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Synthesis and therapeutic potential of a macromolecular prodrug of diflunisal

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Abstract

A macromolecular prodrug of diflunisal was synthesised by linking this anti-inflammatory agent to an α,β-poly(*N*-hydroxyethyl)-DL-aspartamide (PHEA) polymeric carrier via an ester bond. The resulting PHEA-diflunisal conjugate, containing a quantity of linked drug of 16% (w/w) is, like the polymeric carrier, water-soluble. The delayed drug release from the conjugate, in simulated gastric juice, indicates that the adduct PHEA-diflunisal is suitable for peroral applications. Moreover, it was shown that this conjugate subjected to enzymatic hydrolysis releases all the linked drug in the active form, within 24 h. Finally, it was experimentally ascertained that neither PHEA-diflunisal nor PHEA induced platelet aggregation at least up to a polymer concentration of 2% (w/v) in platelet-rich plasma. According to these considerations, the PHEA-diflunisal conjugate can also be employed for parenteral applications.

Key words: Diflunisal; α,β-Poly(*N*-hydroxyethyl)-DL-aspartamide (PHEA); Macromolecular prodrug; Enzymatic cleavage; Aggregometry

1. Introduction

The pharmacokinetics and pharmacodynamics of a drug can be strongly improved by its chemical link to a biocompatible macromolecule (Duncan et al., 1991). These polymer-drug conjugates allow one to achieve a different distribution of the drug in the body, to increase the duration of drug action and to decrease the incidence of side and toxic effects (Kopeček, 1990; Duncan et al., 1991; O'Mullane and Daw, 1991).

Among the polymers able to act as drug carriers, α,β-poly(*N*-hydroxyethyl)-DL-aspartamide (PHEA) has attracted great interest, since it is highly soluble in water, non-toxic, non-antigenic and non-teratogenic (Antoni et al., 1979; Okada et al., 1985). Given these interesting peculiarities, we have recently undertaken a systematic investigation of the synthesis and physico-chemical properties of PHEA conjugates with some nons-

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teroidal anti-inflammatory drugs (NSAIDs). In particular, we have previously shown that, at simulated gastric juice pH, these macromolecular prodrugs are able to release gradually the drug in active form (Giammona et al., 1991). Moreover, in vivo pharmacological tests showed that they have an analgesic and anti-inflammatory activity comparable to that of free drugs (Giammona et al., 1989). Further investigations on the interaction between some of these polymeric prodrugs and liposomes, chosen as biomembrane models, showed that the polymer-drug conjugates interact with these systems more strongly than the free drugs (Castelli et al., 1990, 1991).

Now we have focused our attention on another non-steroidal anti-inflammatory drug, diflunisal. Diflunisal (2′,4′-difluoro-4-hydroxy-[1,1′-biphenyl]-3-carboxylic acid) is a salicylate derivative with analgesic and anti-inflammatory activity commonly used in the treatment of osteoarthritic diseases and for the control of phlogistic and postoperative pain (Kaklamanis et al., 1978; Brodgen et al., 1980). The duration of its analgesic effect is longer than that of aspirin. Diflunisal appears comparable in efficacy to glafenine in chronic pain (Christodoulopoulos and Houssianakou, 1979) and to propoxyphene/paracetamol combinations in musculoskeletal strains and sprains (Jaffe et al., 1978). Gastrointestinal complaints, dyspepsia, giddiness and cutaneous rashes are the most frequently reported side effects (Brodgen et al., 1980).

In this paper we describe the synthesis and characterization of a new macromolecular prodrug obtained by linking diflunisal to PHEA. In addition, we report the experimental results of some in vitro investigations on the potential therapeutical applications of PHEA-diflunisal conjugate. In particular, we studied drug release from the adduct at pH 1.1 in simulated gastric juice and at pH 7.4 buffer solution in the presence of hydrolytic enzymes. Furthermore, since a macromolecule can induce platelet aggregation (Mohammad et al., 1979; O'Mullane and Daw, 1991), whereas diflunisal alone lacks this effect (Mikhailidis et al., 1980), we performed platelet aggregation in vitro testing of PHEA-diflunisal conjugate in order to evaluate whether the attachment of diflunisal to PHEA modifies the behavior of the drug towards platelet function.

2. Materials and methods

2.1. Apparatus

Infrared (IR) spectra were recorded using a Perkin-Elmer 1720 IR Fourier transform spectrophotometer in potassium bromide disks.

Ultraviolet (UV) spectra were recorded in ethanol/water (70:30, v/v) using a Perkin-Elmer 330 instrument equipped with a 3600 data station.

Elemental analysis (C, H, N) was carried out on a Carlo Erba model 1106 analyzer. Compounds were quantitatively dried before analysis on P_2O_5 under reduced pressure (10^{-3} mmHg) at room temperature for 48 h.

Viscosity measurements were performed at 25 $\pm$ 0.01°C using an Ubbelhode micro-viscometer mounted in an AVS 440 automatic viscosity measuring unit (Schott). The micro-viscometer was chosen with sufficiently long flow time in order to minimize the kinetic energy correction.

HPLC was carried out using a Varian 5020 Liquid Chromatograph equipped with a Valco N 60 loop injector (fitted with a 10 μl loop), and a Varian Variable-Wavelength Detector. The samples were chromatographed on a C_{18} Whatman column (300 $\times$ 3.9 mm, particle size 10 m) with methanol-water (70:30, v/v) at 0.6 ml/min. The eluate was monitored at 254 nm for the determination of diflunisal. Quantification was performed with an external standard using a Varian CDS-111 L Chromatograph Data System. This method allows one to check the purity of the PHEA-diflunisal conjugate and to determine the amount of drug released from the adduct by the hydrolysis procedures employed.

Platelet aggregation experiments were performed using an ELVI 840 Aggregometer.

2.2. Materials

DL-Aspartic acid, ethanolamine and 1,1′-carbonyldiimidazole (CDI) were purchased from

Fluka (Switzerland); *N,N*-dimethylformamide (DMF) (Hoechst) was dried using P_2O_5 and distilled under reduced pressure before use.

α,β-Poly(*N*-hydroxyethyl)-DL-aspartamide (PHEA) (**1**) was prepared via the polysuccinimide by polycondensation of DL-aspartic acid in the presence of H_3PO_4 at 180°C followed by reaction with ethanolamine in DMF solution (Neri et al., 1973; Giammona et al., 1987).

Diflunisal (**2**) was obtained from Sigma Chemical Co. (St. Louis, U.S.A.).

Esterase from hog liver was obtained from Boehringer Mannheim (Germany).

Platelets for aggregation in the vitro test were obtained using the following procedure. Blood, obtained from volunteer healthy blood donors, collected into tubes containing sodium citrate (to prevent clotting), was centrifuged at 5000 rpm for 15 min to give a supernatant constituted of platelet-poor plasma (PPP), whereas when it was centrifuged at 1000 rpm for 5 min and at 2000 rpm for 5 min it gave a supernatant constituted of platelet-rich plasma (PRP) (Born, 1962; O'Mullane and Daw, 1991).

2.3. Determination of intrinsic viscosities

Intrinsic viscosities, $[\eta]$, were evaluated by extrapolating reduced viscosities in the range 2–12 mg/ml at zero concentration. Polysuccinimide was dissolved in 0.1 M LiCl in DMF (($M = 42650$, according to the Mark-Houwink relationship: $[\eta] = 1.32 \times 10^{-2} \times M^{0.76} = 43.6$ ml/g (Vlašak et al., 1979) and PHEA in water ($M = 51300$, according to the Mark-Houwink equation: $[\eta] = 2.32 \times 10^{-3} \times M^{0.87} = 29.4$ ml/g (Antoni et al., 1974)).

2.4. Synthesis of PHEA-diflunisal conjugate (3)

A solution of 0.418 g (2.58×10^{-3} mol) of carbonyldiimidazole (CDI) in 2.5 ml of anhydrous DMF was added dropwise, at 0°C, to a solution of 0.538 g (2.15×10^{-3} mol) of diflunisal dissolved in 3.5 ml of dry DMF. To the mixture, kept at 0°C for 30 min, a solution of PHEA (1 g, 1.95×10^{-5} mol) in dry DMF (12 ml) was added dropwise. The reaction mixture was maintained for 8 min at 0°C, for 1 h at 9°C and then set aside at room temperature for 3 days with occasional shaking. Finally, the solvent was evaporated off in vacuo on a bath warmed at 40°C and the solid material obtained was washed several times with acetone. The resulting solid was then solubilised in 25 ml of distilled water and purified by exhaustive dialysis against distilled water using Visking Dialysis Tubing (18/32 inch) with a molecular weight cutoff of 12000–14000. The aqueous solution was concentrated in vacuo and then lyophilised. The pure conjugate (**3**) was obtained in a yield of 98% based on the starting material (PHEA).

The IR spectrum of **3** showed bands at 3300 (OH, broad), 1735 (ester C = 0), 1652 (amide I) and 1540 (amide II) cm^{-1}.

The content of active agent in the adduct was determined by both hydrolysis at pH 10 and elemental analysis.

Analysis: Calculated for $C_{8.08}H_{10.96}F_{0.32}N_2O_{3.32}$ (corresponding to 16% of substitution): C, 32.74; H, 44.41; N, 8.10. Found: C, 32.53; H, 44.27; N, 8.23.

2.5. Hydrolysis of the polymeric conjugate 3 at pH 10

15 mg of PHEA-diflunisal adduct were dissolved in 10 ml of pH 10 buffer solution (H_3BO_3/KCl/0.1 N NaOH). The reaction mix-

Table 1
Release of diflunisal from adduct **3** at pH 1.1 and 37±0.1°C

Time (h)	Release of **2** from **3**	
	Amount [a]	Fraction [b]
3	0.74	4.61
10	1.40	8.73
20	2.73	17.08
39	4.00	25.00
49	5.27	32.92
55	6.59	41.17
63	7.88	49.25
68	8.06	53.75
73	9.09	56.83
80	9.47	59.16
85	9.79	61.13

[a] mg of drug from 100 mg of adduct.
[b] % w/w of drug.

ture was maintained at 60 ± 0.1°C for 30 h after which it was neutralised with 2 N HCl. The amount of diflunisal released by hydrolysis from the adduct was determined by HPLC. The drug loading in **3** was found to be 16% w/w.

2.6. Hydrolysis of the polymeric conjugate 3 at pH 1.1

10 aliquots of 15 mg of adduct were dissolved in 10 ml of pH 1.1 buffer solution (HCl, NaCl and glycine), maintained at 37 ± 0.1°C and sampled at suitable different intervals. Each sample, after neutralisation with 1 N NaOH, was analysed by HPLC. Table 1 reports the rates of release of drug from the conjugate **3**.

2.7. Enzymatic cleavage of polymeric conjugate 3 at pH 7.4

Four aliquots of 10 mg of conjugate **3** were dissolved in 6 ml of pH 7.4 buffer solution (NaCl, Na_2HPO_4, KH_2PO_4) and kept at 37 ± 0.1°C.

After 20 min, 75 μl of esterase solution (13 U/ml) were added for each aliquot which, sampled at suitable different intervals, was analyzed by HPLC.

Table 2 lists the rates of release of drug from the PHEA-diflunisal conjugate as a function of the time.

2.8. Platelet aggregation in vitro test

The platelet aggregation in vitro tests were performed by using the following procedure (Born, 1962; O'Mullane and Daw, 1991). Different amounts of PHEA or of PHEA-diflunisal were dissolved in physiological solution. 20 μl of these solutions were added to 250 μl of PRP obtaining a final concentration of polymer or polymer-drug conjugate of 0.3, 1 and 2% (w/v). Every experiment was repeated three times. Test experiments to verify the normal platelet aggregation function were performed by addition of 20 μl of a 1 μg/ml solution of collagen to PRP.

Table 2
Release of diflunisal from adduct **3** at pH 7.4 buffer solution and 37 ± 0.1°C in the presence of hydrolytic enzymes

Time (h)	Fraction [a]
2	9.17
5	19.24
10	47.37
24	97.6

[a] % w/w of drug.

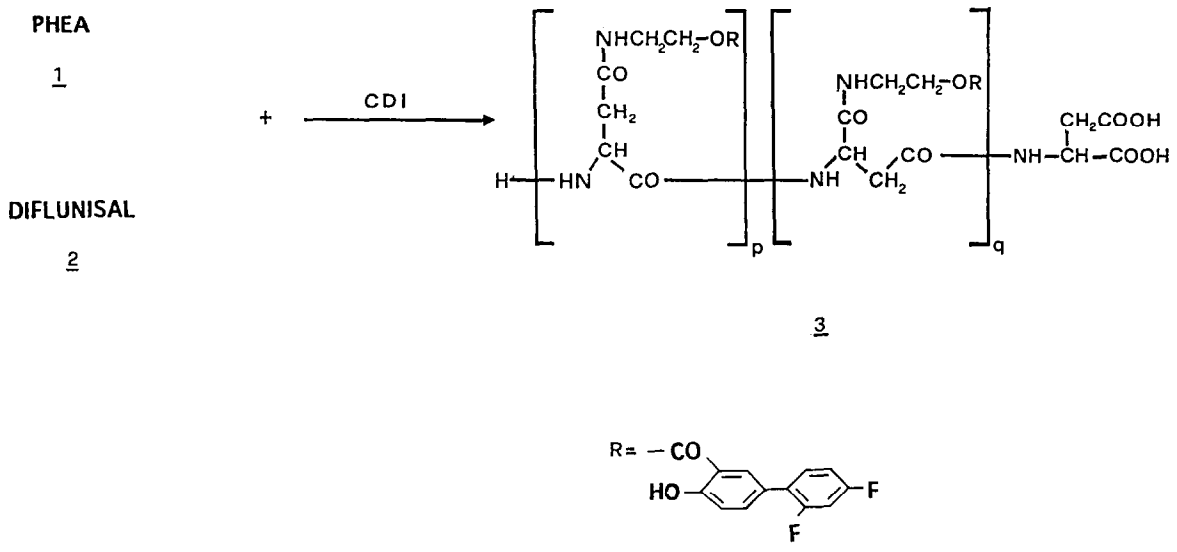
3. Results and discussion

The attachment of diflunisal (**2**) to PHEA (**1**) was performed by means of carbonyldiimidazole (CDI) (Giammona et al., 1987). The synthesis of polymeric prodrug PHEA-diflunisal (**3**) is reported in Scheme 1.

The covalent polymer-drug conjugate bond was demonstrated by UV, IR and elemental analysis. The absence of the free drug in the adduct **3**, purified by washing with acetone (in which only the free drug is soluble) and dialysis (see section 2), was confirmed by HPLC. The PHEA-diflunisal conjugate was water-soluble like PHEA itself. UV analysis of the adduct **3** revealed bands within the range of 230–350 nm, in which the polymeric carrier did not absorb. The IR spectrum of the adduct **3** showed a band typical of the ester C = O group at 1735 cm^{-1} which was not present in the spectrum of PHEA alone, together with two carbonyl stretching bands for the amide groups and the broad band for the hydroxyl groups (see section 2). The amount of diflunisal in the macromolecular prodrug, estimated by elemental analysis and hydrolysis, was found to be 16% w/w. Hydrolysis of the conjugate was performed in a pH 10 buffer solution and at 60 ± 0.1°C for 30 h. After this time interval, hydrolysis reached completion. Free drug under the same reaction conditions did not undergo any degradation.

In order to study the potential use of the PHEA-diflunisal conjugate as a drug delivery system for peroral applications, we investigated the hydrolysis of this adduct in simulated gastric juce (pH 1.1 buffer solution) at 37 ± 0.1°C.

Since the carboxyl group of an anti-inflammatory agent is essential for its therapeutic action, it



Scheme 1.

is necessary that the active agent is released from the polymeric prodrug in the surrounding enviroment by hydrolysis in the intact form. The obtained results demonstrated a delayed relase of the drug in active form from the macromolecular prodrug (see Table 1).

Moreover, in order to evaluate the potential use of PHEA-diflunisal conjugate for parenteral applications as well, we studied drug release from this adduct in pH 7.4 buffer solution in the presence of hydrolytic enzymes. The experimental results are listed in Table 2. As can be seen, in the presence of enzymes nearly all the drug is released in the active form within 24 h. The PHEA-diflunisal conjugate, solubilized in pH 7.4 buffer solution and kept at 37 ± 0.1°C, was shown

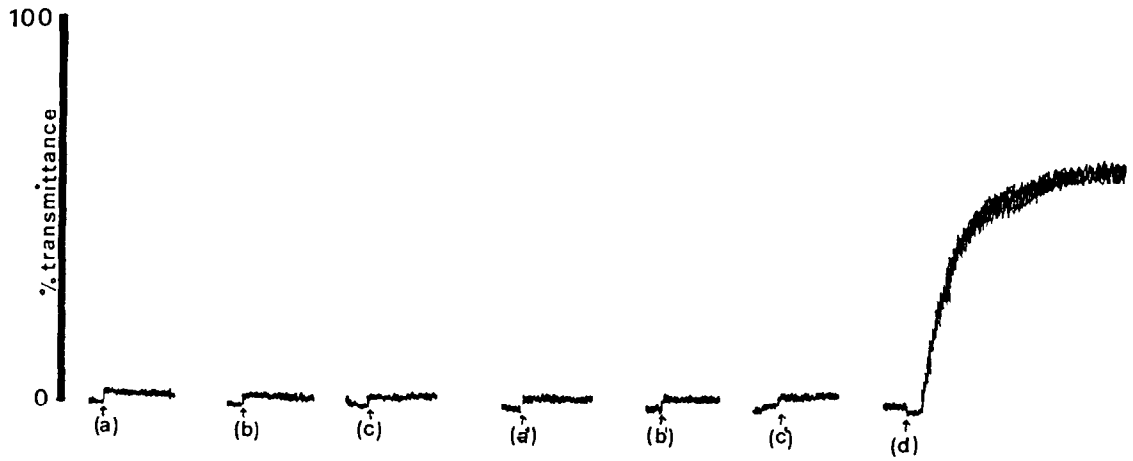


Fig. 1. Transmittance of samples obtained by adding PHEA to PRP in order to attain 0.3% (a), 1% (b) and 2% (c) (w/v) final concentration of polymeric carrier or adding PHEA-diflunisal conjugate to PRP in order to attain 0.3% (a'), 1% (b') and 2% (c') (w/v) final concentration of polymeric prodrug or adding collagen (d).

to be stable during a period of 8 days when analysed by HPLC.

Platelet aggregation in vitro tests of PHEA and PHEA-diflunisal conjugate show that both these polymers, like diflunisal (Mikhailidis et al., 1980) and in contrast to many macromolecules (O'Mullane and Daw, 1991), do not induce platelet aggregation at least up to 2% w/v concentration in PRP. Fig. 1 illustrates these results in detail.

As can be seen, the addition of collagen to PRP produces an increase in light transmission due to the formation of platelet aggregates (Fig. 1d), whereas the presence of PHEA or PHEA-diflunisal adduct at 0.3% (Fig. 1a and a'), 1% (Fig. 1b and b') and 2% (Fig. 1c and c') (w/v) does not cause changes in light transmission. It should be stressed that this behavior, the water solubility and the ability to release totally the linked drug, by enzymatic cleavage of polymer-drug ester bonds, are important features for a possible therapeutic application of this polymeric prodrug.

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5. References

Antoni, G., Arezzini, C., Cocola, F., Gazzei, G. and Neri, P., Pharmacological and toxicological evalutation of polyhydroxyethylaspartamide (PHEA) as a plasma substitute. *Il Farmaco Ed. Pr.*, 34 (4) (1979) 146–156.

Antoni, G., Neri, P., Pedersen, T.G. and Ottesen, M., Hydrodynamic properties of a new plasma expander. Poly(hydroxyethyl)aspartamide. *Biopolymers*, 13 (1974) 1721–1729.

Born, G.V.R., Aggregation of blood platelets by adenosine diphosphate and its reversal. *Nature*, 194 (1962) 927–929.

Brodgen, R.N., Heel, R.C., Pakes, G.E., Speight, T.M. and Avery, G.S., Diflunisal: a review of its pharmacological properties and therapeutic use in pain and musculoskeletal strains and sprains and pain in osteoarthritis. *Drugs*, 19 (1980) 84–106.

Castelli, F., Giammona, G., Puglisi, G., Carlisi, B. and Gurrieri, S., Interaction of macromolecular prodrugs with lipid model membrane: calorimetric study of 4-biphenylacetic acid linked to α,β-poly(*N*-hydroxyethyl)-DL-aspartamide interacting with phosphatidylcholine vesicles. *Int. J. Pharm.*, 59 (1990) 19–25.

Castelli, F., Giammona, G., Raudino, A. and Puglisi, G., Macromolecular prodrugs interaction with mixed lipid membrane. A calorimetric study of naproxen linked to polyaspartamide interacting with phosphatidylcholine and phosphatidylcholine-phosphatic acid vesicles. *Int. J. Pharm.*, 70 (1991) 43–52.

Christodoulopoulos, J. and Houssianakou, E., Diflunisal compared with glafenine in pain associated with carcinoma. In *Diflunisal*, Royal Society of Medicine, International Congress and Symposium no. 6, Academic Press/Royal Society of Medicine, 1979, pp. 125–127.

Duncan, R., Hume, I.C., Yardley, H.J., Flanagan, P.A., Ulbrich, K., Subr, V. and Strohalm, J., Macromolecular prodrugs for use in targeted cancer chemotherapy: melphalan covalently coupled to *N*-(2-hydroxypropyl) metacrylamide copolymers. *J. Controlled Release*, 16 (1991) 121–136.

Giammona, G., Carlisi, B. and Palazzo, S., Reaction of α,β-poly(*N*-hydroxyethyl)-DL-aspartamide with derivatives of carboxylic acids. *J. Polym. Sci. Polym. Chem. Ed.*, 25 (1987) 2813–2818.

Giammona, G., Carlisi, B., Pitarresi, G. and Fontana, G., Hydrophilic and hydrophobic polymeric derivatives of anti-inflammatory agents such as alclofenac, ketoprofen and ibuprofen. *J. Bioact. Compatible Polym.*, 6 (1991) 129–141.

Giammona, G., Puglisi, G., Carlisi, B., Pignatello, R., Spadaro, A. and Caruso, A., Polymeric prodrugs: α,β-poly(*N*-hydroxyethyl)-DL-aspartamide as a macromolecular carrier for some non-steroidal anti-inflammatory agents. *Int. J. Pharm.*, 57 (1989) 55–62.

Jaffe, G.V., Roylance, P.J. and Grimshaw, J.J., A controlled study of diflunisal in sprains and strains. *Curr. Med. Res. Opinion*, 5 (1978) 584.

Kaklamanis, Ph., Sfikakis, P., Demetriades, P., Tsachalos, P., Thouas, B. and Seitanes, B., Double-blind. 12-week comparison trial of diflunisal and acetylsalicylic acid in the control of pain of osteoarthritis of the hip and/or knee. *Clin. Ther.*, 1 (1978) 20.

Kopeček, J., The potential of water-soluble polymeric carriers in targeted and site-specific drug delivery. *J. Controlled Release*, 11 (1990) 279–290.

Mikhailidis, D.P., Freedman, D.B. and Dandona, P., Effect of diflunisal on in vitro platelet aggregation. *Lancet*, (1980) 215.

Mohammad, S.F., Chuang, H.Y.K., Crowther, P.E. and Mason, R.G., Interactions of poly(L-lysine) with human platelets. Correlation of binding with induction of platelets aggregation. *Thromb. Res.*, 15 (1979) 781–791.

Neri, P., Antoni, G., Benvenuti, F., Cocola, F. and Gazzei, G., Synthesis of α,β-poly(*N*-hydroxyethyl)-DL-aspartamide, a new plasma expander. *J. Med. Chem.*, 16 (1973) 893–897.

Okada, M., Matsushima, A., Katsuhata, A., Aoyama, T., Ando, T. and Inada, Y., Suppression of IgE antibody responce

against ovoalbumin by the chemical conjugate of ovalbumin with a polyaspartic acid derivatives. *Int. Arch. Allergy Appl. Immunol.*, 76 (1985) 79–81.

O'Mullane, J. and Daw, A., Macromolecular prodrugs: blood interactions and targeting. *J. Controlled Release*, 16 (1991) 113–120.

Vlašak, J., Rypacek, F., Drobnik, J. and Saudek, V., Properties and reactivity of polysuccinimide. *J. Polym. Sci. Polym. Symp.*, 66 (1979) 59–64.