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International Journal of Pharmaceutics 242 (2002) 115–120

international journal of pharmaceutics

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Poly(lactic acid) microspheres for the sustained release of antiischemic agents

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Received 12 December 2001; received in revised form 19 December 2001; accepted 20 December 2001

Abstract

We report a preliminary study evaluating the encapsulation modalities in microparticles of the antiischemic drug N^6-cyclopentyladenosine (CPA). The effects of release systems have been evaluated on the stability in human whole blood of CPA and its affinity toward human adenosine A_1 receptors. The microspheres were prepared by an emulsion–solvent evaporation method (different CPA amounts and two stirring rates were employed) using poly(lactic acid). Free and encapsulated CPA was incubated in human blood and the drug stability was analyzed. The affinity of CPA to human A_1 receptor was also obtained in the presence and in the absence of unloaded microspheres. The microspheres obtained using 1200 rpm showed a broad size distribution and a mean diameter value of 21 ± 9 μm. Using 1700 rpm the mean diameter decreased to 5 ± 2 μm and a more homogeneous size distribution was obtained. The CPA release changed with the particle size and the different amounts of drug employed during the preparation of the microspheres. The degradation in human whole blood of CPA encapsulated in the microspheres was negligible, with respect to that of free CPA. Affinity values of CPA obtained in the absence and in the presence of unloaded microspheres were the same.

Keywords: Microspheres; Sustained release; N^6-cyclopentyladenosine; Adenosine A_1 receptor; Stability

1. Introduction

Adenosine modulates several physiological functions in the CNS and in peripheral tissues via membrane receptors which have been classified into four adenosine subtypes: A_1, A_{2A}, A_{2B} and A_3 (Fredholm et al., 1994). A_1 activation has been found to produce neuronal and cardiac depression (Jacobson et al., 1991): this inhibition allows adenosine A_1 agonists to produce ischemic tolerance and protection in neuronal and cardiac tissues (Heurteaux et al., 1995; Tucker and Linde, 1993). It has been reported that survival of gerbils

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0378-5173/02/$ - see front matter
PII: S0378-5173(02)00179-5

after ischemic injury is increased by selective A_1 agonists (Von Lubitz and Marangos, 1990). In order to selectively reproduce these effects, several A_1 selective ligands have been synthesised, and a great number of studies have been performed to understand how they interact with the adenosine A_1 receptor (Ralevic and Burnstock, 1998). N^6-monosubstituted adenosine derivatives, as cyclopentyladenosine (CPA) or cyclohexyladenosine, (CHA) are currently identified as a potent and A_1-selective agonist (Muller, 2000).

Despite the great quantity of information available on the adenosine A_1 receptor, the clinical use of A_1 agonists is not allowed (Williams, 1993; Von Lubitz, 1999): unwanted effects are caused by activation of the A_1 receptor, because they are ubiquitous in the body (Ralevic and Burnstock, 1998) moreover selective A_1 agonists appear poorly adsorbed into the brain (Brodie et al., 1987) and can be quickly degraded in vivo or in whole blood (Mathôt et al., 1993; Pavan and Ijzerman, 1998). Several approaches are currently under examination in an attempt to solve these problems. As an example, prodrugs were proposed to increase stability and diffusion through lipid barriers of selective A_1 agonists (Maillard et al., 1994; Dalpiaz et al., 2001a). This paper describes a preliminary study evaluating the encapsulation modalities of CPA in microparticles. Moroever, the effects of release systems have been evaluated on the stability in human whole blood of CPA and its affinity toward human adenosine A_1 receptors.

2. Materials and methods

2.1. Materials

[^{3}H] 1,3 - dipropyl - 8 - cyclopentylxanthine ([^{3}H]DPCPX, 108 Ci/mmol) was obtained from DuPont NEN Research Products (Boston, MA). CPA and CHA were obtained from RBI/Sigma (Natick, MA). Chinese hamster ovary cells transfected with adenosine A_1 human receptors (CHO A_1) were a kind gift from Prof. Peter R. Schofield (Townsend-Nicholson and Shine, 1992; Townsend-Nicholson and Shofield, 1994). All cell culture reagents were obtained from Gibco Laboratories (Life Technologies Italia, Milan, Italy). HPLC grade solvents were purchased from Carlo Erba Reagenti (Milan, Italy). Poly(lactic acid) (PLA, average molecular weight 16 000; Resomer® R203; Boehringer-Ingelheim, Ingelheim am Rhein, Germany) was utilized as biodegradable polymer to prepare the microspheres. Pluronic F-68 (polyethylene–polypropylene glycols; Sigma) was used as the emulsifying agent. All other chemicals and solvents were obtained from standard sources.

2.2. Microsphere preparation and characterization; CPA release studies

Microsphere samples (named A, B, C, and D) were prepared using the 'solvent-evaporation method' (Bodmeier and McGinity, 1986) according the experimental parameters reported in Table 1. PLA (100 mg) and CPA amounts ranging from

Table 1
Effect of the preparative variables on the CPA content of the microspheres (standard deviation in parentheses)

Code sample	PLA (mg)	CPA (mg)	Pluronic F-68 (mg)	Stirring rate (rpm)	Theoretical drug content (mg/100 mg)	Actual drug content (mg/100 mg)
A	100	10	50	1200	9.09	0.11 (0.005)
B	100	5	50	1200	4.55	0.12 (0.010)
C	100	1	50	1200	0.91	0.06 (0.005)
D	100	5	50	1700	4.55	0.11 (0.005)

1 to 10 mg were employed to obtain the loaded microspheres as previously described (Dalpiaz et al., 2001b) The sample B were also recovered by freeze-drying at 2 mbar and −40 °C for 24 h (Liovac GT2, Leybold-Heraus, Hanau, Germany) in order to evaluate the influence of to the recovery procedure (B lyophilized). Unloaded microspheres were prepared following the same procedure used to prepared sample A but without dissolving CPA in the dichloromethane solution. CPA content in the microspheres, the particle size and morphology, and CPA release were analysed as previously described (Dalpiaz et al., 2001b).

2.3. Kinetic experiments, cell culture, membrane preparation and receptor binding assays

The stabilities in human whole blood of CPA encapsulated in the sample A and of free CPA in the absence and in the presence of unloaded microspheres, were investigated as previously described (Dalpiaz et al., 2001b).

Cells were grown in 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium containing 10% foetal bovine serum, streptomycin (50 μg/ml) and penicillin (50 IU/ml) at 37 °C in 5% CO_2. The membranes were prepared as previously described (Dalpiaz et al., 2001b) and their aliquots were incubated in 400 μl of 50 mM TRIS–HCl, pH 7.4, at 25 °C for 90 min. Displacement experiments were performed in the presence of 1 nM [^{3}H]DPCPX as previously described (Dalpiaz et al., 2001b). The experiments were performed in the absence and in the presence of 100 μg/ml unloaded microspheres (sample A). The binding effects of CPA released from 10 μg/ml, 100 μg/ml and 1 mg/ml of loaded microspheres (sample A) were evaluated as previously described (Dalpiaz et al., 2001b).

3. Results and discussion

3.1. Microsphere characterization

According to the SEM analysis (Fig. 1), both the loaded and the unloaded microspheres showed a spherical morphology and a smooth surface

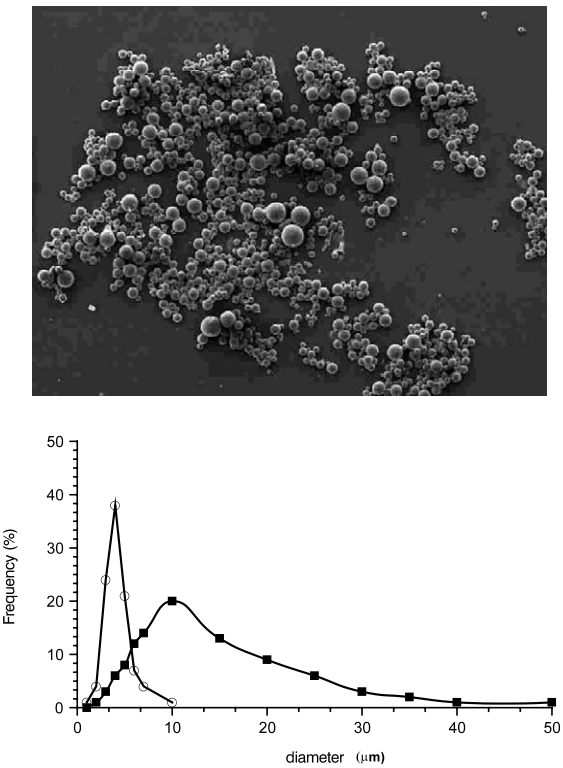

Fig. 1. Scanning electron micrograph of PLA microspheres containing CPA and their particle size distribution according to the stirring rate. Key: (■) 1200 rpm; (○) 1700 rpm.

with no pores or irregular formations due to unencapsulated CPA. The microspheres obtained using 1200 rpm (samples A, B and C) showed a broad size distribution and a mean diameter value of 21 ± 9 μm. Using 1700 rpm (sample D), the mean diameter obviously decreased reaching the value of 5 ± 2 μm and a more homogeneous size distribution was obtained (Fig. 1). The freeze-drying procedure did not modify the morphology and the particles size of the microspheres.

All the microspheres samples showed a low drug content regardless to the conditions and to the CPA amount used in the preparation procedure (0.06–0.12 mg/100mg). From a therapeutic point of view, this low amount of CPA loaded can be enough to achieve a significant effect on adenosine A_1 receptor. From an economical point of view, the yield has to be improved before suggesting the use of microspheres for the CPA

administration. As the drug content is not affected by the amount used for the preparation, it is reasonable to hypothesise the drug content corresponds to the drug solubility in PLA. Therefore, the CPA loaded in the microspheres was molecularly dispersed (dissolved) in the polymer. Our opinion cannot be demonstrated, as the presence of crystalline drug cannot be revealed by the usual analysis method (X-ray diffractometry, thermal analysis) owing to the low concentration of the drug encapsulated.

3.2. CPA release studies

The CPA release from the microspheres is depicted in Fig. 2. From the sample A, the release was practically complete (92%) after 72 h, whereas in the same time the samples B and C released only the 50% of the loaded CPA. From the sample D, the complete CPA release occurred in about 30 h, owing to the increase of the surface area of the microspheres. The rationale of the differences in the release rate from the samples A, B, and C is not easy to explain being the size the same for all the samples and the half the CPA loading in the sample C. The freeze-drying procedure did not modify the release behaviour, as indicated by the release profiles from the sample B and B lyophilized.

The treatment of the experimental data according to the simple power law expression proposed by Korsmeyer et al. (1983) allows the mechanism of the release process to be determined according to the different geometries (Sinclair and Peppas, 1984; Ritger and Peppas, 1987). For all the samples, the values of the kinetics exponent of CPA release (~ 0.4) indicated diffusion-type kinetics.

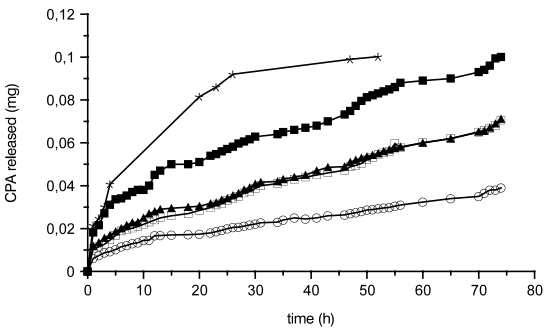

Fig. 2. Release profiles of the PLA microspheres in 25 ml 0.1M pH 7.4 phosphate buffer (USP 24) at 37 ± 2 °C. Key: (■) A; (△) B; (□) B lyophilised; (○) C; (*) D.

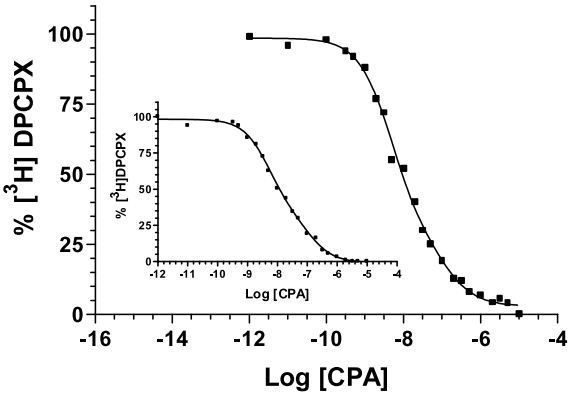

Fig. 3. Competition experiments of CPA on CHO A_1 cell membranes obtained at 25 °C in the absence and in the presence (inset) of 100 μg/ml of unloaded microspheres.

3.3. Kinetic and binding experiments

Free CPA appears degraded in human whole blood according to a first order kinetic with a kinetic constant value (k_d) $= 0.041 \pm 0.003$/min (half life = 17 min). This value does not appear substantially changed for CPA incubation in the presence of unloaded microspheres ($k_d = 0.0043 \pm 0.003$/min; half life = 16 min). On the other hand, only 18% of CPA encapsulated in microspheres appears degraded after 3 h.

Fig. 3 illustrates the inhibition experiments of CPA obtained in the absence and in the presence (inset) of 100 μg/ml of microspheres. According to the computer analysis of inhibition curves, CPA recognized two affinity binding states, with a high affinity state population of approximately 70%. The IC_{50} values obtained in the absence (4.8 ± 0.3 nM for high affinity; 113 ± 8 nM for low affinity) and in the presence (4.9 ± 0.3 nM for high affinity; 111 ± 7 nM for low affinity) of microspheres, suggest that the CPA binding is not modified by the presence of the unloaded release system.

Table 2 reports the percentage of 1 nM [^{3}H]DPCPX bound to CHO A_1 cell membranes in the absence and in the presence of 100 μl of solutions obtained by filtration, at regular time

Table 2
Percentages of 1 nM [^{3}H]DPCPX specific bound to membrane preparation of CHO A_1 cells in the absence (0 min) and in the presence of 100 μl of solutions filtered at regular time intervals from suspensions containing 10 μg/ml, 100 μg/ml or 1 mg/ml of loaded microspheres

Time	Percentages of [^{3}H]DPCPX bound to human adenosine A_1 receptor		
	1 mg/ml of loaded microspheres	100 μg/ml of loaded microspheres	10 μg/ml of loaded microspheres
0 min	100 ± 5	100 ± 5	100 ± 5
10 min	33 ± 3	86 ± 4	96 ± 5
1 h 30 min	16 ± 3	59 ± 3	82 ± 5
3 h	15 ± 2	42 ± 3	78 ± 4
6 h	14 ± 2	39 ± 2	75 ± 4
8 h	11 ± 2	36 ± 3	73 ± 3
24 h	9 ± 2	32 ± 2	69 ± 3
30 h	6 ± 2	31 ± 2	65 ± 3

SEM are reported.

intervals, of suspensions with different amounts of loaded microspheres. One milligram per millilitre of loaded microsphere releases CPA amounts capable of strongly inhibiting the 1 nM [^{3}H]DPCPX binding at all times; 100 μg/ml of microsphere release amount of CPA which can strongly inhibit the [^{3}H]DPCPX binding only after several hours; 10 μg/ml of microspheres allow free CPA concentrations which weakly inhibit the radioligand binding within experimental time range (30 h) to be obtained. It could be hypothesized that similar amounts of loaded microspheres would make it possible to obtain, in vivo, significant CPA activity with weak adverse effects.

Acknowledgements

This research was supported by Italian Ministry of University, Scientific and Technological Research (MURST). The authors thank Prof. Peter R. Schofield of the Garvan Institute of Medical research, Sydney, Australia and Dr Andrea Townsend-Nicholson of the Department of Anatomy and Developmental Biology, University College, London, for providing the CHO A_1 cells. Moreover, the authors thank the Laboratorio Analisi Estense of Ferrara, Italy, for supplying fresh blood.

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