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European Journal of Pharmaceutics and Biopharmaceutics 59 (2005) 449–459
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

Baclofen-loaded microspheres: preparation and efficacy testing in a new rabbit model

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Received 14 June 2004; accepted in revised form 6 August 2004
Available online 9 December 2004

Abstract

Intrathecal baclofen is the reference treatment for severe spasticity. This drug has to be injected chronically in the intrathecal space by implanted pumps which are very expensive, uncomfortable and sometimes lead to side effects. Previous work has been performed by our group to assess the feasibility of encapsulating baclofen into poly(lactide-co-glycolide) (PLGA) microspheres and injecting these preparations in the intrathecal space of rabbits. The aims of the present study were to improve the encapsulation process for industrial application (scale-up), and to set up an animal model to assess the duration of effect of the new formulations. Modifications included the replacement of methylene chloride by a less toxic solvent, ethyl acetate, and the use of high molecular weight polymers to extend the release rate of the drug. The temperature and organic solvent extraction rate were fully controlled during the whole manufacturing process. All these modifications resulted in high quality microsphere batches with a CV inferior to 5% for encapsulation efficiency and drug loading. Encapsulation efficiency and release patterns were dependent on the drug payload and the polymer used. A formulation displaying a sustained release of baclofen over 174 days and a moderate burst effect of 16% in the first day in vitro was evaluated in a new reliable model of baclofen activity based on electrophysiological measurement of *H*-reflex in the rabbit. The activity of a very low dose of baclofen microspheres in vivo was sustained over 35 days. Furthermore, the preparation was well tolerated. These newly developed preparations are a very promising approach for enhancing the efficacy and comfort of patients undergoing spasticity treatment.

Keywords: Spasticity; Intrathecal baclofen; Rabbit; *H*-reflex; Electromyography; Microsphere; Ethyl acetate; Sustained release; Poly(lactide co-glycolide)

1. Introduction

Since the first clinical studies published about 20 years ago [1–3], intrathecal baclofen remains the reference treatment for severe spasticity of spinal or cerebral origin [4–6]. To treat this very disabling syndrome, very small quantities of baclofen have to be continually injected into the intrathecal space as a lifetime treatment. To date, the only solution found to solve this challenging drug delivery issue is to use electronic pumps surgically implanted in the patient's body and connected to indwelling catheters. The costs of surgical implantation and follow-up of these devices are significant [7], thus limiting the number of treated patients. Moreover, the risk of infection or catheter dysfunction is high, often resulting in the discontinuation of therapy [8–11].

The development of long-lasting sustained release intrathecal baclofen dosage forms could solve many of the reported issues related to implanted pumps. These systems should provide a continuous release of baclofen for at least 3 months without any burst effect. Moreover, these formulations have to be well tolerated, biodegradable

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doi:10.1016/j.ejpb.2004.08.013

and easy to administer. Among all the formulations designed for spinal drug delivery [12], poly(lactide-co-glycolide) (PLGA) microspheres could fulfil all these requirements. Our research group has already demonstrated the feasibility of encapsulating baclofen in PLGA microspheres [13] and injecting these preparations in the intrathecal space of rabbits [14]. After these preliminary studies, several problems remained unsolved. In particular, the in vitro release pattern of baclofen from microspheres was biphasic with a burst effect of 10–60% depending on the drug payload. Furthermore, the polymer used (PLGA 50/50) allowed a release over 4 weeks only. Last but not least, the manufacturing process was not consistent with industrial requirements: a chlorinated organic solvent (methylene chloride) was used and its repeatability was not optimised. Methylene chloride is listed as a 'class 2 solvent' ('solvent to be limited'); in contrast, ethyl acetate is listed as a 'class 3 solvent' ('less toxic solvent') in the 'Q3C guidance for industry: residual solvents' edited by the FDA (CDER) in November 2003.

Thus, the main objective of the current investigation was to modify the manufacturing process to adequately address these issues. To do so, two modifications were performed. First, methylene chloride was substituted with ethyl acetate, a less toxic solvent, previously reported for microparticle preparation [15–18]. Second, a polymer with a higher proportion of lactide residues or a higher molecular weight, therefore displaying a slower degradation rate, was used. The manufacturing process was modified in order to obtain an enhanced repeatability. The parameters affecting the overall features of microspheres such as temperature or organic solvent extraction rate and stirring were identified, optimised and fixed.

The second part of the study consisted of developing an animal model for testing baclofen activity and evaluating the efficacy of an optimised batch of microspheres after intrathecal injection. One of the well known pharmacological properties of baclofen is to depress the Hoffmann-reflex (*H*-reflex) [19]. The *H*-reflex is obtained after percutaneous electrical stimulation of the I-a sensitive fibers. The afferent volley then proceeds to the spinal cord leading to a monosynaptic excitation of the alpha motoneurons and the subsequent activation of the muscle fibres [20]. Baclofen inhibits this neural circuitry by acting on presynaptic targets of alpha motor neurons [21]. For sufficient electrical stimuli, the motor neuron is directly stimulated and produces an early signal called the *M*-wave (Fig. 1). In order to standardise the observations, multiple stimulations are performed and the ratio of the *H*-reflex of maximum amplitude over the *M*-wave of maximum amplitude is calculated. The H_{max}/M_{max} ratio has been previously proposed to assess intrathecal baclofen activity in spastic patients [21,22], and has been mostly studied in the rat [23–25] and to a lesser extent in the rabbit [26]. In the latter case, its long term reproducibility has not been determined. Thus, an animal model of intrathecal baclofen activity based on the determination of H_{max}/M_{max} ratio was implemented: the repeatability of *H*-reflex was optimised and the effect of different bolus doses of intrathecal baclofen on H_{max}/M_{max} ratio was investigated. Finally, the activity of new baclofen-loaded microsphere preparations displaying a reduced burst effect was studied with this pharmacological tool.

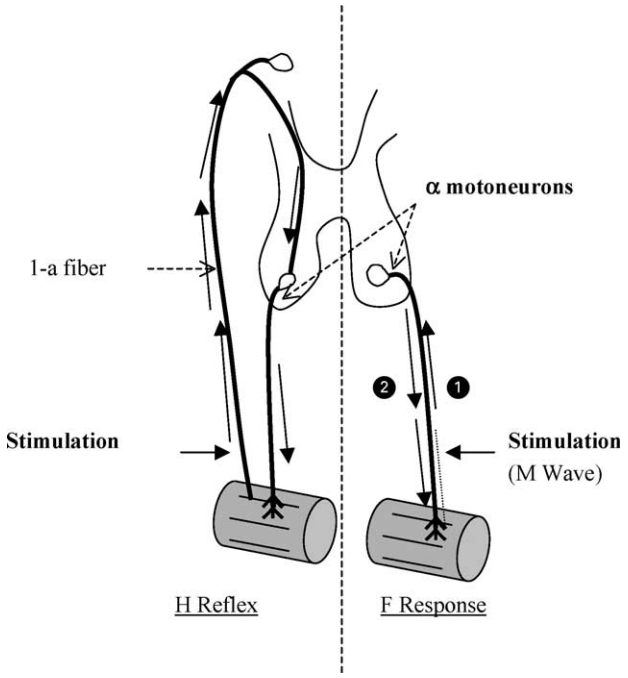

Fig. 1. Pathways and differences between *H*-reflex and *F*-response loops consecutive to sensitive fiber stimulation (*H*-reflex) or motor fiber stimulation (*F*-response). In the latter case the pathway of the signal along the axon of the α motoneuron is first ascendant (1) and then descends back to the muscle following the same axon (2) *M*-waves result from direct muscle stimulation.

2. Materials and methods

2.1. Microsphere preparation

Microspheres were prepared using two different poly(DL-lactide co glycolide), Medisorb®: 50/50 (MW_w 74 kD, inherent viscosity 0.75 dL/g) or 85/15 (MW_w 95 kD, inherent viscosity 0.63 dL/g), obtained from Alkermes® (Cincinnati, OH, USA). Poly(vinyl alcohol) 88% hydrolysed, Rhodoviol® 4/125 was obtained from Merck Eurolab® (Paris, France) and ethyl acetate reagent grade from Sigma Aldrich® (Saint Quantin Fallavier, France). Baclofen powder was purchased from Heumann® (Feucht, Germany).

Microspheres were prepared using a solvent extraction process [18,27], modified to accommodate for polymer solubility in ethyl acetate and to control ethyl acetate extraction speed. Baclofen was first ground using a planetary micro mill (Pulverisette 7, Fritsch®, Idar-Oberstein, Germany) for 20 min at maximum speed in order to

reach 4.7 ± 2.6 μm crystal mean size (light scattering analysis, Mastersizer®, Malvern). The drug (100–150 mg) was then suspended in 4 mL ethyl acetate and homogenised using an Ultra-Turrax® (22,000 rpm, 2 min). The polymer (400 mg) was gently dissolved in the baclofen suspension under magnetic stirring at room temperature 30 min. The resulting organic suspension was emulsified in 40 mL of 5% poly(vinyl alcohol) aqueous solution maintained at 4 °C in a 250 mL reactor under paddle stirring at 1000 rpm. Two milliliters of additional ethyl acetate were used to rinse the vial in which the organic suspension was made. After 2 min, the extraction of ethyl acetate from the dispersed phase of the emulsion was performed by pouring 100 mL distilled water into the emulsion over 1 min. Finally, the extraction was completed by transferring the emulsion into 2 L of water at room temperature under paddle stirring (500 rpm). Microspheres were then isolated by filtration under nitrogen pressure using ethylcellulose membranes with a 3 μm porosity (Millipore®, Guyancourt, France). Finally, the microspheres were freeze-dried overnight at −37 °C under 5 Pa and sterilised under gamma irradiation at atmospheric pressure with a dose of 20 kGy.

2.2. Microsphere characterisation

2.2.1. Size distribution analysis

Microsphere size analysis was performed using a Coulter® particle size analyser (Multisizer®, Coultronics, Margency, France). Samples of 5 mg microspheres were suspended by sonication (5 min) in 2 mL of an aqueous solution of polysorbate 80 (0.02% w/v) and size was monitored after dilution in Isoton® II (Coultronics®, Margency, France) using a 200 μm diameter probe.

2.2.2. Microscopy studies

Optical microscopy was performed using an Olympus® BH2 microscope (OSI, Paris, France). The surface of the microspheres was observed by scanning electron microscopy (SEM). The microspheres were vacuum-coated with a carbon film and directly analysed under SEM (JSM 6301F, Jeol®, Paris, France) with an electronic acceleration of 2.6 keV for surfaces analysis and 15 keV for overall batch observation.

2.2.3. Baclofen content

The baclofen content was determined by liquid chromatography tandem mass spectrometry (LC/MSMS), after dissolution of 10 mg of microspheres in 1 mL chloroform under slight stirring for 10 min followed by liquid/liquid extraction of the drug by 3 mL of distilled water under gravimetric rotational stirring for 15 min. A 20 μL aliquot of the extracted aqueous phase was diluted (1 in 500) prior to analysis in order to remain in the linear range of the calibration curve. Electrospray tandem mass spectrometry analysis was performed on a Sciex® triple quadrupole API 300 mass spectrometer (Courtaboeuf, France) equipped with an atmospheric ionization source via an ionspray interface. For liquid chromatography, two micropumps and a Perkin Elmer® autosampler series 200 (Courtaboeuf, France) were used. Baclofen was separated on a Waters X-Terra® MS C8 5 μm 100 × 2.1 mm column (St Quentin-en-Yvelines, France). The mobile phase was a mixture of water (60%) and acetonitrile (40%). Solvent flow was set at 250 μL/min and 20 μL of sample was injected. The elecrospray evaporation process produced a protonated molecular ion (mass = 214.1 a.m.u.) selected in the first quadrupole Q1 and fragmented in the collision cell Q2 with nitrogen gas. The product ion spectra were obtained in the second quadrupole Q3. To increase specificity, one resulting daughter ion (mass = 151.2 a.m.u.) of the protonated molecular ion was monitored in Q3. The quantification was thus based on the transition from 214.1 to 151.2. Before analysis, the samples were diluted (1 in 10) to limit the perturbation of the buffer salts during the evaporation process. Prior to sample analysis, this analytical procedure was validated using SFSTP guidelines [28]. More particularly, limit of quantification (LOQ = 1 μg/L, absolute error < 15%), limit of detection (LOD = 0.2 μg/L, peak to peak signal to noise ratio > 3) and linearity (1 μg/L–1 mg/L, $r^2 > 0.999$) were assessed.

2.2.4. In vitro release studies

In vitro release testing was performed with Sotax® (Basel, Switzerland) continuous flow cells placed in a 37 °C bath and connected to a IPCN Ismatec® (Merk Eurolab, Paris, France) peristaltic pump to form an open circuit. All release measurements were made in PBS buffer (pH 7.35). The pH of the buffer was monitored twice a week during the release procedure. The flow of the PBS buffer release medium was set at 85 μL/min and controlled by a peristaltic pump previously calibrated at this flow rate. Weighed baclofen–PLGA microsphere samples (20–50 mg) were inserted in the cells and baclofen content was determined in collected fractions by LC/MSMS as previously described except that the samples were diluted 1 in 10 to fit the linear calibration range. A specific calibration curve was thus determined by using 10 times diluted PBS buffer. The key parameters of this analytical method (LOQ, LOD and linearity) were similar to those obtained with a 500-fold diluted buffer. Experiments were performed in triplicate.

2.2.5. Residual ethyl acetate determination

The determination of residual ethyl acetate in the microparticle batches was assessed after freeze-drying. A weighed amount of around 65 mg of microparticles was dissolved in 5 g of *N*,*N*-dimethylformamid in a sealed vial. The solution was then allowed to equilibrate at 80 °C. The head space was injected on a Gas chromatography line and separated along a capillary Varian DB5 Column ($l = 3$ m, $d = 0.32$ mm, 1 mm thickness) at 130 °C. A flame Ionisation Detector at 250 °C was used for detection. The retention

time of ethyl acetate was 3.7 min. Quantification was performed using a calibration curve.

2.3. Efficacy testing

2.3.1. Animals

The study was approved by the local ethics committee of University of Angers and performed in accordance with the European guidelines on animal experimentation. Experiments were conducted on New-Zealand rabbits (four males and six females) weighing 2.7–4 kg and obtained from Charles River (L'arbresle, France).

2.3.2. Electromyography

Anaesthesia was conducted using a mask with 2–5% isoflurane (Belamont®, Paris, France) while checking the cardiac or respiratory functions and pupillary dilatation. Various stimulations and recording locations were tested using monopolar needles or surface electrodes connected to an electromyograph machine (MS92B, Neurostar®, Paris, France). Constant current square wave stimuli, 0.05 ms in duration, were administered following a simple or double stimulation procedure with an intensity ranging from 0 to 300 V.

Ten *H*-reflex and *M*-wave peak to peak amplitudes were measured within 5 min and the maximal values observed were selected for calculating the *H*/*M* ratio. The H_{max}/M_{max} ratios were determined from three consecutive series of measurements in a 15 min interval (intraday repeatability). These ratios were determined once a week for 10 weeks (interday repeatability) with electrodes located on the medial edge of the right foot of four healthy rabbits of both gender. During three additional weeks, other locations of the electrodes were tested: the left foot and the lateral edge of the right foot.

2.3.3. Catheterisation of the intrathecal space and baclofen intrathecal injection

Four rabbits were anaesthetised with a mixture of ketamine 35 mg/kg (Ketalar®, Parke Davis®, Courbevoie, France) and xylazine 5 mg/kg (Rompun®, Bayer®, Puteaux, France) injected in the quadriceps. A 4F catheter (Soft-flow®, Nycomed®, Pantin, France) was inserted as described by Kroin et al. [29]. One of the four rabbits died during the procedure, the other rabbits received 0.1, 1 and 8 μg/kg of baclofen in a sterile solution (Lioresal®, Novartis®, Basel, Switzerland), respectively. The doses were diluted in order to fix the injected volume at 400 μL. H_{max}/M_{max} ratios were determined 30 min and 1 h after injection whilst the animal remained under anesthesia. Then the rabbit was allowed to regain consciousness under surgical supervision. One day and 4 days after injection, the H_{max}/M_{max} ratio was determined using the procedure previously described in the text.

2.3.4. Microsphere implantation and pharmacological assay

The efficacy of baclofen-loaded microspheres taken from the pooled batches prepared with PLGA 85/15 (10.95% encapsulated drug), was tested in two rabbits after intrathecal surgical implantation. On the day prior to surgery, reference H_{max}/M_{max} ratios were determined for each rabbit following the procedure previously detailed and the animals were fasted. To avoid any hazard of microsphere spreading or leakage, implantation was made by open surgery under aseptic conditions. Rabbits were anaesthetized with intramuscular injections of xylazine 5 mg/kg (Rompun®, Bayer, Puteaux, France) and ketamine 35 mg/kg (Ketalar®, Parke Davis, Courbevoie, France).An incision was made between the fifth lumbar and the first sacral spinous process and, after dissection of paravertebral muscles, a laminectomy of the sixth lumbar vertebrae was performed. Under optical magnification, a polyurethane catheter (outer diameter of 1 mm and inner diameter of 0.5 mm) purchased from Vygon® (Ecoven, France) was inserted under the *dura mater* 2 cm in the caudal direction. A reflux of cerebrospinal fluid confirmed the correct position of the catheter in the subarachnoïd space. A suspension of 1.5 or 4 mg of microspheres was made extemporaneously in 1 mL of sterile injectable water and half of this suspension volume was injected into each of the rabbits. The resulting doses were thus 0.75 mg of microspheres for the first rabbit and 2 mg of microspheres for the second rabbit, i.e. 81 μg (28 μg/kg) and 216 μg (76 μg/kg) of encapsulated baclofen, respectively. The catheter was then carefully removed and the *dura mater* was closed by a patch of subcutaneous fascia. Finally the skin and muscles were stitched. For the next 4 days, 5 mg/kg marbofloxacine (Marbocyl® FD, Vetoquinol®, Lure, France) were injected daily in the muscles of the back. The rabbits were allowed to regain consciousness under infrared lamp warming and with medical supervision. After waking up, the animals were gently placed on the floor and their gaits and postures were observed to detect any limb paralysis. The H_{max}/M_{max} ratios were then determined as described before under isoflurane anaesthesia.

2.4. Statistical analysis

The intra-individual and inter-individual variations of H_{max}/M_{max} ratio were determined with one way ANOVA and the Bonferroni test. The Student *t*-test was used to compare the variations of the H_{max}/M_{max} ratio after baclofen injection or microsphere implantation. The Mann–Whitney *U*-test was used to compare the different sites of electrode insertion.

3. Results

3.1. Microsphere characterization

Microscopy showed that in all the cases, microspheres obtained were spherical with smooth surfaces and almost no

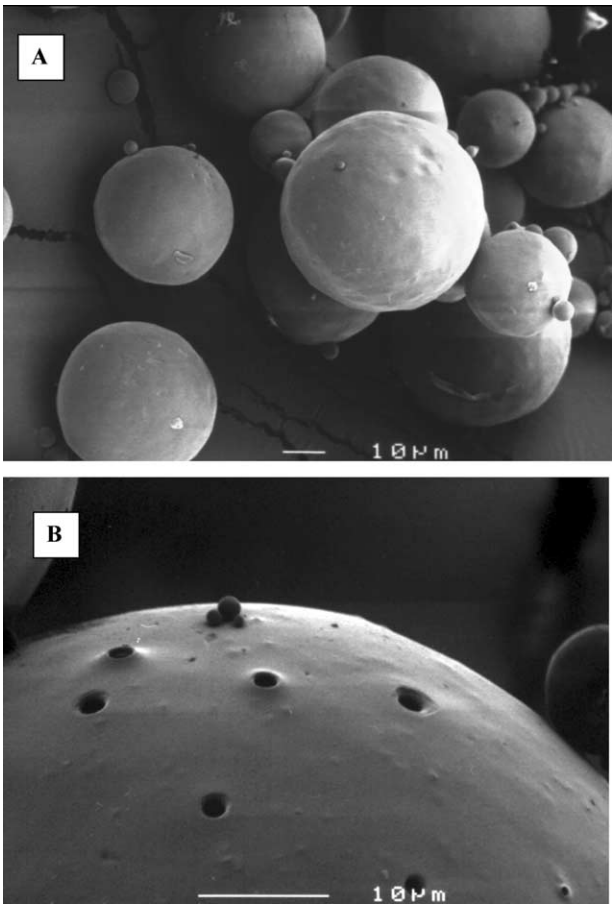

Fig. 2. SEM photography of a baclofen loaded microsphere batch. (A) Overview (electronic acceleration 15 keV), (B) surface details (electronic acceleration 2.6 keV).

polymer remnants (i.e. polymer not involved in forming microparticles). Pores of 0.2–1 μm were observed on particle surfaces with a reduced electronic acceleration of 2.6 keV in the SEM observations (Fig. 2). Their volumetric size distributions (Table 1) were found to be monomodal and gaussian (0.9 < mean/mediane ratio < 1.1). Encapsulation efficiency and in vitro release behaviour varied with the nature of the polymer used and the drug loading as shown in Table 1 and Fig. 3. The preparations were replicated five times and coefficients of variation of baclofen loading were 4.72, 3.43, and 7.73 for 10.83 and 15.30% baclofen-loaded 85/15 PLGA batches and 13.09% baclofen-loaded 50/50 PLGA batches, respectively. Residual ethyl acetate was determined in the five batches of 10.83% loaded 85/15 PLGA microparticles and was found to be 12,570 ± 3810 ppm.

3.2. H-reflex study and H_{max}/M_{max} variation in healthy rabbits

The *H*-reflex and *M*-wave responses were obtained for a stimulation electrode located near the tibial nerve at the popliteal fossa (stimulated muscles: *interosseus)* and for an active electrode located on the dorsum of the foot. The *H*-reflex was constantly obtained only when needle electrodes were used and a double 50 μs stimulation with a 2.4 ms interval was performed (Fig. 4). The H_{max} value was obtained for low stimulation intensity (50 V), although it was frequently observed for supramaximal stimulation intensity (300 V).

In healthy rabbits, the mean H_{max}/M_{max} ratios measured three times a week for 10 weeks in four rabbits varied from 19 to 32% (Fig. 5). No statistical differences were observed in the same gender ($P = 0.793$ for males and $P = 0.905$ for females), but the ratio was statistically lower in females compared to males ($P < 0.001$). No statistical differences were observed for intraday repeatability and interday reproducibility testing ($P > 0.05$). There were also no statistical differences between the two different positions (lateral edge or center of the foot) of the reception electrodes tested ($P > 0.05$) and also between the left and the right limbs ($P > 0.05$).

3.3. Evolution of H_{max}/M_{max} ratio after intrathecal injection of baclofen

The *H*-reflex disappeared 30 min after injection (Fig. 6) of the medium and high dose intrathecal baclofen solutions (0.8 and 8 μg/kg) and after 1 h for low dose intrathecal baclofen (0.1 μg/kg). After 24 h, the *H*-reflex reappeared but the H_{max}/M_{max} ratio remained significantly lower for

Table 1
In vitro characterization of PLGA baclofen-loaded microsphere batches with different theoretical drug loadings and different polymers

Polymer (drug loading)	Encapsulated drug (%)	Encapsulation efficiency	Volume size ± SEM (μm)	% released after 24 h	Mean release rate after 72 h (ng/d/mg)	Time to reach 100% release (day)
100% PLGA 85/15 (20.8%)	10.83% ± 0.54	52.61% ± 2.48	30.05 ± 1.759	15.97 ± 0.99	530 ± 37	174 ± 2.1
100% PLGA 85/15 (28.2%)	15.30% ± 0.43	55.04% ± 1.89	29.21 ± 1.817	66.56 ± 7.53	2200 ± 107	85 ± 6.2
100% PLGA 50/50 (20.7%)	13.09% ± 0.44	62.69% ± 2.59	34.52 ± 1.954	43.62 ± 5.59	1730 ± 82	40 ± 2.7

Encapsulated drug and encapsulation efficiency are presented as mean ± SD of five batches. Size and release in vitro were tested after batch pooling. Mean volume size is given as mean ± SEM calculated by Coultronics software. The release parameters are expressed as mean ± SD of three in vitro samples of the pooled batches.

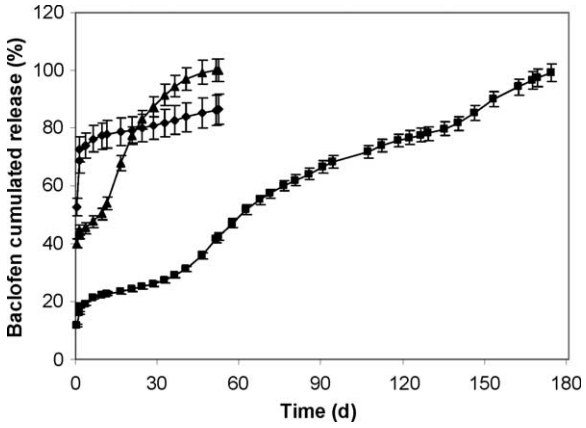

Fig. 3. In vitro release testing of different microspheres batches in continuous flow cell apparatus. The release medium was a PBS buffer pH 7.4, with a flow rate of 85 μl/min. The batches tested were PLGA 85/15 microspheres with drug loadings of 10.83% (–■–) or 15.30% (–◆–) and PLGA 50/50 microspheres with a drug loading of 13.09% (–▲–).

the three groups ($P<0.01$). After 4 days the H_{max}/M_{max} ratio returned to baseline in all groups. Hind limb paralysis was observed for all animals for a duration of 1–4 days according to the injected dose. Paralysis was reversible and not associated with disturbances of bowel or bladder functions.

3.4. Microsphere efficacy and safety profile

Because of the narrow therapeutic index of baclofen, the batch displaying a reduced burst effect after 1 day (15.97%) and having a low drug loading (10.95%) was selected for the first two experiments reported in this paper. The safety profile of PLGA 85/15 microsphere implantation was good, since the two rabbits presented no hind limb paralysis after waking-up from the surgical procedure. Whilst, the gait was subnormal, the movements of forelimb were normal and the movements of hind limb displayed only minor paralysis, which disappeared 16 h after microsphere implantation. Respiration and body temperature were normal. The rabbits displayed no drowsiness and were fully reactive to visual stimulation. After 24 h the weight loss was $5.3 \pm 1.6\%$ and returned to normal after 6 or 8 days which was consistent with the performed surgical procedure. For both rabbits, the H_{max}/M_{max} ratio remained significantly lowered for 39 days (Fig. 7) suggesting a sustained release of baclofen at an efficient dose in vivo.

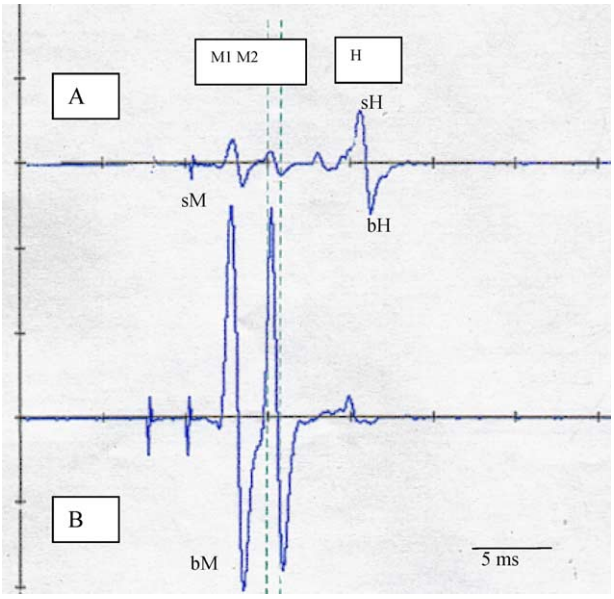

Fig. 4. Double stimulation (2.4 ms interval) leading to *M*-waves (M1 and M2) followed by *H*-reflex for low stimulation intensity (A; 50 V) or high stimulation intensity (B; 300 V). The *H*/*M* ratio is calculated peak to peak, i.e. between summit and bottom for each signal (distance from *s* to *b*). $H/M = (sH–bH)/(sM–bM)$. This calculation is performed for M1 and M2 waves and the maximal value obtained is considered.

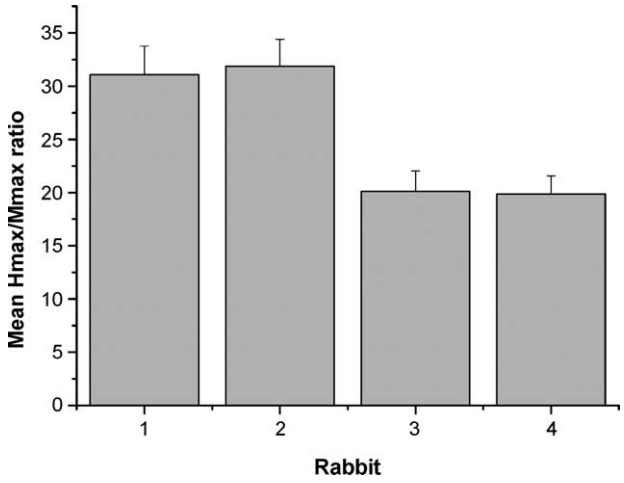

Fig. 5. H_{max}/M_{max} ratio measured on four rabbits of both gender (1 and 2 male, 3 and 4 females) three times a day during 10 weeks. Data expressed as mean ± SEM of 30 measurements.

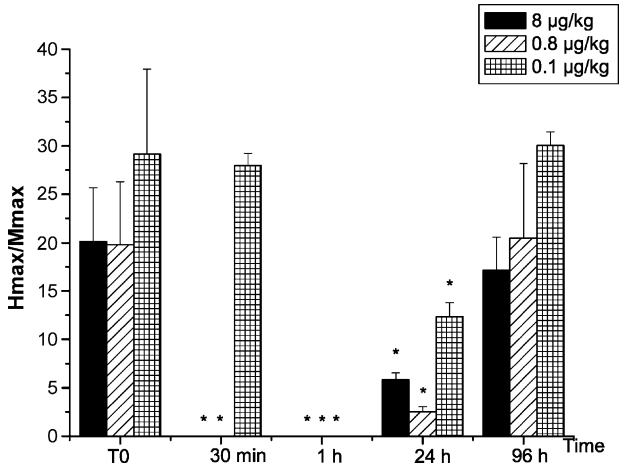

Fig. 6. Variation of the H_{max}/M_{max} ratio after injection of three doses of intrathecal baclofen in three different rabbits. Data expressed as mean ± SEM of four measurements. Significative inhibition of H_{max}/M_{max} ratio (*) was considered if $P<0.01$ after Student *t*-test.

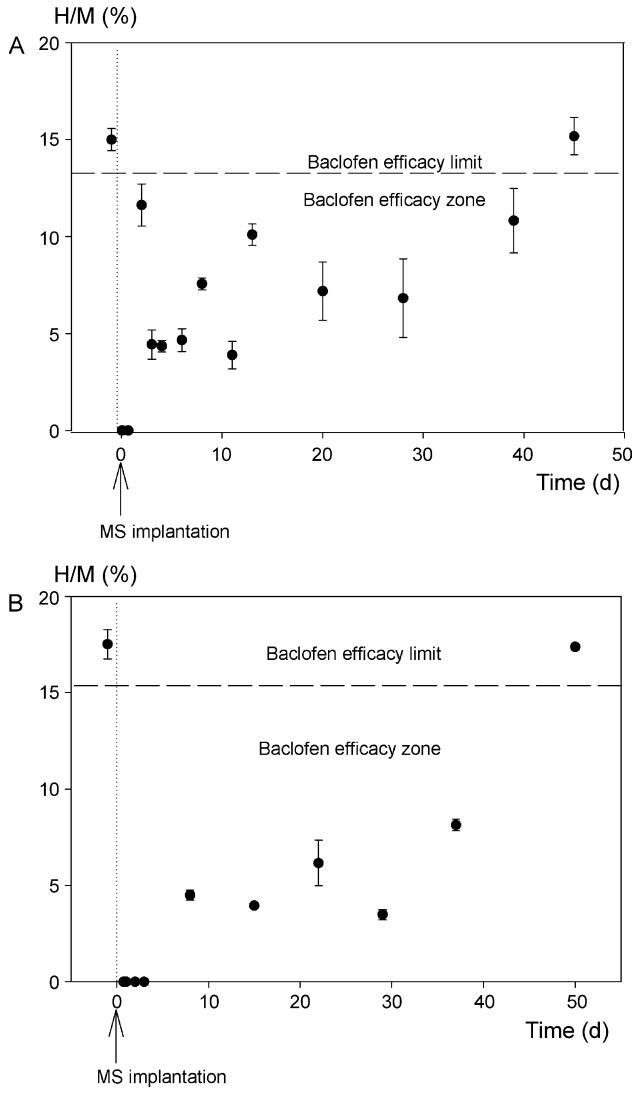

Fig. 7. Microsphere efficiency after intrathecal implantation on two rabbits. Batch PLGA 85/15, drug loading 10.95%. Total baclofen encapsulated dose of 28 μg/kg (A), and 76 μg/kg (B), i.e. 1.0 and 2.7 mg. Baclofen efficacy limit corresponds to the mean H_{max}/M_{max} ratio measured previously to baclofen implantation with a decrease of three standard deviations. If H_{max}/M_{max} is under this limit then the sustained release of baclofen preparation has a pharmacological effect (baclofen efficacy zone).

4. Discussion

The aim of this study was firstly to improve baclofen microsphere formulation by changing key parameters of the manufacturing process previously published [13], and secondly to set up an animal model to evaluate the efficacy of this new sustained release baclofen dosage form.

4.1. Improvement of the manufacturing process

The first improvement in microsphere preparation protocol was to substitute methylene chloride with a less toxic solvent: ethyl acetate. Microsphere preparation using ethyl acetate is delicate and often leads to polymer remnants and high size variability if the rate of solvent removal is not controlled [18,30–32]. In order to control this parameter, overnight evaporation is often used instead of solvent extraction [16,17] but this process is only suitable for non water soluble drugs. In other studies, the aqueous external phase is saturated with ethyl acetate to slow its extraction speed [30,31,33]. To encapsulate water soluble drugs such as proteins, a W/O/W double emulsion technique is used. However, baclofen solubility in water (6 mg/mL) does not allow a drug loading higher than 1% (w/w) using this encapsulation process. An extraction process where baclofen crystals were suspended in the internal organic phase of the emulsion had thus to be used in order to quickly form the microparticles limiting the drug loss in the aqueous phase and maximising encapsulation efficiency.

It was demonstrated that the key parameter to take into account for high quality microsphere preparation was the O/W ratio [18]. If this ratio was less than 1/10, this author showed that the polymer precipitated, as soon as the two phases were in contact, and the resulting preparation contained polymer remnants. The claimed optimal ratio in this paper, was between 2/5 and 4/25. In another study, a reduced O/W ratio was shown to reduce the burst effect [34]. As demonstrated with methylene chloride [35–37] and confirmed for ethyl acetate [34], the rate of solvent removal had dramatic consequences on microsphere properties. A higher solvent removal speed often leads to hollow core formation, a lower surface area, higher residual solvent and a slower drug release. To minimize the burst effect, it would have been preferable to accelerate the solvent removal and to use a low O/W ratio. But under these conditions, the precipitation of microdroplets into microspheres may not be homogeneous and often results in non-spherical particles, highly variable in size and drug loading. To compromise between these opposite requirements, reduced O/W ratio of 3/20 was selected; but, in order to obtain a controlled precipitation of the polymer, its precipitation into microspheres was performed as slowly as possible by pouring 100 mL water in the emulsion under vigorous stirring. After addition of this amount of water, the O/W ratio was 6/140 and thus the polymer precipitated into microspheres without forming any polymeric remnants. This procedure allowed us to minimize the burst effect while keeping a high quality (sphericity, homogeneity, absence of polymer residues) of microsphere batches as discussed later.

The second major improvement of the encapsulation process over the technique of Cruaud et al. [13], was the enhancement of reproducibility, which remains a major issue for microsphere manufacturing process [15]. To obtain reproducible batches without polymer remnants, the emulsion was made in a temperature-controlled apparatus, as it was very clear that temperature was a process parameter determining the overall quality of the microspheres and thus the release patterns [38]. Microsphere preparation was performed at low temperature because it has previously been shown that it narrowed the mean volume size of

the microparticles and lowered the burst effect [38]. This is due to an increase of both the inner organic phase viscosity and the solvent removal rate, as methylene chloride or ethyl acetate are more soluble in water at low temperature and are thus more rapidly extracted by water. The vigorous stirring of the emulsion at 1000 rpm allowed a fine and homogeneous emulsion, which is the primary condition for high quality microsphere batches. The hardening of this fine emulsion in two steps (100 mL water pouring into the emulsion and then rapid transfer in a large volume of water) allowed the preparation of microparticles without any polymer remnants in a reproducible manner. The use of two steps to solidify embryonic microparticles has already been shown to produce better microsphere preparations [34]. In this case, saturating or doping the external aqueous phase with organic solvent was not necessary.

The bathing of the microparticles in 2 L of water under paddle stirring was used to remove the maximum ethyl acetate from the microspheres. This step contributed to limit the aggregation of the particles after vacuum freeze-drying as it was previously demonstrated [18]. This multi-step process, with progressive hardening of the emulsion microdroplets into particles under temperature control and vigorous stirring, allowed us to obtain reproducible batches consistent with a future industrial scale-up.

Another way to enhance the overall quality of microsphere batches, is to use high molecular weight polymers [16,17]. While Cruaud et al. [13] used 40 kDa polymers, in the current studies 74 and 95 kDa polymers were used. In addition to an improvement in batch quality, in terms of particle shape and aggregation (data not shown), it allowed us to increase baclofen release period.

4.2. Modification of the release pattern

Switching from methylene chloride to ethyl acetate has been shown [39] to impact on the release profile. The diffusion of ethyl acetate into the external aqueous phase was promoted by the solubility of this organic solvent in water, especially at low temperature (solubility is 12% (w/w) at 4 °C). Even if the solvent removal can be controlled, microparticles prepared using ethyl acetate, have a higher porosity and a greater burst effect [39] than particles prepared in the same conditions with methylene chloride. Hermann and Bodmeier also demonstrated that the encapsulation efficiency of a water-soluble peptide (somatostatin) was reduced when ethyl acetate was used in a W/O/W process [15,39].

To restrict the burst effect, we chose to grind the baclofen crystals. Indeed, it has been previously shown that an optimised milling process had an important impact on the release profile [40] and, in particular on the burst effect. Also, the drug loading was kept low, as this parameter greatly influences the burst effect. Indeed, in the present work, the comparison of the release of PLGA 85/15 microsphere batches showed a dramatic increase of the burst effect followed by a zero order release rate (Fig. 3) as the drug loading increases from 10.8 to 15.3% (Table 1). This could be related to a percolation mechanism as previously reported for microparticles loaded with hydrophilic crystals, such as cisplatin, in a sufficient amount ($>18\%$ w/w) [41]. In this latter case, the release was controlled by the dissolution of the embedded drug crystals, thus forming a network instead of their diffusion through the polymer matrix. The use of high MW polymers permitted an increase in the release period of baclofen in comparison to the work of Cruaud et al. [13]. This effect was more pronounced when PLGA 85/15 was used: the baclofen release was sustained for 6 months with this polymer.

4.3. Animal model of baclofen activity

To assess baclofen activity, a choice between an animal model of spasticity or a non injured animal model was required. Spasticity was defined by Lance [42] as "a motor disorder characterized by a velocity-dependent increase in tonic stretch reflexes ('muscle tone') with exaggerated tendon jerk, resulting from hyperexcitability of the stretch reflex, as one of the component of the upper motor neurone syndrome". Spasticity is first of all a clinical symptom having multiple components and belonging to the upper motor neurone syndrome. Thus, in an animal model, it is very difficult to obtain all the components of spasticity after a surgical lesion [43]. For that reason and for ethical issues, the study of baclofen activity in healthy, non-injured, animals, was undertaken. Baclofen has been described to inhibit *H*-reflex in healthy animals or humans and spastic patients [21,22,29,44–50]. To set up a model for evaluation of baclofen activity of sustained release dosage forms, we had to find a procedure to easily obtain *H*-reflex and to assess its long term reproducibility. The only stimulation protocol leading constantly to an *H*-reflex was a double 50 μs stimulation with 2.4 ms interval associated with needle electrodes. This difficulty in obtaining the *H*-reflex was likely due to the isoflurane depressive effect on the motor neurone excitability [51]. This type of anesthesia was nevertheless chosen for its flexibility. Under these precise conditions, the H_{max}/M_{max} ratio was reproducible for the same rabbit. However, a difference between the mean ratios was observed when comparing males to females. To our knowledge, this peculiarity has not been reported in the literature, but these results should be confirmed with a higher number of animals. The persistence of *H*-reflex for supramaximal stimulations was observed. In fact, in many cases, the H_{max} and M_{max} were obtained for the same intensity of stimulation. This was also described by Cliffer and co-workers on Sprague–Dawley rats [23]. As represented in Fig. 1, the delayed signal obtained after the *M*-wave can be an *H*-reflex or an *F*-response. The *F*-response has a variable latency. In contrast, the *H*-reflex has a regular latency for the same neural circuitry. In our model, the *H*-reflex appeared 5 ms after the second *M*-wave

(Fig. 4). If a delayed signal appeared with a latency different from 5 ms, it was considered as an *F*-response. If the signal had a 5 ms latency, the constancy of its peak to peak amplitude was checked for a similar stimulation intensity to interpret this signal as an *H*-reflex.

After establishing a reproducible *H*-reflex animal model, the activity of intrathecal baclofen was studied using this model. Results with three different doses are reported, showing a constant and reversible depression of *H*-reflex following intrathecal baclofen injection (Fig. 6). The rate of this depression was comparable to the rate observed in spastic patients [52]. The time necessary for the return to a normal H_{max}/M_{max} ratio was between 1 and 4 days. Our study was not aimed at precisely determining the rate of *H*-reflex reappearance. The point to note is the reversibility of *H*-reflex depression after 24 h. After 4 days, the H_{max}/M_{max} ratio returned to normal in the three groups. A depression lasting more than 4 days would thus be the sign of a sustained pharmacological action of a baclofen dosage form. The electrophysiological measurement of baclofen activity is then independent from clinical observations in comparison to the reference method developed by Kroin and co-workers [29]. In this latter method, the intrathecal effect of baclofen was assessed by the inhibition of a contralateral polysynaptic reflex. The stimulation intensity (constant current pulse of 1 ms duration occurring once every 5 s) was increased until a reflex crossed extension was seen in the contralateral limb. The level of reflex threshold was considered as a sign of baclofen activity and could be more subjective than the H_{max}/M_{max} ratio.

4.4. Efficacy testing

The animal model of intrathecal baclofen activity was used to evaluate sustained pharmacological activity of the microspheres. As baclofen is a very effective drug when injected intrathecally, it was important to probe the effect of low dose sustained release dosage forms first. Furthermore, testing low doses was a good way to assess the sensitivity of our new pharmacological tool. The first rabbit received the lowest dose of encapsulated baclofen, i.e. 81 μg (28 μg/kg). This dose, if released immediately, would kill the rabbit or would lead to hind limb paralysis as assessed by clinical observation following the bolus doses of 0.1–8 μg/kg. But no paralysis was observed after microsphere implantation. The surgical procedure using a catheter allowed us to ensure that microspheres were effectively injected in the intrathecal space. Hence, it can be postulated that the absence of clinical signs of a toxicological effect of baclofen associated with evidence of its pharmacological activity (diminution of H_{max}/M_{max} ratio), was proof of a reduced burst effect in vivo relative to what was observed in vitro. This promising result was also observed in the second rabbit after receiving a dose almost three times higher than the first rabbit (76 μg/kg). In this latter case, the 16% burst observed in vitro would have corresponded to a released baclofen dose of 13 μg/kg which was shown to be in the toxic range in both our study and other previously published work [29]. This suggests that the burst effect was reduced in vivo in comparison to in vitro experiments. The duration of H_{max}/M_{max} ratio reduction was the same for the two rabbits. At first glance, it could be concluded that the duration of baclofen effect was dose independent within the dose range studied, but baclofen activity is known to be subject dependent. A complete dose study with groups of animals is also necessary to conclude on the influence of the dose on H_{max}/M_{max} ratio. Our model showed a duration of baclofen activity over 5 weeks. This does not appear to be consistent with the baclofen release in vitro which was found to be sustained for 6 months. Two hypotheses can be ventured to explain these discrepancies. Firstly, the release rate during the plateau observed in vitro, is very low (Fig. 3.) and could be even lower in vivo as suggested by the results obtained in the burst effect evaluation (reduced burst effect in vivo in comparison to in vitro). In this case, the released amounts of baclofen could become insufficient to significantly depress the *H*-reflex. Secondly, tolerance to baclofen acquired after a few weeks could be responsible for a *H*-reflex return to a normal value. Indeed, an acquired tolerance mechanism on muscle tone has been described for baclofen [53–55], requiring an increase in the dose after a few months. The long term effect of baclofen on *H*-reflex has still to be investigated, in order to study possible induced mechanism of recovery of this reflex.

5. Conclusion

In the present study, the manufacturing process of baclofen-containing microspheres was improved in comparison to a previous study by Cruaud et al. [13]. The baclofen release was extended to 6 months by using a polymer with a slower degradation rate. The organic solvent was changed to one presenting a favourable toxicological profile: ethyl acetate. However, the partial water solubility of ethyl acetate necessitated a redesign of the manufacturing process. Using the protocol discussed, high quality microparticles, i.e. spherical, smooth, narrow sized, non-aggregated microspheres without any polymer residues were obtained. The rate of solvent removal was controlled during the hardening of microdroplets into microparticles by slowly adding water directly to the emulsion. The encapsulation efficiency was maintained above 50% by shortening the overall duration of the encapsulation process to under 15 min. During this process, the temperature was fully controlled. All these improvements permitted a decrease in the coefficient of variation with regard to encapsulation efficiency, passing from 11.6% in methylene chloride batches to less than 5% in ethyl acetate batches, even though it has often been claimed that it is difficult to obtain repeatable batches using ethyl acetate as the dispersed solvent. The burst effect was maintained to 16%

at 24 h by grinding the baclofen crystals and using relatively low drug loads.

After passing this challenging formulation milestone, an animal model of baclofen activity was set up to evaluate the resulting microparticles. The repeatability of the model was consistent with pharmacological tests. Moreover, the kinetics of disappearance and reappearance of *H*-reflex after baclofen bolus injection in the rabbit was comparable to the observations made in the clinic. Two different doses of the same microsphere batch were evaluated using this model and showed no toxicological effect. A sustained *H*-reflex depression lasting more than 1 month was observed. To our knowledge, this is the first study of sustained release intrathecal dosage form of baclofen in vivo. The next step will consist of evaluating the biopharmaceutics of these preparations in a larger animal model such as sheep or goat.

Acknowledgements

The authors wish to thank Pr Longchamp, Unité d'explorations fonctionnelles neuro-musculaires, centre Universitaire Hospitalier d'Angers for his advices on the pharmacological model of baclofen activity and Dr D.C. Bibby for rereading this manuscript.

The authors are also grateful to Dr Filmon, SCIAM, University of Angers, for his assistance on electronic microscopy. This work has been partly granted by the French ministery of research and technology.

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