protons of chloro-substituted aromatic ring exhibit also strong interaction with γ-CD. Moreover, the main difference with the β-CD/midazolam complex is the mode of entry of the guest into the CD cavity: with γ-CD only protons of the narrower rim side (i.e. H-5 and H-6) exhibit strong interactions with protons of midazolam. The proposed structure of the γ-CD/midazolam inclusion complex is displayed in Fig. 5.

3.3. Influence of the formulation on the stability of the inclusion complex

In order to investigate the effect of the formulation on the interaction behavior with γ-CD, we prepared a D_2O solution containing midazolam, γ-CD and citric acid in agreement with the dedicated formulation in which, we added increasing amounts of orange aroma. Following these additions, it should be pointed out that a precipitate appeared leading us to consider that competitive process had to be taking account to rationalize this observed data. To better understand this phenomenon, we studied the potential interactions between γ-CD and orange aroma using ^{1}H NMR experiments. We have compared ^{1}H NMR spectra of γ-CD in the formulation in the absence and in the presence of orange aroma after addition of 10 and 20 μL into the solution. From these experiments, chemical shifts variations of γ-CD's protons have been observed indicating that an interaction occurred with the terpenes of orange aroma, consistent with the formation of one or several inclusion complexes. It is reported that some terpenes can form inclusion complex with CD (Decock et al., 2006). As a consequence, these complexes led to a displacement of the equilibrium of γ-CD/midazolam complex formation inducing midazolam precipitation in the solution. It should be concluded that addition of several components in the final formulation leads to competitive inclusion phenomenon those can be circumvented by an excess of γ-CD.

3.4. Effect of sterilization on midazolam stability

Microbiological quality requirements for oral solutions are mentioned in European Pharmacopeia (2007). In this context, an important aspect in the development of a pharmaceutical formulation is the choice of the sterilization process. Since the use of some preservatives such as parabens (de Vries and Caira, 2008; Qian et al., 2008) or benzalkonium chloride (Echezarreta-Lopez et al., 2002) induce competitive complexation towards cyclodextrins, steam sterilization (120 °C, 35 min) has been considered. The UV chromatogram (220 nm) obtained for the LC–HRMS experiment is presented Fig. 6 (bottom chromatogram). It clearly appeared that steam sterilization alter midazolam formulation by the formation of two degradation products detected at R_t = 7.5 and 7.8 min. These two peaks revealed protonated molecules $[M+H]^+$ at m/z 518.1 and 500.1, respectively.

Moreover, their isotopic patterns (Fig. 6) are similar to those of midazolam open and closed forms indicating the presence of one atom of chlorine in the structures of these two compounds. For the structural characterization of low concentration components in complex mixtures, the combination of the high resolution capabilities of the quadrupole orthogonal time-of-flight mass spectrometer (Q-TOF) with on-line liquid chromatography is now a

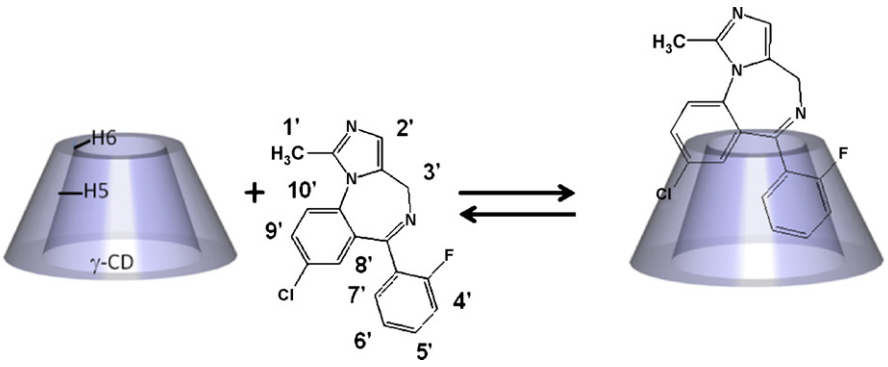

Fig. 5. Proposed model of γ-CD/midazolam inclusion complex.

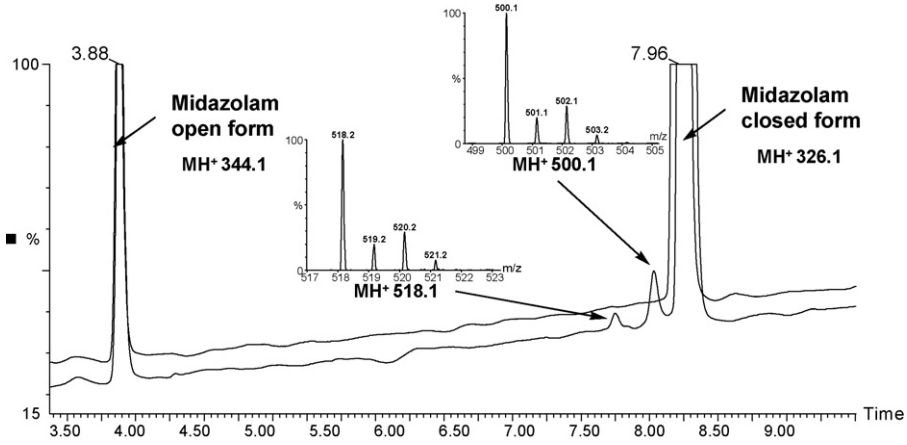

Fig. 6. LC–UV (220 nm) chromatogram of the γ-CD/midazolam formulation following steam (bottom) and filtration (top) sterilization process.

Table 2
Results of the three day assay.

Introduced concentration (g/L)	Measured concentration		Recovery (%)	Bias	
	Mean (g/L)	SD (g/L)		Absolute (g/L)	Relative (%)
1.606	1.634	0.023	101.772	0.028	1.772
2.008	2.030	0.030	101.073	0.022	1.073
2.412	2.440	0.035	101.175	0.028	1.175

SD: standard deviation of measured concentration.

well establish tool (Lee et al., 1999). Accurate mass measurements were carried out on the $[M+H]^+$ ions of the two degradation products allowing their respective elemental composition determination: $C_{24}H_{22}N_3O_7FCl$ (found 518.1151; requires 518.1130) and $C_{24}H_{20}N_3O_6FCl$ (found 500.1031; requires 500.1025). These results showed that the degradation product at m/z 500.1 was probably resulting from a condensation ($-H_2O$) between midazolam ($C_{18}H_{13}N_3FCl$) and a molecule of citric acid ($C_6H_8O_7$). If it is the case, the more polar open form should correspond to the m/z 518.1 ($+H_2O$). In order to obtain unambiguous assignment of these degradation product structures, deeper structural analysis experiments, including NMR and MS–MS, are under active investigations in our laboratory.

To avoid that chemical reactions occur in final batches, a sterile filtration was investigated. The UV profile obtained for the filtered solution (Fig. 6, top chromatogram) clearly evidenced that none of the previously described degradation products was detected. This was confirmed by the negative result of a specific MS search of their respective $[M+H]^+$ ion. Following these observations, the final microbial preservation was realized in a 20 mL vial using a 0.22 μm sterile filter. This pharmaceutical preparation must be discarded 12 h after it has been opened.

3.5. Quantification of midazolam in the formulation

For the control of midazolam concentration in pharmaceutical batches, a validated quantification method was required. During the validation of the HPLC–DAD method we assessed the linearity, the trueness, the precision and the repeatability; the matrix effect was also evaluated.

3.5.1. Calibration curve

The calibration graph over the concentration range of 0.5–4 g/L was found to be linear. This was confirmed by ANOVA ($p < 0.001$). Moreover residuals plots revealed no systematic deviation.

3.5.2. Linearity and trueness

Quantification results of the three day assays are shown in Table 2. Control batches concentrations were calculated using accurate weighted amounts of midazolam (these exact concentrations are called "introduced concentration"). Measured concentrations are concentrations calculated from the calibration curve. The quantification method is linear between 1.606 and 2.412 g/L, this was demonstrated by ANOVA ($p < 0.001$).

Trueness or absolute bias is expressed by the difference between the introduced concentration and the measured one (Table 2). These absolute biases are low and relative biases are <5%. Recoveries are within a [95; 105]% range.

3.5.3. Intermediate precision and repeatability

Intermediate precision is assessed with the between series standard deviation. The repeatability is represented by the within series standard deviation. All values are in acceptable ranges (Table 3).

3.5.4. Matrix effect

Absolute bias for the matrix effects is equal to 0.010 g/L and relative bias is equal to 0.502%. Matrix has no or little effect on the dosage of midazolam in pharmaceutical solution (2.020 g/L vs. 2.030 g/L, $p = 0.623$).

We decided to fix the acceptance limits for the result of the dosage of the pharmaceutical solution at 2.00 ± 0.10 g/L (i.e. ±5%). All solutions outside this range will be discarded.

Table 3
Between and within series standard deviation.

Introduced concentration (g/L)	Between series SD (g/L)	Within series SD (g/L)
1.606	0.015	0.009
2.008	0.019	0.012
2.412	0.021	0.019

SD: standard deviation.

4. Conclusion

Based on clinician's requests, a new 0.2% (w/v) midazolam solution for oral routes including γ-CD was developed. We have demonstrated using NMR that γ-CD offers two advantages: the masking of the solution bitterness and the enhancement of midazolam solubility. This is the first report to our knowledge of a γ-CD/midazolam complex. The ^{1}H NMR studies of midazolam and γ-CD in citric acid (40 mM) D_2O solution confirmed the formation of 1:1 γ-CD/midazolam inclusion complex. Fluoro-substituted aromatic ring penetrates deeply into the γ-CD from the narrower rim side leading to a relatively stable complex. The value of the associate constant of γ-CD/midazolam inclusion complex (283 M^{-1}) is three times higher than the one calculated for β-CD (108 M^{-1}) (Ali and Upadhyay, 2008), this is in agreement with the enhancement of the solubility and the decrease of the bitterness.

The strategic choice, in term of drug stability, of the formulated solution microbial preservation was determinate by LC–HRMS. Minor byproducts were detected and characterized when using steam sterilization leading us to prefer a sterile filtration procedure. Finally, a robust and no-matrix affected HPLC–DAD quantification method was proposed for the determination of global midazolam amount in the pharmaceutical preparation. Further studies are needed to finalize this project. In order to determine an expiration date for the preparation, we have already start stability studies following the ICH (International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use) guidelines. A clinical trial is in preparation to determine pharmacokinetics properties of this γ-CD/midazolam solution and to highlight γ-CD effect on oral bioavailability of midazolam. Once these studies completed, the 0.2% (w/v) midazolam solution proposed in this paper could easily be used in hospitals.

References

AFSSaPS, 2007. Bonnes Pratiques de Fabrication. Direction des Journaux Officiels, Paris.

Ali, S.M., Upadhyay, S.K., 2008. Complexation study of midazolam hydrochloride with β-cyclodextrin: NMR spectroscopic study in solution. Magn. Reson. Chem. 46, 676–679.

Amato, M.E., Djedaïni-Pilard, F., Perly, B., Scarlata, G., 1992. High field NMR and molecular modelling study of the inclusion complexes of the nootropic drug tenilsetam in cyclodextrins. J. Chem. Soc. Perkin Trans. II, 2065–2069.

Amorim, C.G., Araújo, A.N., Montenegro, M.C.B.S.M., Silva, V.L., 2008. Cyclodextrin-based potentiometric sensors for midazolam and diazepam. J. Pharm. Biomed. Anal. 48, 1064–1069.

Armijo, J.A., Herranz, J.L., Pena Pardo, M.A., Adín, J., 2004. Intranasal and buccal midazolam in the treatment of acute seizures. Rev. Neurol. 38, 458–468.

Bar, R., Ulitzur, S., 1994. Bacterial toxicity of cyclodextrins: luminuous *Escherichia coli* as a model. Appl. Microbiol. Biotechnol. 41, 574–577.

Benesi, H.A., Hildebrand, J.H., 1949. A spectrophotometric investigation of the interaction of iodine with aromatic hydrocarbons. J. Am. Chem. Soc. 71, 2703–2707.

Davies, F.C., Waters, M., 1998. Oral midazolam for conscious sedation of children during minor procedures. J. Accid. Emerg. Med. 15, 244–248.

de Vries, E.J.C., Caira, M.R., 2008. A structural and thermal investigation of the inclusion of parabens in heptakis(2,6-di-O-methyl)cyclomaltoheptaose. Carbohydr. Res. 343, 2433–2438.

Decock, G., Fourmentin, S., Surpateanu, G.G., Landy, D., Decock, P., Surpateanu, G., 2006. Experimental and theoretical study on the inclusion compounds of aroma components with β-cyclodextrins. Supramol. Chem. 18, 477–482.

Djedaini-Pilard, F., Lin, S.Z., Perly, B., Wouessidjewe, D., 1990. High field NMR techniques for the investigation of a beta-cyclodextrin-indomethacin inclusion complex. J. Pharm. Sci. 79, 643–646.

Dodziuk, H., 2006. Cyclodextrins and their Complexes: Chemistry, Analytical Methods, Applications. Wiley-VCH, London.

Echezarreta-Lopez, M.M., Perdomo-Lopez, I., Estrada, E., Vila-Jato, J.L., Torres-Labandeira, J.J., 2002. Utility of nuclear magnetic resonance spectroscopy to characterize the structure of dexamethasone sodium phosphate inclusion complexes with cyclodextrins in solution and to analyze potential competitive effects. J. Pharm. Sci. 91, 1536–1547.

European Pharmacopoeia, 2007. 6th ed., Council of Europe, Strasbourg, pp. 567–583.

Hubert, P., Nguyen-Huu, J.J., Boulanger, B., Chapuzet, E., Chiap, P., Cohen, N., Compagnon, P.A., Dewe, W., Feinberg, M., Lallier, M., Laurentie, M., Mercier, N., Muzard, G., Nivet, C., Valat, L., Rozet, E., 2007a. Harmonization of strategies for the validation of quantitative analytical procedures. A SFSTP proposal—part II. J. Pharm. Biomed. Anal. 45, 70–81.

Hubert, P., Nguyen-Huu, J.J., Boulanger, B., Chapuzet, E., Cohen, N., Compagnon, P.A., Dewe, W., Feinberg, M., Laurentie, M., Mercier, N., Muzard, G., Valat, L., Rozet, E., 2007b. Harmonization of strategies for the validation of quantitative analytical procedures. A SFSTP proposal—part III. J. Pharm. Biomed. Anal. 45, 82–96.

Higuchi, T., Connors, K., 1965. Phase solubility techniques. In: Reilly, C. (Ed.), Advances in Analytical Chemistry and Instrumentation. Wiley Intersciences, New York, pp. 117–212.

Job, P., 1925. Spectrographic study of the formation of complexes in aqueous solution. Compt. Rend. 180, 928–930.

Ko, Y.P., Huang, C.J., Hung, Y.C., Su, N.Y., Tsai, P.S., Chen, C.C., Cheng, C.R., 2001. Premedication with low-dose oral midazolam reduces the incidence and severity of emergence agitation in pediatric patients following sevoflurane anesthesia. Acta Anaesthesiol. Sin. 39, 169–177.

Lee, M.J., Monté, S., Sanderson, J., Haskins, N.J., 1999. A preliminary study using fast liquid chromatography coupled to a quadrupole orthogonal time-of-flight mass spectrometer. Rapid Commun. Mass Spectrom. 13, 216–221.

Loftsson, T., Duchêne, D., 2007. Cyclodextrins and their pharmaceutical applications. Int. J. Pharm. 329, 1–11.

Loftsson, T., Gudmundsdóttir, H., Sigurjónsdóttir, J.F., Sigurdsson, H.H., Sigfússon, S.D., Másson, M., Stefánsson, E., 2001. Cyclodextrin solubilization of benzodiazepines: formulation of midazolam nasal spray. Int. J. Pharm. 212, 29–40.

Loftsson, T., Vogensen, S.B., Brewster, M.E., Konrádsdóttir, F., 2007. Effects of cyclodextrins on drug delivery through biological membranes. J. Pharm. Sci. 96, 2532–2546.

McGraw, T., Kendrick, A., 1998. Oral midazolam premedication and postoperative behaviour in children. Paediatr. Anaesth. 8, 117–121.

Orive, M.M., Gallo, B., Alonso, R.M., Vicente, F., Viré, J.C., Patriarche, G.J., 1989. Spectrophotometric study of the acid-base equilibria of an imidazobenzodiazepine, midazolam, and its determination in pharmaceutical formulations. Microchim. Acta 97, 181–190.

Péan, C., Creminon, C., Djedaïni-Pilard, F., Pradelles, P., Perly, B., 1999. NMR investigation of the inclusion of thyroxine and derivatives in natural and chemically modified cyclodextrins. J. Inclusion Phenom. 33, 307–319.

Qian, L., Guan, Y., Xiao, H., 2008. Preparation and characterization of inclusion complexes of a cationic β-cyclodextrin polymer with butylparaben or triclosan. Int. J. Pharm. 357, 244–251.

R Development Core Team, 2008. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna.

Szejtli, J., 1992. The properties and potential uses of cyclodextrin derivatives. J. Inclusion Phenom. Macrocyclic Chem. 14, 25–36.