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Physicochemical stability of compounded midazolam capsules over a one-year storage period

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Abstract

Objectives: In patients suffering from chronic liver disease, the hepatic metabolism of drugs is perturbed and the metabolic capacity is difficult to assess. Midazolam could be used as a phenotypical probe to predict the metabolic capacity of CYP3A to adjust dosages of drug substrates of this cytochrome. In this context, a prospective clinical trial is going to be conducted in our institution and a hospital preparation of midazolam capsules suitable for the clinical trial was developed. The objective of the present work was to assess the physicochemical stability of the formulation over 12 months to set shelf life.

Methods: Three batches of 1 mg capsules were prepared using midazolam hydrochloride and microcrystalline cellulose as a diluent. The capsules were stored at ambient temperature and protected from light. To measure the evolution of the capsules content, a stability-indicating high-performance liquid chromatography (HPLC) method was developed with ultraviolet (UV) detection at 254 nm. Data were confirmed using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) analytical method.

Results: After one year, midazolam hydrochloride content remained higher than 95% of the initial concentration in capsules.

Conclusions: The results show that 1 mg midazolam capsules are stable for 12 months at room temperature and under dark conditions.

Keywords: CYP3A; hepatic failure; midazolam capsules; personalized medicine; phenotypic probe.

Introduction

The liver plays an important role in the metabolism of most drugs. Also, liver failure following chronic hepatic disease is a major source of pharmacokinetic variability. This inter- and intra-individual variability is difficult to predict and lead to drug-related iatrogenic. An inadequate dosage could lead to adverse events in 30% of cirrhotic patients [1]. Biological markers may help to evaluate the secretion and synthesis functions of the liver. However, these markers may assess hardly its ability to metabolize drugs that appears crucial to minimize the iatrogenic risk during treatments because of the role of the liver in detoxifying drugs. Thus, the assessment of the metabolic capacity in patients could prevent iatrogenic events in this population particularly sensitive to drug toxicity [2].

Midazolam, a benzodiazepine, is selectively metabolized by cytochrome P450 (CYP) 3A4/5, so it is the gold-standard phenotypical probe of the CYP3A4/5 activity. CYP phenotyping consists of measuring in blood the ratios between the concentrations of midazolam and its metabolite (1-hydroxymidazolam). It can be administered by oral route or by intravenous route [3–6]. The most common practice is to use a low oral dose of midazolam to assess the CYP3A activity [7]. However, midazolam as a probe is not suitable for routine use because pharmaceutical products are not adapted for this indication.

To better assess metabolic capacity in patients with liver failure, pharmacologists and physicians in our institution are going to conduct a clinical trial. The subjects, divided into three groups (healthy volunteers and patients with Child-Pugh grade A or B hepatic impairment) will receive a low dose of midazolam (1 mg as free base).

In this context, the department of pharmacy was solicited to help with the study design. To reduce inter-subject variability and to minimize the pharmacologic effect, an oral liquid formulation was not selected. Indeed, midazolam can pass rapidly across the oral mucosal, with an upper bioavailability than by oral route and a lower first-pass effect [8]. Moreover, midazolam has a bitter taste hardly to mask [9]. Oral formulations masking its taste such as a tablet (e.g. Dormicum®) or syrup exist but there are not available in our

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country [10, 11]. For these reasons, it was decided to compound midazolam capsules.

The study aimed to prepare 1 mg midazolam capsules and to assess their stability during one year by combining two analytical methods.

Materials and methods

Chemicals and reagents

Midazolam hydrochloride (Figure 1) powder and microcrystalline cellulose PH 102 powder were provided by INRESA (Bartenheim, France). Lactose monohydrate was purchased from Fagron (Thiais, France). Hard gelatin capsules (size 4, ivory color) were bought from LGA (La Seyne-sur-Mer, France).

Midazolam certified standard (purity >99.9%) was obtained from LGC Standards (Molsheim, France) and was used for analytical validation to ensure the identity of the active product ingredient (API).

Acetonitrile and water were of high-performance liquid chromatography (HPLC) or liquid chromatography-mass spectrometry (LC-MS) grade and were purchased from Fisher Scientific (Illkirch-Graffenstaden, France). Ammonium acetate and glacial acetic acid were respectively bought to Merck Millipore (Molsheim, France) and Fisher Scientific (Illkirch-Graffenstaden, France). Hydrochloric acid (HCl) 0.1 and 1 M, sodium hydroxide (NaOH) 0.1 and 1 M were procured from VWR (Fontenay-sous-Bois, France) and hydrogen peroxide solutions (H_2O_2) 3 (w/w) and 30% (w/w) were respectively acquired from Gifrer (Decines-Charpieu, France) and Cooper (Melun, France).

Development of a solid oral formulation

To select the excipient to use for midazolam capsules (size 4), a preliminary test was performed by comparing properties of two powder mixtures: midazolam hydrochloride and lactose monohydrate vs. midazolam hydrochloride and microcrystalline cellulose. The mass of powder required for capsule filling was firstly assessed. The visual aspect, the chemical stability of the mixture for 70 days, and the safety of the excipient were criteria taken into account to select the excipient for the finished product.

Preparation of midazolam hydrochloride capsules

Midazolam hydrochloride capsules were compounded and quality controlled according to good manufacturing guidelines [12].

Figure 1: Chemical structure of midazolam (A) (Molecular weight (MW): $325.77 \text{ g}\cdot\text{mol}^{-1}$) and its hydrochloride salt (B) (MW: $362.23 \text{ g}\cdot\text{mol}^{-1}$).

The API and excipient powders were mixed with a mortar and a pestle gradually to homogenize the mixture. The powder mixture was conditioned in hard gelatin capsules (size 4) using a capsule-filling machine with a loader from LGA (La Seyne-sur-Mer, France). Each unit contained 1.11 mg of midazolam hydrochloride with 85 mg of microcrystalline cellulose as diluent.

After batch release, the capsules were next packaged in an individual blister made of polyvinyl chloride (PVC) film with aluminum foil. The primary packaging was ultraviolet (UV) resistant to prevent photodegradation because midazolam is photosensitive [13, 14]. The units were also stored in a safe local at ambient temperature ($23 \pm 2^\circ\text{C}$). This parameter and relative humidity (RH) were continuously monitored with a datalogger 0172SI (IHM Moineau Instruments, Chef-Boutonne, France). The RH of the local was about $45 \pm 15\%$.

Quality control of midazolam hydrochloride capsules

According to the European Pharmacopeia (EP), uniformity of mass (Monograph 2.9.5) was checked. Then, uniformity of content (Monograph 2.9.6) and uniformity of dosage units tests (Monograph 2.9.40) were performed for batch release [15].

Analytical method development

Separative methods like Liquid Chromatography must be preferred to perform a stability study to differentiate the analytes of a mixture. A stability-indicating HPLC-UV method was developed, on the one hand, to assess the uniformity of content of batches of midazolam capsules prepared, and on the other hand, to perform the stability study of midazolam capsules. The peak purity analysis was not perfect because the detection was not done with a Diode Array Detector (DAD). Thereby, a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed and validated to get additional data for the stability study [16].

Instrumentation and chromatographic conditions

HPLC method with UV detection: Midazolam concentrations of each sample were determined by HPLC with UV detection. The system included an HPLC 515 pump, a 717 plus autosampler, and a 2487 UV detector (Waters Corporation, Milford, MA, USA).

Contrary to midazolam, midazolam hydrochloride has no monograph in EP although German Drug Codex (DAC) has one [14, 15]. The midazolam hydrochloride assay described in the DAC was similar to the midazolam assay in EP. However, it was not appropriate for this present work regarding the retention time too long of the drug in this assay (17 min), chromatographic conditions from the DAC were adapted.

Chromatographic separation was performed with a RP18 Xterra column ($150 \times 4.6 \text{ mm}$, $5 \mu\text{m}$) (Waters Corporation, Milford, MA, USA). The mobile phase was prepared by a mixture of 10 mM ammonium acetate buffer, adjusted to pH 5.4 with glacial acetic acid, with acetonitrile (55:45, v/v). The flow rate was adjusted at 1 mL/min and the run time was 12 min. The injection volume was $10 \mu\text{L}$ and detection was done at 254 nm. The autosampler and the oven temperature was kept at 25°C . Data were acquired and processed using EMPOWER Software (Waters Corporation, Milford, MA, USA).

After each analysis, all samples were frozen and stored at -20°C .

HPLC method with MS/MS detection: An Ultimate 3000 HPLC system (Thermo Fisher Scientific, San Jose, CA, USA) was used. The autosampler was set at 15 °C during analysis. Separation of the analyte was realized by a C18 Hypersil Gold column (50 mm × 2.1 mm, 3 µm) fitted with a guard column (10 mm × 2.1 mm × 3 µm) (Thermo Fisher Scientific, Dreieich, Germany) maintained at a constant temperature of 40 °C. The mobile phase composition was the same as used for the HPLC-UV method and the flow rate was kept at 0.4 mL/min. The run time was 2 min. The volume injection of the sample was 2 µL.

The chromatographic system was connected with an ENDURA® triple quadrupole mass spectrometer system (Thermo Fisher Scientific, San Jose, CA, USA). Positive electrospray ionization interface parameters were as follows: spray voltage 3.5 kV, sheath gas and auxiliary gas (N₂) 20 and 2 arbitrary units respectively, ion transfer tube temperature 325 °C and vaporizer temperature 275 °C. A mass range from m/z (0–1000) was covered to see the mass spectrum of midazolam and to detect degradation products.

The MS run was also performed in selected reaction monitoring (SRM) scan mode to confirm midazolam concentrations in samples. Midazolam was monitored by the transition of quantification m/z 326.1 [M+H]⁺ → 291.1 [M–Cl]⁺ [17] (collision energy at 28.6 eV and RF lens value at 167 V) and by the transition of confirmation 326.1 → 249.1 (collision energy at 39.6 eV and RF lens value at 167 V).

Data acquisition and analysis were performed with Xcalibur and LCQuan Software (Thermo Fisher Scientific, San Jose, CA, USA).

Preparation of stock and working solutions: Calibration and quality control (QC) stock solutions were independently prepared in 20 mL volumetric flasks by dissolving 22.2 mg of midazolam hydrochloride powder with the mobile phase to obtain a concentration of midazolam of 1 mg/mL (as free base). Each calibration solution and each QC solutions were next daily diluted with the mobile phase to obtain working solutions at five concentration levels (30, 40, 50, 60, and 70 µg/mL) and three concentration levels (30, 50, and 70 µg/mL) for calibration samples and QC samples respectively. An additional QC sample was prepared at 50 µg/mL with the certified standard solution from LGC.

Extraction procedure of midazolam from capsules: The powder of each capsule was transferred to a 10 mL volumetric flask and dissolved in and dilute with the mobile phase. The sample was ultrasonicated for 10 min and centrifuged at 4000 rpm for 5 min to sediment microcrystalline cellulose. A final dilution (1:2 v/v) of supernatant with mobile phase was performed to obtain a solution having a concentration of 50 µg/mL. Then, the samples were analyzed by HPLC-UV and after each analysis, all samples were stored at –20 °C before analysis by HPLC-MS/MS. For LC-MS analysis, samples were thawed and diluted with the mobile phase (1:50 v/v) before injection to prevent signal saturation. Thus, the final concentration was about 1000 ng/mL for LC-MS/MS analysis.

Analytical method validation

The HPLC method was validated according to the International Conference on Harmonization (ICH) guidelines (Q2R1) [18]. Also, the stability-indicating capacity of the HPLC-UV method was assessed according to the Group of Evaluation and Research for Protection in Areas under Control (GERPAC) guidelines [16].

System suitability testing: The system suitability testing is an essential element that ensures the performance of the chromatographic method. This test was performed by using five replicates of a standard solution. Several factors like injection repeatability, retention time of the midazolam, capacity factor (k'), number of theoretical plates, and symmetry factor (A_s) were assessed [19]. For A_s , the value had to be between 0.8 and 1.5 [15]. The data of peak area from replicate injections of the analyte were used to calculate the relative standard deviation (RSD), and the threshold value for acceptability was 2% [20].

Linearity: The linearity was evaluated by three calibration curves performed on three different days with calibration samples. The assay range was set at 60–140% of the target concentration (50 µg/mL). A final dilution (1:2 v/v) of supernatant with mobile phase was performed to obtain samples ranging from 30 to 70 µg/mL. The method was considered as linear if: (i) the coefficient of determination (R^2) was over 0.99 for the mean standard curve, (ii) the variances on each concentration were uniform and, (iii) the slope was significantly different than 0 [16, 18].

Matrix effect: The matrix effect was assessed by preparing two types of calibration ranges samples: the first containing API alone and the other one composed of API reconstituted with excipient [16]. To perform that, microcrystalline cellulose suspension at 8.5 mg/mL was added in calibration samples to reproduce the composition of the finished pharmaceutical form. Then, samples were ultrasonicated for 10 min and centrifuged at 4000 rpm for 5 min to sediment microcrystalline cellulose.

Accuracy: The accuracy was assessed using nine determinations of the QC samples (realized in triplicate each day, for three days). For each QC, the accuracy was determined by calculating the bias between theoretical and calculated concentrations. This bias, for each concentration, had to be less than 5% to be accepted.

Precision: The repeatability was studied by measuring a 50 µg/mL QC solution six times (100% of the sample preparation). The intermediate precision was checked by measuring six times this concentration each day, for three days.

Repeatability and intermediate precision were performed by two operators over three days and were determined using RSD and the threshold value for acceptability was 5%.

Stability of the processed samples: In order to check whether samples could be re-analyzed after the first analysis, the stability of the processed samples was carried out. Sample stability in the autosampler was also performed in triplicate at room temperature for 12 h. Moreover, analyte stability was assessed in triplicate on calibration samples stored at –20 °C for two months after one freeze/thaw cycle.

Stability-indicating capacity: To study the stability-indicating capacity of the HPLC-UV method, working solutions at a concentration corresponding to four times the concentration of the middle of the calibration range (200 µg/mL), were prepared with or without the presence of the excipient. There were next exposed to forced degradation under several conditions: dry heat, acid, base, and oxidative conditions [16]. The percentage of degradation of the midazolam was quantified and the degradation products were observed.

Thermal, acid, base, and oxidative degradation were assessed with midazolam solutions exposed in a water-bath heated to 60 °C for 3 h. Acid hydrolysis was studied by adding 1 mL of HCl solutions at 0.1 and 1 M in 1 mL of midazolam solution. After exposure time, the reaction was stopped and neutralization was performed with 1 mL of NaOH solutions at 0.1 and 1 M respectively. The alkaline hydrolysis was assessed by adding 1 mL of NaOH solutions at 0.1 or 1 M and neutralization with 1 mL of HCl at the same concentration after exposure time. Finally, the oxidative degradation was carried out by adding 3 mL of H₂O₂ 3 or 30% in 1 mL of midazolam solution.

Blank samples of HCl, NaOH, and H₂O₂ solutions without midazolam hydrochloride were also prepared and analyzed to identify their signal.

Degradation products were researched at 254 nm using the UV detector. Moreover, they were highlighted in the mass spectrum after infusion of the solutions from degradation studies in the MS detector. Beforehand, they were diluted with the mobile phase (1:20 v/v) to prevent signal saturation.

LC-MS/MS method validation: All samples of midazolam solution with the excipient used in HPLC-UV method validation were next used for LC-MS method validation. To perform that, samples were diluted with the mobile phase (1:50 v/v) before injection. Thereby, the calibration curves included five concentrations of midazolam (600, 800, 1000, 1200, and 1400 ng/mL) and QC solutions were at 600, 1000, and 1400 ng/mL. System suitability parameters, repeatability, accuracy, and precision were also assessed.

Stability study of midazolam hydrochloride capsules

Three batches of hundred capsules were prepared at day 0. At each time point of the study (days 0, 15, 45, 90, 180, 270, and 360), physical and chemical parameters were observed on the three batches according to the ICH guidelines [21].

Physical stability: The physical stability was evaluated by organoleptic examination to detect a color change of the shell or the content and other macroscopic manifestations (e.g. smell change). The preparation was considered physically stable if those characteristics were not changed throughout the study.

Chemical stability: At each analysis time and for each batch, 10 units of midazolam hydrochloride capsules were weighted full, then opened and emptied of their content. The content extracted from the capsules was also weighted before preparing samples to avoid uncertainty on the capsules filling.

The chemical stability was assessed by calculating the percentage of the nominal value remaining at each time point. Midazolam capsules were considered stable if API contents were more than 95% of the initial value with a 95% confidence interval around that mean (95% CI) and if no degradation product appeared.

Statistical analysis

Statistical analysis was performed by Excel[®] software (Microsoft Office, USA, 2019). All statistical tests were undertaken with an authorized risk α of 5% [16].

Results

Development of a solid oral formulation

The mass of excipients powder required for capsule filling was about 100 mg for lactose monohydrate and 85 mg for microcrystalline cellulose. The compatibility of the two excipients powder, lactose monohydrate, and microcrystalline cellulose, with midazolam hydrochloride, was acceptable for at least 70 days (Table 1). Eventually, microcrystalline cellulose was the excipient chosen.

Quality control of midazolam hydrochloride capsules

The capsules, which had an average mass lower than 300 mg, were uniform in weight. The percentage of deviation of all units was less than 10% (RSD < 5%).

Midazolam content was within the limits of 85–115% of the average content. Furthermore, for the test of uniformity of dosage units, the acceptance value for each batch was less than 15 (14.3, 11.7, and 11.1) [15]. Thereby, the batches were released, which meant the beginning of the stability study.

Analytical method validation

System suitability testing

The test showed that the retention time of midazolam was approximately 4.9 min. After five injections of the standard solution, the mean peak area was about 1,191,509 μ V·s with RSD of 0.4%. Capacity factor k' was assessed to 2.2 and the number of theoretical plates was estimated to 8057. The peak was well defined and the symmetry factor was calculated to 1.07.

Table 1: Evolution of relative midazolam content (%) in the two mixtures.

	Mixture of midazolam hydrochloride (1.11 mg) + lactose monohydrate (100 mg)		Mixture of midazolam hydrochloride (1.11 mg) + microcrystalline cellulose (100 mg)	
	Residual concentration %	RSD % (n=3)	Residual concentration %	RSD % (n=3)
Day 0	100.0	0.5	100.0	3.3
Day 35	93.0	1.3	96.0	3.3
Day 70	96.2	1.5	96.9	0.5

Linearity

The results showed that the method was linear (Table 2). There is a linear relationship between concentration x and the detected signal y (area under the curve): $y=23,686.8x + 997.9$. The coefficient of determination was higher than 0.99.

Matrix effect

No excipient effect was observed between curves with and without microcrystalline cellulose. The slopes and intercepts did not show significant differences between calibration ranges sample set composed of API alone and the one composed of midazolam reconstituted in excipient ($p=0.991$). Moreover, the method was linear in both sample sets (Table 2). These results allow further preparing calibration range samples with API diluted only with the mobile phase with no need to add excipient.

Accuracy

The method was accurate since the bias between the observed and the theoretical value were 2.4, 1.5, and 1.5% for the 30, 50, and 70 $\mu\text{g/mL}$ QC levels respectively.

Table 2: Linearity data of HPLC-UV method.

	API	Reconstituted form (RF)
Coefficient of determination R^2 (mean, $n=3$)	0.991	0.996
Slope (mean, $n=3$)	23,686.8	23,912.9
Standard deviation of the slope (mean, $n=3$)	909.0	565.4
Intercept (mean, $n=3$)	997.9	-8,863.0
Standard deviation of the intercept (mean, $n=3$)	47,234.5	29,378.7
Homogeneity of variances	Confirmed (C calculated = 0.39)	Confirmed (C calculated
<i>Cochrane test</i> ($\alpha=5\%$, $k=5$, $df=2$)	< C critical value = 0.68)	= 0.37 < C critical value = 0.68)
Slope significance	Significant	Significant
<i>Student test</i> ($\alpha=5\%$, $df=13$)	(t calculated = 26.06 > t critical value = 2.16)	(t calculated = 42.29 > t critical value = 2.16)

Precision

The RSD of repeatability and the intermediate precision were inferior to 5% and were estimated to 0.5 and 2.9% respectively.

Stability of the processed samples

As shown in Table 3, at room temperature, the samples stored in the automatic injector remained stable for 12 h. Moreover, after storage at -20°C for two months and one freeze/thaw cycle, the concentrations of the samples remained within $\pm 5\%$ of the nominal value.

Stability-indicating capacity

The chromatogram of midazolam solution which was not exposed under stressed conditions is presented in Figure 2A. No thermal degradation was observed at 60°C (Figure 2B). In this method, acidic, alkaline, and oxidative conditions lead to the emerging of degradation products observed in chromatograms (Figure 2C–F). The degradation products were well-resolved from the midazolam peak. For acid degradation, the percentage of degradation obtained was 16 %. After NaOH 1 M and H_2O_2 3% exposure, the percentage of degradation was 7 and 9% respectively. Midazolam was almost completely degraded when it was exposed in presence of H_2O_2 30%.

Regarding the MS detection, the mass spectrum of a reference solution showed a top peak (m/z 326) that corresponded to the midazolam ion and its isotopic species $[\text{M}+\text{H}]^+$ (Figure 3A). The signal of the MS spectrum and its interpretation were consistent with the theoretical value and with the chemical formula respectively. Degradation products formed after oxidation were detected. The parent ion was reduced in several degradation product ions

Table 3: Stability of midazolam processed samples at room temperature and after one freeze/thaw cycle.

Concentration levels	12 h at room temperature % Recovery ^a ($n=3$)	One freeze/thaw cycle at -20°C , 2 months % Recovery ^a ($n=3$)
30 $\mu\text{g/mL}$	99.8	100.1
40 $\mu\text{g/mL}$	99.7	101.6
50 $\mu\text{g/mL}$	100.3	102.1
60 $\mu\text{g/mL}$	100.3	103.3
70 $\mu\text{g/mL}$	100.9	103.2

^aRecovery compared to standard samples freshly prepared.

Figure 2: Representative HPLC-UV chromatograms of a reference midazolam hydrochloride solution at 200 µg/mL (A) and after stressed conditions for 3 h: heat (B), HCl 1 M (C), NaOH 1 M (D), H₂O₂ 3% (E) and H₂O₂ 30% (F).

including the m/z 273, m/z 289, m/z 305, m/z 342, and m/z 383 (Figure 3B, C).

LC-MS/MS method validation

This method was validated in terms of linearity, precision, and accuracy. The data regarding LC-MS method validation are summarized in Table 4. The retention time of midazolam was approximately 0.6 min (Figure 4).

Stability study of midazolam hydrochloride capsules

Physical stability

No variation of the color neither odor of the content nor capsules was observed in the three batches. Moreover, the

capsule shells did not show brittleness throughout the stability study.

Chemical stability

Regarding chemical stability, midazolam concentrations were within $\pm 5\%$ of the nominal value (Table 5). Moreover, no degradation products were detected in capsules regardless of the types of the detector (UV or MS/MS).

Discussion

In the present study, we assessed the stability of midazolam hydrochloride- microcrystalline cellulose mixture in compounded capsules.

Microcrystalline cellulose was chosen as diluent for several reasons such as safety and lack of metabolism activity.

Figure 3: Positive ion mass spectrum of midazolam hydrochloride reference solution at 10 µg/mL (as free base) obtained in full scan mode (0–1000 m/z range) without degradation (A) and after exposure to H_2O_2 30% for 3 h (0–1000 m/z range (B) and 275–425 m/z range (C)).

Indeed, in opposition to the lactose, this is not an excipient known to have a recognized action or effect [22]. Moreover, like lactose, it is present in midazolam tablets marketed that suggested physicochemical compatibility with the API [10].

The pharmaceutical excipients are frequently considered to be pharmacologically inert, and they are often chosen for their safety and their physicochemical compatibility with the APIs. However, many studies had demonstrated that they may affect the pharmacokinetics by influencing absorption, disposition, metabolism, or elimination of the drugs [23–25]. Their effects on pharmacodynamics and pharmacokinetics of drugs must therefore be evaluated to avoid health situations like the “Australian Phenytoin Disaster” that occurred in the sixties where the change of the excipient in the capsules brought the modification of the pharmacokinetics in phenytoin and generated an outbreak [26].

It is well known that some excipients may affect the CYP and interfere with the metabolism of midazolam [25, 27]. For the formulation to be suitable for the clinical study intended, the chosen excipient had to be effect-less

on CYP. Microcrystalline cellulose is not absorbed in humans following oral administration, and its potential for hepatic metabolism is low [28]. Moreover, its potential effects on the expression of CYP3A4 in colon and liver cells were investigated *in vitro* and no modulation of the CYP3A4 mRNA and protein expression was evidenced [29].

Eventually, all these arguments emphasize that cellulose was the best choice that lactose to formulate midazolam capsules.

Regarding HPLC-UV method validation, all the parameters fulfilled the acceptance criteria. Linear regression of the model was validated because the coefficient of determination of the mean was over 0.99, the variances on each concentration were similar and statistical verification of the regression showed a relationship between concentration and detected signal. Moreover, no interference of the microcrystalline cellulose was observed. The accuracy, the repeatability, and the intermediate precision were validated because values were inferior to 5%. The method is stability-indicating, and reliable to detect potential degradation products. Other stability-indicating HPLC methods were reported in the literature

Table 4: Data of LC-MS/MS method validation.

Parameters assessed		Values (RF)
<i>System suitability test</i>		
Injection repeatability	Average peak area	33,136,086
	RSD %	0.4%
Capacity factor k'		1.4
Number of theoretical plates		573
Symmetry factor		1.06
Signal to noise ratio		4,333
<i>Linearity</i>		
Coefficient of determination R^2 (mean, $n=3$)		0.997
Slope (mean, $n=3$)		5,330.1
Standard deviation of the slope (mean, $n=3$)		121.8
Intercept (mean, $n=3$)		162,719.7
Standard deviation of the intercept (mean, $n=3$)		126,530.6
Homogeneity of variances		Confirmed
<i>Cochrane test</i> ($\alpha=5\%$, $k=5$, $df=2$)		(C calculated = 0.61 < C critical value = 0.68)
		Significant
Slope significance		(t calculated = 43.78 > t critical value = 2.16)
<i>Student test</i> ($\alpha=5\%$, $df=13$)		
<i>Accuracy</i>		
QC1: 600 ng/mL (bias, %)		2.7%
QC2: 1000 ng/mL (bias, %)		1.4%
QC3: 1400 ng/mL (bias, %)		1.4%
<i>Precision</i>		
Repeatability (RSD %)		2.1%
Intermediate precision (RSD %)		3.3%

Table 5: Evolution of relative midazolam content (%) over time, expressed as 95% CI of the mean ($n=3$, 10 units per batch).

Analysis time (Days)	HPLC-UV quantification	LC-MS/MS quantification
D0	99.7 < X < 100.3	97.6 < X < 102.4
D15	97.1 < X < 100.5	98.4 < X < 102.8
D45	96.0 < X < 98.6	99.1 < X < 103.1
D90	99.9 < X < 103.1	101.2 < X < 105.9
D180	97.3 < X < 101.6	98.6 < X < 108.9
D290	99.8 < X < 101.7	99.1 < X < 105.6
D365	98.3 < X < 101.8	98.6 < X < 105.2

Day 0 corresponds to the day of production.

but their mobile-phase were composed of orthophosphoric acid or sodium buffer which is not LC-MS compatible [30].

In this work, degradation products were found in acid, base, and oxidative conditions with UV detection. Under oxidative conditions, midazolam may be hydroxylated in 1-hydroxymidazolam, 4-hydroxymidazolam, or 1,4-dihydroxymidazolam; which are the metabolites of midazolam [17, 31]. With MS detection, only degradation products were found after H_2O_2 exposure. The product ions at m/z 273; m/z 289 and m/z 342 could be the impurities H, F, and D described in the midazolam monograph [14, 15]. The product ion m/z 342 could also be the 1'-hydroxymidazolam or the 4-hydroxymidazolam, which are the metabolites of midazolam [17]. However, additional experiments should be realized to confirm this hypothesis.

Figure 4: Representative LC-MS/MS chromatograms of midazolam hydrochloride solution at 1,000 ng/mL (as free base): transition of confirmation (m/z 326 $\rightarrow$ 249) and transition of quantification (m/z 326 $\rightarrow$ 291).

De Diego et al. reported degradation products after acid exposure but none after alkaline exposure [32]. This peak could be the open-ring benzophenone form of midazolam [32–34]. Geiger et al. described stability of midazolam after acid, heat, and oxidation exposure but a slight degradation in alkaline conditions while Costa et al. showed that midazolam was not stable after exposition to stress conditions (acid, alkaline, neutral hydrolysis, oxidation, UV light) during a few days [35, 36]. In alkaline conditions, midazolam precipitated. After neutralization, the peak of the degradation product observed had a retention time of 8.4 min, which meant it was more apolar than midazolam. We tried to identify the product with the mass-to-charge ratio with the LC-MS/MS method without success. The base-mediated degradation mechanism may be due to the fixation of OH⁻ ions on the benzodiazepine cycle [16, 37].

Otherwise, midazolam is a photosensitive drug that produces degradation products when it was exposed to UV [13, 35, 36]. However, because of a shortage of equipment, no photodegradation assay could be performed in our work. To prevent photosensitivity, capsule shells contained titanium dioxide, which is an opacifier agent [38]. Moreover, they were packaged in amber blister and stored under dark conditions.

Besides, the UV detector used in the study was not a DAD which means that the peak purity was not fully accessed [16]. Indeed, degradation products that do not absorb at the chosen wavelength could be present in the preparation. For this reason, an LC-MS/MS method was developed and validated to improve the specificity of the study and to quantify content in midazolam capsules. Several methods with LC-MS/MS technology were developed for midazolam quantification in patients [5, 31, 39–42]. However, to our knowledge, there are no published data about the use of the LC-MS/MS technique in pharmaceutical formulations except for the degradation mechanisms and the studies of new carriers like cyclodextrins [13, 34, 43]. In the stability studies, DAD and even better mass spectrometer permit the assessment of the peak purity [16]. Moreover, mass spectrometry uses a universal detector that enables to detect and characterize the degradation products [44]. Tandem mass spectrometry increases even greater the specificity by fragmenting the precursor ion (midazolam) into fragment ions. In our study, the sensibility and the specificity of the method were correct with two product ions (m/z 291.1 and 249.1).

After one year, no variation of the midazolam content in capsules was observed with the two quantification methods. This content was higher than 95% of the initial value and no macroscopic changes have been detected. These results are in agreement with the absence of degradation products which

could be seen in the chromatograms. For the LC-MS method, the concentrations of the analyte seemed higher than those measured by HPLC-UV. This slight difference between the two results could have been reduced if an internal standard was added to the samples.

Disintegration and dissolution testing could not be carried out according to the EP (Monographs 2.9.1 and 2.9.3 respectively) because no apparatus was not yet available in our department. Midazolam is a biopharmaceutical classification system (BCS) class 1 drug that is soluble in water (10.3 mg at pH 3.4) [45, 46]. Moreover, the certificate of analysis of the empty capsules indicated their conformity to the Japanese Pharmacopeia monograph for capsules, which reports a dissolution time within 10 min [47]. In summary, the dissolution testing should be acceptable despite it must be checked in the future.

In the absence of a climate chamber in our department, the stability study could not be performed in the conditions as recommended by the ICH guidelines (25 ± 2 °C, RH $60 \pm 5\%$) [21]. The capsules were thus kept in an environmentally controlled room, which is the storage local for clinical trial material.

Conclusion

Midazolam capsules were compounded in this study using excipient free of potentially harmful effects. A rapid and accurate HPLC-UV stability-indicating method was developed and validated for the quality control of the batches and the stability study. To increase the specificity and to confirm the results, MS detection was coupled with UV detection.

The results show that 1 mg midazolam capsules are stable for one year at room temperature and under dark conditions. Thus, this preparation of midazolam capsule is suitable for the clinical trial. These data will be included in the Investigational Medicinal Product Dossier (IMPD) of the clinical trial.

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