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PHYSICOCHEMICAL AND MICROBIOLOGICAL STABILITY OF A NEW ORAL CLONIDINE SOLUTION FOR PAEDIATRIC USE

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Stability of oral clonidine solution for paediatric use

Abstract

Background: As many drugs are unavailable for paediatric use, hospital pharmacies are often required to develop suitable formulations themselves. Clonidine is commonly used in paediatrics (in severe hypertension, in opiate withdrawal syndrome, in tics and Gilles de la Tourette syndrome or in anaesthetic premedication) but no appropriate formulation has been drawn up.

The aims of this work were to develop an oral solution of clonidine dedicated to children and to assess its physicochemical and microbiological stability.

Methods: Formulation of an oral solution of clonidine hydrochloride suitable for neonates and paediatrics was developed using the active pharmaceutical ingredient (API), with as few excipients as possible and without any complex excipient vehicle. A stability study was made according to GERPAC-SFPC guidelines. At each point in time (D0, D1, D7, D15, D29, D60 and D90), visual aspect (limpidity), pH and osmolality were established. Clonidine concentration was quantified using a stability-indicating HPLC-UV-DAD method previously developed from a forced degradation study and validated according to SFSTP Pharma. Microbiological stability was also tested according to the European Pharmacopeia monograph with the best adapted method (by comparing membrane filtration and inclusion). Solutions were stored in amber glass bottles with an oral adapter for up to 3 months in two different conditions: 5°C +/- 3°C and at 25°C +/- 2°C with 60% residual humidity (climatic chamber).

Results: The formulated oral solution is composed of API at a concentration of 10 µg/mL and of potassium sorbate (0.3%), citric acid, potassium citrate (pH 5 buffer) and sodium saccharine (0.025%). Forced degradation highlighted six degradation products and the method was validated in the acceptance limits of ± 5%. On D29, the mean percentages of the initial clonidine concentrations (+/-standard deviation) were 92.95+/-1.28% in the solution stored at 25°C +/- 2°C and 97.44+/-1.21% when stored at 5°C +/- 3°C. On D90, means were respectively 81.82+/-0.41% and 93.66+/-0.71%. The visual aspect did not change. Physical parameters remained stable during the study: pH varied from 4.94 to 5.09 and osmolality from 82 to 92 mOsm/kg in the two conditions tested here. Membrane filtration appeared to be the more sensitive method. Whatever the storage conditions, <1 micro-organism/mL was identified (only environmental) with no detected *E.coli*.

Conclusions: This formulation is stable for at least 3 months at 5°C +/- 3°C in amber glass bottles and for one month when stored at room temperature. Microbiological stability was proven in accordance with the European Pharmacopeia.

Stability of oral clonidine solution for paediatric use

Keywords: clonidine, oral solution, drug stability, pediatric preparation

Introduction

Among drugs available on the European market, only a few have a formulation or dosage adapted to paediatric use. In this context, off-label drug use is frequent. In hospital, it is estimated to vary from 11% to 80% [1] due to the absence of paediatric clinical trials or adapted formulations for children. In 2006, a European regulation [2] encouraged pharmaceutical industries to develop specific paediatric formulations [3]. As they are still lacking, hospital pharmacies are required to develop and prepare paediatrically-adapted forms [4].

Clonidine is a partial agonist of the alpha2 adrenergic receptors and central imidazoline receptors. Pharmacologically, it acts centrally to reduce sympathetic tone, resulting in a fall in diastolic and systolic blood pressure and a reduction in heart rate [5]. In the adult population, clonidine is licenced for the treatment of hypertension. In paediatrics, clonidine is frequently used, still off-label, in paediatric intensive care and anaesthesia. It has been used in attention-deficit hyperactivity disorder associated with methylphenidate, in opiate withdrawal syndrome in newborn infants [6-8], or in tics and Gilles de la Tourette syndrome [9]. Other uses are as an analgesic in neuropathic pain, in continuous epidural infusion or in severe non-treatable oncologic pain, combined with opiates [10]. Clonidine has also been considered for children in premedication before anaesthesia, rather than midazolam, because of potentially better post-operative analgesia [11].

The marketed formulations in Europe are tablets (25 µg), ampoules for injection (150 µg/1 mL) and transdermal patches providing between 0.1 and 0.3 mg of clonidine per day. Tablet formulation is not suitable for paediatric use (especially under six years of age) [12], as swallowing tablets is unsafe or impossible, and is not a flexible form for dose adjustment [13]. Furthermore, the recommended therapeutic range of clonidine in children implies grinding tablets, which increases the risk of dose imprecision. In this population, solution is the most acceptable pharmaceutical form [14]. Some formulations have already been specifically developed to obtain suitable liquid forms [15-20]. The number and proportion of excipients must be as low as possible as risks about certain excipients, especially ethanol, propylene glycol, benzyl alcohol and parabens have been identified [21-25]. These excipients are sometimes found in ready-to-use commercial complex excipient vehicles (for example, parabens in Ora®) which are still not available worldwide, are subject to shortages, and their composition is susceptible to modification. Quality control for these vehicles in hospital pharmacies do not correspond to compendial methods.

Therefore, the aim of this study was to develop with raw materials, a paediatric formulation of clonidine hydrochloride without any identified risks for children and to evaluate its

physicochemical and microbiological stability in two different conditions (storage at room temperature and at 5°C +/- 3°C).

Materials and Methods

Materials:

Clonidine hydrochloride, citric acid monohydrate (Inresa, Bartenheim, France), tripotassium citrate, potassium sorbate and sodium saccharin (Cooper, Melun, France) used in the formulation were of pharmaceutical quality. Sterile water (Fresenius Kabi, Sevres, France) was used as solvent.

Acetonitrile, potassium dihydrogen phosphate, sodium hydroxide and 37% hydrochloric acid were supplied by VWR (Fontenay-sous-bois, France). 30% hydrogen peroxide was purchased from Cooper (Melun, France). Potassium hydrogen phosphate was obtained from Merck (Darmstadt, Germany). Clonidine hydrochloride was supplied by CRS European Pharmacopoeia. All reagents used were at least of analytical grade except acetonitrile and water which were HPLC grade.

Formulation:

All compounding steps were carried out in a dedicated non-classified zone, without any specific protection, according to National Good Compounding practices. The operator respected the regulatory hygiene protocol for clothing (surgery mask, gloves, gown and cap) and hand-washing.

Solutions of 10µg/mL clonidine hydrochloride (HCl) were prepared in two steps. First, a solution of 1mg/mL clonidine HCl was made by dissolving 50 mg Clonidine HCl in 50 mL of sterile water.

The final solution was then obtained by dissolving 200 mg of citric acid monohydrate and 346 mg of potassium citrate in water to obtain a pH value of 5. Once the components were dissolved, 300 mg potassium sorbate and 26 mg sodium saccharine were added. Finally, 1 mL of 1 mg/mL solution and water were added to reach a volume of 100 mL.

Final solutions were stored in amber type II glass bottles with Low Density Poly Ethylene (LDPE) white oral adapter (Baxter, USA), and an LDPE stopper.

One batch of 66 vials of 15 mL was prepared on day 0.

Chromatographic conditions

The chemical stability of the Clonidine HCl solution was assessed using an HPLC method consisting of a ThermoFisher Ultimate 3000 system (Villebon-sur-Yvette, France). Separation was obtained using a C18 HyperSil Gold guard column (10x3mm, 3µm) and a C18 HyperSil Gold column (100x3mm, 3µm) conditioned at 40°C. The mobile phase was composed of a mixture of acetonitrile and phosphate solution (25mM, pH=4.5) (5/95 v/v). The injection volume was 10 µL and the flow rate 0.5 mL/min. UV detection was performed at 210 nm with a DAD system (DAD 3000).

Development of stability-indicating method

Forced degradation was performed to ensure complete separation between clonidine, excipients and clonidine degradation products. Thus, Clonidine HCl was stressed in acidic, alkaline, oxidative and neutral hydrolytic conditions according to the GERPAC-SFPC standard [26]. One volume of a 400 µg/mL clonidine solution was mixed with one volume of 5N HCl, 5N NaOH and 30% hydrogen peroxide in turn, prior to being heated at 100°C for up to 3 days. Samples were then withdrawn at several times, and diluted to 100 µg/mL, taking care to neutralise media before injection, in an attempt to obtain a 20% decrease in the main active ingredient. For each sample, the UV spectrum was analysed with the PDA detector to highlight the possible appearance of new peaks and clonidine peak decreases. Method specificity was assessed by comparing chromatograms of stressed clonidine solutions with a standard clonidine solution and with a blank solution spiked with excipients.

Validation method

The validation method was carried out according to the validation guidelines established by the French Society of Pharmaceutical Sciences and Techniques – SFSTP pharma [27-30]. Each day and for three consecutive days, five calibration standard sets and three sets of 4 validation standards were prepared from 10 µg/mL independent aqueous clonidine solutions with appropriate dilutions in the mobile phase to obtain a calibration range at 1.5; 2; 2.5; 3 and 3.5 µg/mL and 4 validation standard levels at 1.75; 2.25; 2.75; 3.25 µg/mL. A preliminary study of the absence of matrix effect had been made.

The Limit Of Quantification (LOQ) and Limit Of Detection (LOD) according to the International Conference on Harmonization (ICH) [31] were calculated respectively as the

ratios 10×σN/S and 3.3×σN/S where σN is the standard deviation of the noise obtained with six blanks and S, the slope of the calibration curve. Selectivity, response function linearity, trueness, precision (repeatability and intermediate precision) were studied according to the validation requirements of the SFSTP and accuracy profile was assessed by taking into account the ± 10% acceptance limits for quantification (maximum tolerated error), an alpha risk of 5% and ß expectation tolerance limits (ß = 95%).

Physico-chemical stability study:

Samples were stored in amber glass bottles, protected from light in two different storage conditions:

- at 25°C +/- 2°C, with 60% residual humidity (climatic chamber) and,
- at 5°C +/- 3°C.

On the day of the analysis, two samples were tested twice for each condition to limit variability. Parameters were measured on days 0, 1, 7, 15, 30, 60, 90 and 120.

Vials were stored protected from light and horizontally so that all the conditioning materials were in contact with the solution.

At each point in time, the percentage of the remaining clonidine HCl concentration was compared to the concentration at T0 and the four validation standards were analysed. Stability was defined as a percentage of at least 90% of the initial clonidine HCl concentration.

Physical parameters such as colour, odour, limpidity, osmolality and pH value were measured.

At each sampling time, the pH was measured three times in two vials with an HI223 HANNA Instrument pH-meter (Tanneries, France).
The osmolality of a vial was measured using an Advanced Micro Osmometer® Model 3300 (Series 03010058P, Advanced Instruments, Norwood, USA).
Again the measurement was repeated three times for the two vials. Statistical analysis on osmolality and pH was made at different times with the Kruskall Wallis test (alpha risk of 0.05).

Finally, at each sampling time, and for each storage condition, a visual inspection of the vial was made by the same operator for the appearance of colouring, blurring, haze, particles, gas or precipitate.

Validation of microbial recovery method for bacteria and fungi

As the formulated solution is designed for the oral route, sterility is not necessary but the absence of contamination has to be validated.

A study of the artificial microbiological contamination of the solution was conducted, adapted from 5.1.4 “microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use”, 2.6.12 “microbiological examination of non-sterile products: microbial enumeration tests ” and 2.6.13 “microbiological examination of non-sterile products: tests for specified micro-organisms” monographs in the EP [32-34].

The preservative effect of potassium sorbate was neutralised by diluting the clonidine solution with pH 7.2 phosphate buffer (1:10), and by increasing its pH to over 6 [33-35].

Reference strains (*Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *Aspergillus brasiliensis* ATCC 16404, *Candida albicans* ATCC 10231) were used for neutralisation tests.

Briefly, micro-organisms isolated from agar plates were resuspended in sterile phosphate saline buffer. Serial dilutions of bacterial and fungal suspensions were made to obtain standardised microbial suspensions of 10^3 micro-organisms/mL. 5 mL of the suspensions were then added to 45 mL of the studied solutions (i.e. pure clonidine, 1/10 diluted clonidine and neutralising buffer), yielding 10^2 micro-organisms/mL suspensions.

Validation of microbial recovery was carried out by comparing the inclusion and filtration methods recommended by EP guidelines. On the one hand, 1 mL of the 10^2 micro-organisms/mL suspensions was covered by 19 mL of agar medium. After solidification, the agar plates were incubated at 37 °C (bacteria) or 30°C (fungi). On the other hand, 10 mL of the 10^2 micro-organisms/mL suspensions was filtered through 0.45 μm membranes (Millipore, Billerica, USA) rinsed or not with 5 ml of pH 7.2 phosphate buffer. Membranes were set on appropriate agar media: Chapman and Tergitol (Oxoid, Wesel, Germany) for *S. aureus*, and *E. coli*, respectively, and Sabouraud for *C. albicans* and *A. brasiliensis*.

Microbiological count was made after an incubation period of three days at 37°C for bacteria and five days at 30°C for fungi.

Fertility tests were made beforehand for all agar plate media.
Each experiment was performed in duplicate.

Analysis of the data

The efficacy of neutralisation was considered for a microbial recovery ratio > 0.5 between the neutralised solution and the negative control.

Microbiological quality control study

Analysis was performed on clonidine solutions stored for 75 days at room temperature or 5°C +/- 3°C. The results were interpreted according to EP requirements for oral aqueous preparations which are: total aerobic microbial count (TAMC) of less than 100 CFU/mL, total combined yeast and moulds (TYMC) of less than 10 CFU/mL and absence of *E. coli* [32].
Each studied solution was diluted at 1:10 with phosphate buffer pH = 7.2.
Ten milliliters of the diluted solutions were then filtered on 0.45 μm membranes rinsed with 5 mL of phosphate buffer. The membranes were then set in duplicate on PCA, Chapman, Tergitol or Sabouraud media. Agar plates were incubated for 3 days at 37°C for bacteria and 5 days at 30°C for fungi. Identification of bacterial colonies was made through MALDI TOF mass spectrometry (Brüker, Wissembourg, France).

Results

Validation of the stability-indicating method

The specificity of the method was assessed between clonidine and excipients (figure 1) and clonidine and degradation products (figure 2). With these elution conditions, complete separation between Clonidine and excipients (figure 1a) and a convenient clonidine retention time (around 6 min) were obtained (figure 1b). The stress degradation study led to the formation of degradation products, of which six were detected at the following retention times: 2.9 – 3.4 – 4.2 - 6.5 – 7.6 and 10.9 min. Degradation peaks appeared in the 3 conditions (acid, alkaline and oxidative) but there was a 20% reduction in clonidine only in alkaline media (figure 2). A comparison of chromatograms indicated the absence of co-

elution between the degradation products, clonidine and excipients and ensured the specificity of the method (figures 1 and 2).

LOD and LOQ were 0.07 µg/mL and 0.21 µg/mL, respectively. The response function obtained by using the calibration curves was y= 1.8762x+0.0406 and the determination coefficient (R^2) of over 0.999 attested to the validity of the model. Linear models (R^2>0.995) were obtained by plotting the introduced concentration to the back-calculated concentration; the slope close to one and y-intercept approaching zero showed the linearity of the method. The accuracy profile displayed in figure 3 shows a tolerance interval included within the acceptance limits of ± 5% and so respects acceptance criteria set at ± 10%. Consequently, the method was able to quantify clonidine from 1.5 to 3.5 µg/mL with a total error of ±5% and an alpha risk of 5%.

Physico-chemical stability

Table 1 shows the percentage of initial concentration +/- SD of clonidine HCl remaining on each day, for the two storage conditions. A solution stored at 25°C +/- 2°C was stable for one month whereas one stored at 5°C +/- 3°C was stable for at least 3 months.

An unidentified product appeared on the chromatogram (Tr: 2.9 min) at 2 months, corresponding to none of the components in the formulated solution when stored at 25°C.

Osmolality and pH were also measured and are presented in Table 2. No statistical change in pH value was observed during the study. Even if a statistical difference is found in osmolality, threshold or link with the degradation process is unclear. No change in colour, odour or limpidity was observed.

Validation of microbial recovery method for bacteria and fungi

Results are presented in Tables 3 and 4 for bacteria and fungi respectively.

In all samples, bacterial and fungal load was inferior to 100 UFC/mL with similar values between the positive (pure clonidine) and the negative (phosphate solution at pH 4.5) controls. All micro-organisms grew, regardless of the method. Although slightly higher recovery rates were observed with the inclusion method for some organisms (*i.e. E. coli* and *C. albicans*), lower bacterial or fungal concentrations are to be expected in the clonidine suspension and larger volumes should be used to increase sensitivity. However, in this experiment, the filtration method was not adapted to *A. brasiliensis* numeration owing to a rapid confluence of the colonies. In any case, the additional step of membrane washing did

not alter the final microbiological count, enabling the elimination of residual preservative components. In these experiments, membrane filtration appeared as a sensitive and appropriate method for highlighting low bacterial or fungal concentrations. Hence, this method was chosen for further quality control assessment.

Microbiological quality control study

When assessing microbial contamination after 75 days' storage at room temperature or at +2-8°C, bacterial counts varied between 1 and 25 CFU in both. No fungal colony was found after filtering the neutralised clonidine solution (Table 5). Mass spectrometry identification performed on bacterial colonies indicated environmental or cutaneous species (*Micrococcus* sp., coagulase negative *staphylococci*, *Methylbacterium* sp., *Bacillus* sp.). It is Important to note that no *E. coli* was detected in any sample. These results were in accordance with EP thresholds.

Discussion

To the best of our knowledge, this is the first description of a ready-to-administer oral clonidine solution, compounded without any parabens or any complex excipient vehicle, adapted to children and stable for at least 3 months.

Most formulations use clonidine tablets as raw materials [16-19]. Clonidine powder is more accurate and reduces the variability of tablet sourcing. Preparing in conditions as described above eliminates the introduction of microbiological contamination, although it is necessary to validate this data in the specific compounding location. Adding an antimicrobial preservative to an aqueous-based oral solution contained in a multi-dose vial is mandatory. Potassium sorbate appears to be the candidate of choice, as it does not cause any side effects and its concentration in the solution is higher than the minimal inhibitory concentration (MIC) for some bacteria (*E. coli* and *S. aureus*) and fungi [35]. To be effective, it is necessary to add a buffer to adjust pH to around 5; citrate buffer was chosen because of its good tolerance when used orally, but it can modify the taste [3] and so a sweetener was added to the solution.

Neither sugar nor dextrose was added, because of cariogenic or metabolic risk; furthermore, they potentially increase the osmolality and viscosity of the solution. The taste of similar solutions, with various saccharin concentrations, and without clonidine, was assessed by a group of adult volunteers and led to using the lowest concentration tested (0.025%). The

solution is now regularly dispensed in the wards of our hospital and accepted without complaints.

Two clonidine liquid formulations dedicated to paediatrics have previously been developed, without parabens [19-20]. Sauberan *et al.* [19] reported a liquid oral form at 20 µg/mL with simple syrup NF as vehicle, stable for 35 days. This compounded liquid form was highly osmolar, at around 3180 mOsm/kg, tenfold higher than what is physiologically acceptable; this justified, according to Sauberan et al, diluting it with breast milk, without establishing its stability or acceptability in this context. Simple syrup in Europe generally contains parabens as preservatives [15].

Potier A *et al.* [20] developed a clonidine liquid formulation dosed at 10 µg/mL, compounded with inorpha°, a complex excipient vehicle, available firstly in France. This excipient vehicle has many advantages, for example it is alcohol- and paraben-free. Also, data increasingly shows its advantages such as convenience in compounding liquid forms, with microbiological and chemical stability for various APIs. However, it is not available worldwide, is susceptible to composition alterations and its impact on the compounded forms of API is unknown. Inorpha° supplies have recently been disrupted, which is potentially detrimental to paediatric patients. Furthermore, some raw materials are not well defined in terms of pharmaceutical quality, such as taste-masking agents. All these reasons have led us to conclude that the formulation we develop is useful in practice, is easy to implement and it is available. Our data indicates longer stability between +5 +/- 3°C (at least 90 days, when stocked) compared to 60 days for Potier *et al*. They showed however that the concentration of sorbate potassium, similar to our formulation, is physico-chemically stable.

Potier *et al.* [20] hypothesised that degradation at ambient temperature is potentially due to an adsorption on the PET container. As we use glass for containers and reach a similar decrease (86.8% of initial concentration *vs* 84.1% for Potier A *et al.*), the degradation of clonidine is due to another phenomenon that should be investigated.

Other developed formulations appear to be stable for 9 months at ambient temperature. The origin of instability needs to be studied, and hypotheses to be explored are concentration, pH or interaction with excipients. Injectable solution specialties dosed at 150 µg/mL clonidine with extensive stability are at a pH of 4 whereas the pH of our oral solution is around 5. It was not possible to buffer the solution at pH 4 because of blurring and physical change. A formulation dosed at 50µg/mL and with methylparaben, expiring at 9 months is at pH 5 [15]. It is also necessary to identify, by an adapted analytical method (nuclear magnetic resonance, mass spectrometry), the substance that induced a peak (previously mentioned) during a forced degradation study and increased during a stability study. Thus the mechanism of formation should be studied, and potentially reduced.

Several forced degradation studies have already been reported [18, 20, 36-38]. However none have highlighted degradation products, notably those indicating a loss of clonidine. Our results describing 6 degradation products can be explained by our weaker eluting analytical conditions which were able to detect polar degradation products. Moreover, degradation product detection was improved with drastic stress conditions and a concentrated clonidine solution at 100 µg/mL.

Other HPLC stability-indicating methods have previously been developed [15-18, 20, 39-41], usually with ammonium salt and methanol as mobile phase. In these methods, the sample is often centifuged before analysis, especially when using complex vehicles in formulations or when formulating suspensions. The simplicity of our formulation meant we did not have to filter samples. Indeed, we have shown that filtration induces a potential adsorption of clonidine on the media.

The validation method according to SFSTP is based on the study of « accuracy », also called « total error » which results from the sum of systematic error (trueness) and random error (precision) obtained with validation standards. The total error of the analytical method is represented by an accuracy profile indicating total error according to the concentration level of the validation standard studied. The accuracy profile is therefore a decision-making tool ensuring the quality of the analytical procedure with a tolerance error (acceptance limits) and a risk (β-expectation tolerance interval) previously defined by the analyst. Hence, β represents the probability that measurements are included within the acceptance limits.

Two inoculation methods were tested: inclusion (with deep seeding) and membrane filtration. The inclusion seeding method has the disadvantage of having a small test sample (1 mL) compared to membrane filtration (10 mL). Since a low inoculum of microorganisms was expected in the clonidine solution to be tested, a large volume was necessary to ensure a sufficiently sensitive result. The deep seeding method makes it possible to search in particular for anaerobic and microaerobic flora. Moulds and yeasts are aerobic microorganisms and so inclusion is not suitable for them. During the inoculation of *A. brasiliensis* and *C. albicans* in depth, we noticed that these microorganisms had developed mainly on the surface and not within the agar. Moreover, microorganisms cannot be identified with the inclusion method. As regards the microbiological assessment of the clonidine solution, the contamination level after 75 days' storage is in accordance with EP criteria, with a number of CFUs below the accepted limits, and absence of pathogenic bacteria or fungi. The efficacy and advantage of using sorbate potassium in our formulation has yet to be shown, whereas it has already been proven [33]. In view of these results,

conditions of environmental and raw-material contamination in the compounding of the clonidine solution are acceptable. It is important to note that according to the EP, the microbiological qualities of pharmaceutical grade substances are controlled and must be stored in accordance with good practices. There is a limitation as the analysis was made once on each vial and not repeated after they were opened. The study does not provide data on the re-use or contamination of the vials. With volumes of 15 mL there can be no prolonged use of the solution. Potier A *et al.* [20] used a similar concentration of clonidine and preservative, and did not find any stability variation when stocked or in use.

Even if this work offers information about formulation and stability, data to document bioavailability, pharmacokinetics, pharmacodynamics, efficacy, and tolerance profile is still lacking. The aqueous solubility of clonidine of about 77 mg/mL (Merck index) ranks it in either Class I (high solubility/high permeability) or Class III (high solubility/low permeability), under the Biopharmaceutics Classification System [42]. Even if permeability is reduced, which should in theory reduce absorption, oral bioavailability is reported to be high when used in solid oral dosage form for adults [43-44].

Conclusion

This work led to the development of a 10 µg/mL clonidine solution, suitable for pediatric use, and easily quantifiable through the use of a stability-indicator dosing method to determine the active ingredient in a pharmaceutical preparation.

According to preliminary results, this solution is stable for at least 3 months when stored at 5°C +/- 3°C and for 1 month at room temperature.

The study of the microbiological contamination of a non-sterile preparation was also carried out. The microbiological data obtained is satisfactory according to current recommendations.

The advantage of this solution as anaesthetic premedication in children must now be assessed. Clinical evaluation will have to be initiated to obtain pharmacokinetic, tolerance and acceptability data to establish its efficacy. The role of oral clonidine should then be compared to other means, making use of other reference strategies (placebo or midazolam) and / or other routes of administration (e.g.nasal route).

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Conflict of interest statement

The authors declare that they have no conflict of interest.

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Tables

Table 1: content of clonidine over 90 days at two different storage conditions

	Storage conditions	Day 0	Day 1	Day 7	Day 15	Day 29	Day 61	Day 90
% initial concentration of clonidine (+/- SD)	25°C	100 +/- 1.28	99.66 +/- 0.29	98.35 +/- 0.71	95.99 +/- 0.28	92.95 +/- 1.28	86.80 +/- 0.37	81.82 +/- 0.41
	2-8°C	100 +/- 1.28	99.41 +/- 0.22	99.89 +/- 0.62	98.68 +/- 1.58	97.44 +/- 1.21	95.96 +/- 0.48	93.66 +/- 0.71

Table 2: Osmolality and pH value over 90 days at two different storage conditions, with kruskal wallis statistical analysis

Storage conditions		Day 0	Day 1	Day 7	Day 15	Day 29	Day 61	Day 90	Statistical analysis
25°C	Osmolality (mOsm/kg)	89.83	89.83	84.67	84.67	83.83	82.83	82.33	p<0.0001
	pH	4.97	4.94	5.09	5.02	5.01	5.02	5.02	p=0.193
2-8°C	Osmolality (mOsm/kg)	89.83	92.33	84.00	83.83	83.33	82.50	81.50	p=0.002
	pH	4.97	4.96	5.06	5.02	4.99	5.04	5.04	p=0.065

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Table 3: Validation of microbial recovery method for bacteria (CFU: colony-forming unit)

Microorganisms		*E. coli ATCC 25922*						*S. aureus ATCC 29213*					
Recovery method		*Membrane filtration (10 mL)*				*Inclusion (1mL)*		*Membrane filtration (10 mL)*				*Inclusion (1mL)*	
Clonidine solution		Pure	Pure rinsed	Diluted 1/10	Diluted at 1/10 and rinsed	Pure	Diluted 1/10	Pure	Pure rinsed	Diluted 1/10	Diluted at 1/10 and rinsed	Pure	Diluted 1/10
Bacterial load in the solution	Mean CFU values	316	268	284	261	46	43	292	309	318	285	34	34
	CFU / mL	**31,6**	**26,8**	**28,4**	**26,1**	**46**	**43**	**29,2**	**30,9**	**31,8**	**28,5**	**34**	**34**

Table 4: Validation of microbial recovery method for fungi (CFU : colony-forming unit; NQ : not quantifiable)

Microorganisms		*A. brasiliensis* ATCC 16404					*C. albicans* ATCC 10231				
Recovery method		*Membrane filtration (10 mL)*				*Inclusion (1mL)*	*Membrane filtration (10 mL)*				*Inclusion (1mL)*
Clonidine solution		Pure	Pure rinsed	Diluted 1/10	Diluted at 1/10 and rinsed	Pure	Pure	Pure rinsed	Diluted 1/10	Diluted at 1/10 and rinsed	Pure
Fungal load in the solution	Mean CFU values	NQ	NQ	NQ	NQ	48	337	355	367	342	84
	CFU / mL	NQ	NQ	NQ	NQ	**48**	**33,7**	**35,5**	**36,7**	**34,2**	**84**

Table 5: Microbial recovery after filtration of aqueous clonidine solution stored for 75 days at room temperature or at +2-8°C

Media	2-8°C storage	25°C storage
	Microbiological count (CFU)	
Sabouraud	0	0
PCA	25	1
Chapman	0	25
Tergitol	15	1

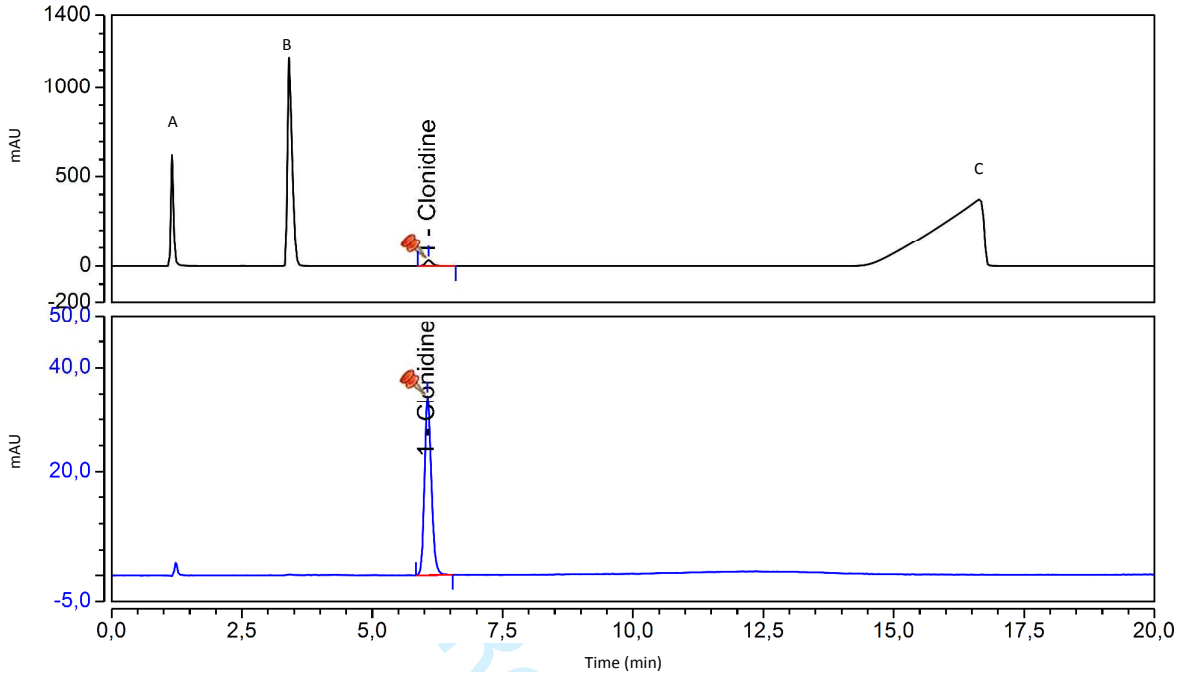

Figure 1: Specificity of clonidine and formulation excipients

Figure 1A : chromatogram of formulation excipients (A-Potassium Citrate/Citric acid (t_r: 1.2 min); B-Saccharine (t_r: 3,4 min); C-Potassium sorbate (t_r: 16,5 min)) and clonidine (t_r: 6.06 min).

Figure 1B : Chromatogram of clonidine alone at 2.75 µg/mL (A) (t_r = 6 min).

Separation was performed according to the analytical conditions described in the Material and Methods section.

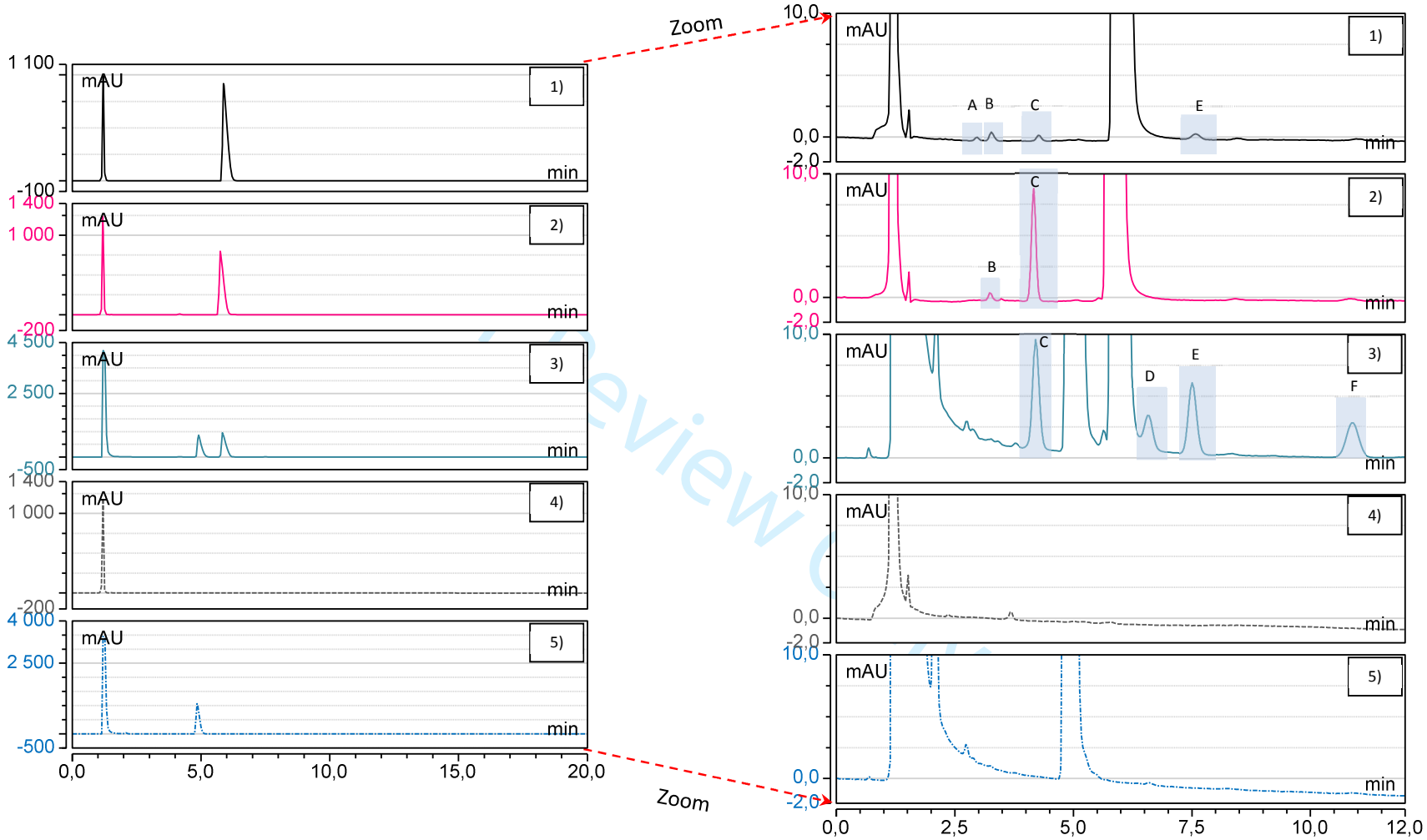

Figure 2: Forced degradation

Chromatograms were obtained according to the optimal analytical conditions described in Chromatographic conditions in Materials and Methods section.
1) 100 µg/mL clonidine solution stocked in HCl 5N at 100°C for 3 days ; 2) 100 µg/mL clonidine solution stocked in NaOH 5N at 100°C for 3 days; 3) 100 µg/mL clonidine solution stocked in 30% H_2O_2 at 100°C for 3 days; 4) NaOH and HCl blank; 5) H_2O_2 blank – A (tr = 2.9 min), B (tr = 3.4 min), C (tr = 4.2 min), D (tr = 6.5min), E (tr = 7.6 min) and F (tr= 10.9 min) are degradation products highlighted with the 100 µg/mL clonidine solutions stressed in the three different conditions (HCl 5N, NaOH 5N and 30% H_2O_2).

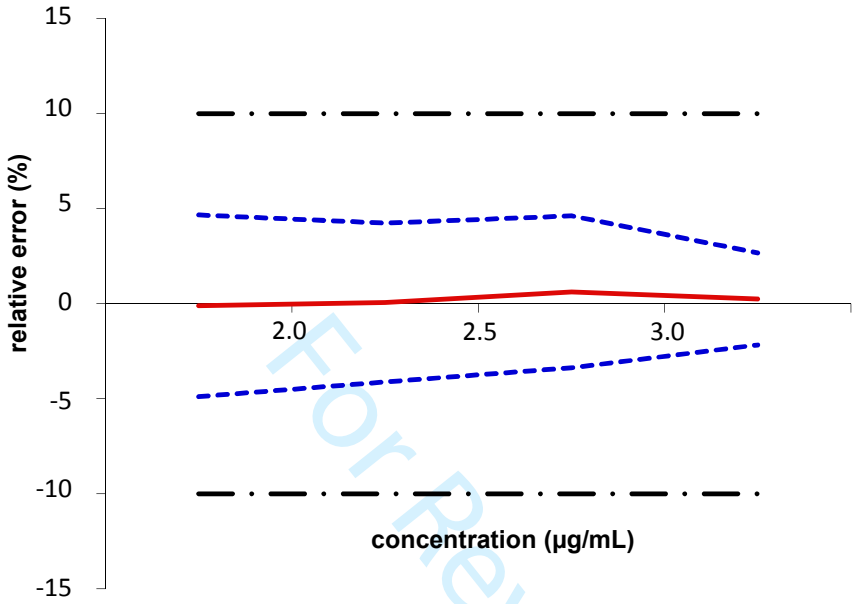

Figure 3: Accuracy profile of Clonidine.

The plain line corresponds to the bias. The dashed lines and dotted lines represent acceptance limits of 10% and the tolerance interval for a risk of 5%, respectively.