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In vitro release performance and analgesic activity of endomorphin-1 loaded nanoparticles

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Endomorphin-1 normally has a short half-live in blood and brain and has difficulty in penetrating the blood-brain barrier when given intravenously. To transport endomorphin-1 across the blood-brain barrier, the peptide was adsorbed onto the surface of butylcyanoacrylate nanoparticles and coated with polysorbate 80. The release properties of the drug *in vitro* were demonstrated. The central analgesic effect of the drug was measured by tail flick test. The results of the *in vitro* release study show that there is a burst release effect at first and a slow and continuous release then followed. A longer analgesic effect was shown when the nanoparticles coated with polysorbate 80 were intravenously injected into mice than with the other groups including endomorphin-1, nanoparticles uncoated with polysorbate 80, and a simple mixture of the three components (drug, nanoparticles, and surfactant) mixed directly. The results showed that the way we used to promote endomorphin-1 penetration of the blood-brain barrier was useful. These results suggested that nanoparticles coated with polysorbate 80 were useful for delivery of EM-1 loaded nanoparticles to target the brain.

1. Introduction

The blood-brain barrier (BBB) is considered to be an insurmountable obstacle to a large number of drugs, particularly peptides and proteins. Drugs or compounds that are un-ionized at physiological pH, lipophilic and of low molecular weight can cross the BBB by diffusion mechanisms. Peptides and proteins can cross the BBB by saturable transport systems (Banks et al. 1991). For many years attempts have been made to overcome the diffusion limiting BBB by various procedures, including enhancement of brain blood vessel membrane fluidity (Bigon et al. 1979; Tokes et al. 1980), chemical modification, or opening of the blood-brain barrier by osmotic methods (Rapoport 1996). Despite these attempts, no procedure is currently available which promotes the passage of inherently non-penetrating drugs through the intact brain blood vessel endothelium in an unmodified form. Drug carriers for targeted drug delivery have been examined in this context such as liposomes (Zhou and Huang 1992) and nanoparticles (Kreuter et al. 1995; Schroeder et al. 1998). Endomorphin-1 (H-Try-Pro-Trp-Phe-NH_2, EM-1) and endomorphin-2 (H-Try-Pro-Phe-Phe-NH_2, EM-2), two tetrapeptides, were discovered and isolated from the bovine in 1997 (Zadina et al. 1997). They proved to have very high affinity and selectivity for the μ-opioid receptor, and can influence a wide range of physiological and pharmaceutical activity. Endomorphins have a short half-life in blood and brain, where they are rapidly degraded (Gentry CL et al. 1999; Hau et al. 2002), and they have difficulty in penetrating the BBB when given intravenously. Somogyvari-Vigh et al. (2004) and Kastin et al. (2001) found that endomorphin-1 and endomorphin-2 are saturably transported from brain to blood, which was established by self-inhibition of transport of each radiolabeled peptide by an excess of that peptide. To improve the CNS entry of endomorphin-2, Hau et al. (2002) studied the effect of Pro (4) substitution and cationization on its physico-chemical characteristics, BBB transport, and analgesic profile. The study demonstrated that Pro(4)-substitution can promote BBB permeability and enhance i.v. analgesia.

Some years ago Tröster et al. (1990) observed that coating poly(methyl methacrylate) nanoparticles with surfactants, especially with polysorbate 80, led to a significantly higher total brain concentration after intravenous injection to rats. In the present study, endomorphin-1 was used as a model drug. The endomorphin-1-polyisobutylcyanoacrylate-nanoparticles (EM_1-PIBCA-NP) were prepared by an adsorption method with polyisobutylcyanoacrylate (PIBCA) and coated with polysorbate 80 (Tween-80). Their morphology and particle size were examined by transmission electron microscope. The *in vitro* release curve of EM_1-PIBCA-NP followed a biexponential model. Testing by mouse tail flicking in warm water showed that EM_1-PIBCA-NP coated with Tween-80 had an analgesic effect of $118 \pm 18\%$ at a dose of 20 mg/kg, which was much more effective than EM_1-PIBCA-NP uncoated with Tween-80, EM-1 alone and a simple mixture of the three components (EM-1, PIBCA-NP, and Tween-80).

2. Investigations, results and discussion

The nanoparticles were spherical and homogeneous in appearance and the average diameter was 27 ± 9 nm. Fig. 1 shows a TEM picture of EM_1-PIBCA-NP.

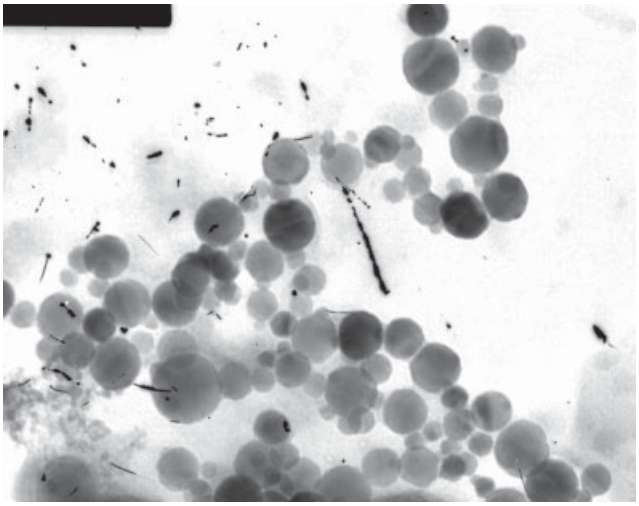

Fig. 1: TEM micrograph of EM_1-PIBCA-NP (× 100,000)

At periodic intervals, we determined the concentration of EM-1 in PBS dialysed from the EM_1-PIBCA-NP and obtained the release profile of EM_1-PIBCA-NP *in vitro* as seen in Fig. 2.

The relationship between the percentage relative release (Q) and time (t) was described by the Higuchi, Weibull Monoexponential and Biexponential equation. The regression equations and correlation coefficients (r) are given in Table 1.

In vitro release studies showed that the release behavior of EM_1-PIBCA-NP followed a biexponential model. In the first 2 h 5% to 15% of the drug was released, while 15% to 20% of the drug was released in the following 30 h. The longest release time was up to 32 h. The results demonstrated a burst effect followed by slow and continuous release. EM-1 release from the nanoparticles was rapid, probably because the drug was adsorbed on the nanoparticle surfaces rather than entrapped into the polymeric core.

As seen in Fig. 3 and Table 2, EM-1 dissolved in PBS, as

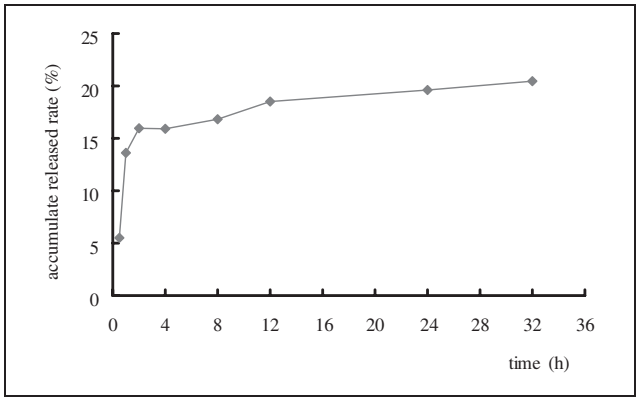

Fig. 2: Release curve of EM_1-PIBCA-NP *in vitro*

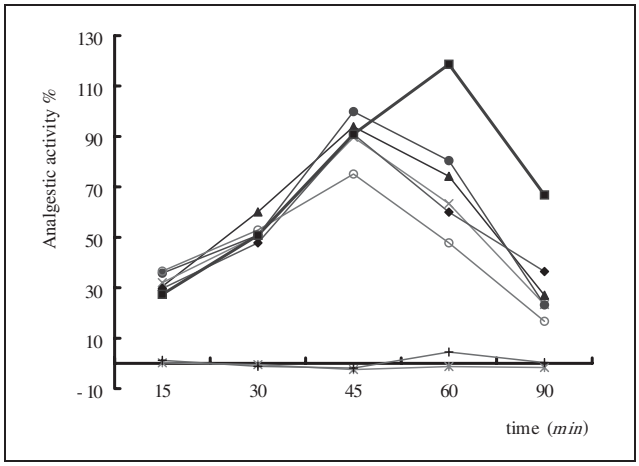

Fig. 3: Results of analgesic activity determined by the tail flick test
* – Empty NP (100 mg/kg); + – Tween-80 (1%, 100 mg/kg); ○ – EM-1-loaded NP (300 μg/kg); ◆ – EM-1 (300 μg/kg) + empty NP (100 mg/kg); × – EM-1 (300 μg/kg) + Tween 80 (100 mg/kg): ● – EM-1 (300 μg/kg) + empty NP (100 mg/kg) + Tween-80 (100 mg/kg); ▲ – EM-1 solution (300 μg/kg); ■ – Tween-80-coated EM-1 NP (30 mg/kg)

well as the other groups 1, 4, 5, 6 and 7, led to a drastic enhancement of analgesia at a time of 45 min after i.v. injection, however, polysorbate 80-coated nanoparticles induced a drastic central analgesic effect after 60 min. These results demonstrate that the EM-1 nanoparticles coated with polysorbate 80 could produce a longer lasting analgesic effect than the other groups including endomorphin-1, nanoparticles uncoated with polysorbate 80, and a simple mixture of the three components (drugs, nanoparticles, and surfactant) mixed directly.

The main finding is that endomorphin-1-loaded nanoparticles coated with polysorbate 80 (Tween 80) are able to induce a more continuous and drastic analgesic effect at 60 min after i.v. injection than the other control groups.

The *in vitro* release behavior showed an initial burst effect in the first 2 h followed by a slower rate stage of 30 h with nearly 20% of the drug released. These results suggest that the EM-1 loaded nanoparticles may stay in the blood and brain longer and induce a continuous analgesic effect.

The analgesic effect measured after intravenous administration offers new possibilities for targeting of potent active drugs to the CNS when they are bound to such nanoparticles. These results suggest that nanoparticles coated with polysorbate 80 are useful for delivery of EM-1 loaded nanoparticles to the brain. The mechanism whereby endomorphin-1 is released from the nanoparticle surface is not yet known and we can only speculate on the basis of the literature (Schroeder et al. 1998). Whatever the mechanism may be, the main finding of our studies is that EM-1 entrapped into coated with polysorbate 80 is stable in blood and brain, and can induce continuous analgesia. Therefore, nanoparticles represent a novel tool to deliver drugs across the BBB.

Table 1: Regression equations of EM_1-PIBCA-NP *in vitro* release

Model	Regression equation	r
Higuchi	$Q = 0.0208t^{1/2} + 0.1007$	0.8049
Weibull	$\ln\ln(1/1 - Q) = 0.2478\ln t - 2.2114$	0.8270
Monoexponential	$\ln(1 - Q) = -0.0034t - 0.1380$	−0.7298
Biexponential	$1 - Q = 0.0536e^{-0.2973t} + 0.8270e^{-0.0012t}$	$r_1 = -0.9990$ $r_2 = -0.9781$

Table 2: Results of analgesic activity determined by the tail flick test

Group	Analgesic activity % ($\bar{\chi} \pm s$)				
	15 min	30 min	45 min	60 min	90 min
1. EM-1 solution (300 μg/kg)	30.56 ± 11.30	60.11 ± 12.33	93.89 ± 16.40	74.23 ± 10.24	27.00 ± 6.74
2. Empty NP (100 mg/kg)	0.21 ± 3.68**	−0.49 ± 8.67**	−2.42 ± 8.30**	−1.27 ± 1.98**	−1.61 ± 6.09**
3. Tween-80 (1%, 100 mg/kg)	1.22 ± 7.58**	−1.19 ± 6.33**	−1.86 ± 8.84**	4.50 ± 10.09**	0.29 ± 6.40**
4. EM-1 (300 μg/kg) + Tween 80 (100 mg/kg)	32.04 ± 13.30	50.70 ± 7.32	89.69 ± 20.17	63.37 ± 24.03	23.37 ± 14.42
5. EM-1 (300 μg/kg) + empty NP (100 mg/kg)	29.68 ± 5.72	47.82 ± 8.73	90.89 ± 12.53	59.99 ± 10.50	36.43 ± 8.90
6. EM-1 (300 μg/kg) + empty NP (100 mg/kg) + Tween 80 (1%, 100 mg/kg)	35.78 ± 15.22	50.83 ± 9.42	99.87 ± 11.26	80.39 ± 14.69	23.24 ± 4.06
7. EM-1-loaded NP (300 μg/kg)	36.56 ± 9.53	52.91 ± 7.63	75.11 ± 14.10	47.83 ± 10.77	16.70 ± 3.40
8. Tween-80-coated EM-1 NP (10 mg/kg)	22.75 ± 8.19	48.10 ± 11.11	87.99 ± 15.25	108.92 ± 11.92**	63.74 ± 7.58**
Tween-80-coated EM-1 NP (20 mg/kg)	27.37 ± 5.70	50.77 ± 15.87	90.88 ± 19.42	118.62 ± 18.86**	66.77 ± 10.09**
Tween-80-coated EM-1 NP (30 mg/kg)	46.72 ± 12.09	58.58 ± 11.73	98.72 ± 19.22	124.92 ± 22.54**	68.61 ± 13.38**

note: n = 10, compared wih EM-1 solution, $^{**}p < 0.01$

3. Experimental

3.1. Nanoparticle preparation

Nanoparticles were prepared in an acidic polymerization medium. According to the method described by Schroeder et al. (1998), we selected polysorbate 85 (Tween-85) as stabilizer (1% stabilizer in 0.01N HCl). Isobutylcyanoacrylate was added to obtain a 1% nanoparticle suspension. The mixture was stirred magnetically at 600 rpm for 4 h to facilitate nanoparticle formation. The resulting suspension was neutralized with 0.1 N sodium hydroxide solution, and was then ultracentrifuged. Nanoparticles (40 mg) were resuspended in 25 mL phosphate buffered saline (PBS). EM-1 was added in a concentration of 0.2 mg/mL suspension. The peptide was allowed to adsorb onto the nanoparticle surface for 3 h. Then 0.01% Tween-80 (relative to the total suspension volume) was added and incubated for 30 min. After ultracentrifuging the sediment was lyophilized and the particle size was determined by transmission electron microscopy.

3.2. In vitro release of EM-1

During the *in vitro* release studies the concentration of EM-1 was determined by reverse phase HPLC with a C_{18} column (Kromasil ODS-1, 250 mm × 4.6 mm, 5 μm). The HPLC system was equipped with pumps (SP8800, Spectra-Physics), a variable wavelength detector (UV2000, Spectra-Physics) and an integrator (SP4400, Spectra-Physics). The mobile phase comprised a binary mixture of varying proportions of acetonitrile and water containing 0.08% (v/v) trifluoroacetic acid (TFA). Gradient elution was carried out from 5% to 95% of organic modifier in 20 min. EM-1 was detected at a retention time of 11 min.

In the *in vitro* release study of EM-1, 10 mg EM_1-PIBCA-NP were added to PBS buffer, which was incubated at 37 ± 1 °C. At periodic intervals, samples were taken and analyzed by HPLC for EM-1 released from the EM_1-PIBCA-NP. The *in vitro* release profile was obtained by the dialysis method. To provide a more reliable prediction of *in vivo* performance, the release data were treated with the Higuchi, Weibull Monoexponential and Biexponential equations.

3.3. Analgesic studies

3.3.1. Test groups

The nanoparticle suspension was diluted with PBS to obtain concentrations of 10, 20, or 30 mg/mL (groups 8–10, respectively) and injected into the tail veins of mice.

3.3.2. Control groups

Group 1: endomorphin-1 solution in PBS (300 μg/kg). Group 2: suspension of empty nanoparticles (100 mg/kg). Group 3: polysorbate 80 solution in PBS (1% w/v, 100 mg/kg). Group 4: mixture of endomorphin-1 solution (300 μg/kg), and polysorbate 80 (1% w/v, 100 mg/kg). Group 5: mixture of endomorphin-1 solution (300 μg/kg), and empty nanoparticles (100 mg/kg). Group 6: mixture of endomorphin-1 (300 μg/kg), empty nanoparticles (100 mg/kg), and polysorbate 80 solution in PBS (1% w/v, 100 mg/kg). Group 7: endomorphin-1 loaded onto empty nanoparticles without polysorbate 80 (300 μg/kg).

3.3.3. Analgesia studies

Analgesic effect was measured using the tail-flick test, in which a response from the tails of mice was evoked by water at 55 ± 1 °C and the time for tail withdrawal was recorded. The tail flick latency was tested 15, 30, 45, 60 and 90 min after intravenous injection.

3.3.4. Statistics

Statistical significance was determined by one-way ANOVA and subsequent post hoc Tukey comparison.

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