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A Hydrogel Based on a Polyaspartamide: Characterization and Evaluation of In-vivo Biocompatibility and Drug Release in the Rat

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Abstract

This paper deals with the characterization of a new microparticulate hydrogel obtained by gamma irradiation of α,β-poly[*N*-(2-hydroxyethyl)-DL-aspartamide] (PHEA).

When enzymatic digestion of PHEA hydrogel was evaluated using various concentrations of pepsin and α-chymotrypsin no degradation occurred within 24 h. In-vivo studies showed that this new material is biocompatible after oral administration to rats. PHEA hydrogel was also studied as a system for delivery of diflunisal, an anti-inflammatory drug. In-vitro release studies in simulated gastrointestinal juice (pH 1 or 6·8) showed that most of the drug was released at pH 6·8. In-vivo studies indicated that diflunisal-loaded PHEA microparticles significantly improved the gastric tolerance and oral bioavailability of the drug in comparison with free diflunisal.

These results suggest the potential application of PHEA hydrogel as a new delivery system for the oral administration of anti-inflammatory drugs.

Oral dosage forms are particularly suitable in prolonged therapy because they result in better patient compliance than other routes of administration (Park 1988; Couvreur et al 1995; Kost 1995; Pabon Gonzales et al 1995; Rubinstein 1995; Sarciaux et al 1995; Russel-Jones, 1996).

Non-steroidal anti-inflammatory drugs such as diflunisal have been proposed for the long-term treatment of rheumatoid arthritis (Brouwers & De Smet 1994). Diflunisal is a salicylic acid derivative with remarkable analgesic and anti-inflammatory activity. The duration of its analgesic effect is longer than that of aspirin, it is not metabolized to salicylic acid, and it causes less platelet aggregation than aspirin in-vivo (Brodgen et al 1980; Shen 1983).

Like other non-steroidal agents, diflunisal induces gastrointestinal side-effects, for example haemorrhages and ulceration of stomach and small intestine (Langman et al 1994; Smith et al 1994).

Although these side-effects are closely connected with the mechanism of action of non-steroidal anti-inflammatory drugs (i.e., inhibition of cyclooxygenase which catalyses the prostaglandin biosynthesis), the administration of diflunisal loaded on to a microparticle system could provide active distribution over a wide surface of the gastric and duodenal mucosa. This would avoid localized drug concentrations and would result in better gastric tolerability (Swinyard 1990; Chiou & Liu 1996). In addition, microparticles, because of their reduced dimensions, could rapidly cross the pyloric canal, irrespective of gastric emptying (Meyer et al 1985).

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Our study has focused on the development of new hydrogel microparticles based on biocompatible polymers such as α,β-poly[*N*-(2-hydroxyethyl)-DL-aspartamide] (PHEA) (Giammona et al 1987, 1992, 1995). In a previous paper we described the experimental conditions used to obtain crosslinked PHEA microparticles by gamma irradiation using a ^{60}Co source (Spadaro et al 1996). In this work we report the characterization of PHEA microparticles by aqueous swelling measurements at various pH values, and stability studies in simulated gastrointestinal fluids in the presence of enzymes. We have also evaluated the biocompatibility of PHEA microparticles after oral administration to rats. Diflunisal was loaded on to PHEA microparticles and the in-vitro release profile in simulated gastrointestinal fluid was studied. Finally, we studied gastric damage and the bioavailability of diflunisal loaded on to PHEA microparticles after oral administration to rats and compared the results with those obtained for free diflunisal.

Materials and Methods

Materials

DL-aspartic acid, ethanolamine and *N,N*-dimethylformamide (DMF) were from Fluka (Switzerland). Pepsin from porcine stomach mucosa, 3500 units (mg protein)$^{-1}$ α-chymotrypsin from bovine pancreas, 58 units (mg protein)$^{-1}$ and diflunisal (2′,4′-difluoro-4-hydroxy-[1,1′-biphenyl]-3-carboxylic acid) were purchased from Sigma (USA). All the reagents used were of analytical grade.

α,β-Poly[*N*-(2-hydroxyethyl)-DL-aspartamide] (PHEA; molecular weight 56 900, polydispersity index, M_w/M_n, 1·89) was prepared by reaction of a polysuccinimide (PSI) with ethanolamine in DMF solution and purified according to a procedure reported elsewhere (Giammona et al 1987).

Apparatus

The weight-average molecular weight and polydispersity index of PHEA were measured by light scattering using a Dawn DSP-F Laser Spectra Physics Spectrometer. FTIR spectra were recorded using a Perkin-Elmer 1720 Fourier Transform Spectrophotometer. UV spectra were recorded on a Perkin-Elmer 330 instrument equipped with a 3600 data station.

High-performance liquid chromatography (HPLC) was performed with a Varian 5000 chromatograph equipped with a Rheodyne 7125 injector (fitted with a 10-μL loop) and a Kontron 432 HPLC detector on line with a computerized Hewlett-Packard workstation. Separations were performed on a 250 mm × 4·6 mm i.d. × 10 μm (Bondapack reversed-phase C_{18} column (Waters) equipped with a direct-connect 10-μm Bondapack C_{18} guard column (Waters).

Gamma irradiation was performed by means of the IGS-3, a panoramic 3000 Ci ^{60}Co irradiator (Spadaro et al 1996). The dose rate, measured by use of a Fricke dosimeter was 5·24 kGy; a variance of ±5% in the absorbed dose was considered acceptable.

Particle-size distribution and the aqueous swelling process were studied by use of a Leica Quantimet 500 image-processing and analysis system equipped with a Leica Wild 3B stereomicroscope. This image processor calculated the particle area and converted it to an equivalent circle diameter. The apparatus also calculated the roundness index, which was always less than 1·3.

Centrifugation was performed with an International Equipment Company Centra MP4R equipped with a 854 rotor and temperature control.

Preparation of PHEA microparticles

An aqueous solution of PHEA (40 mg mL^{-1}) was irradiated at 604·8 kGy in the presence of air and at room temperature according to a procedure reported elsewhere (Spadaro et al 1996). The microparticulate PHEA network obtained was immersed in a concentrated ethanolic solution of diflunisal and left to soak for 5 days at room temperature. The microparticles were filtered, rapidly washed with ethanol and dried to constant weight. The amount of drug loaded on the microparticles was determined by extraction with ethanol, the samples being assayed by UV spectrophotometry at 252 nm and the absorbance being converted into concentration using an $E^{1\%}$ value of 541·23. The drug loading was found to be 16·7% w/w.

Samples with and without loaded drug were ground. Particle fractions between 20 and 100 μm were separated by sieving and stored in a desiccator over P_2O_5.

Swelling studies

Swelling of PHEA microparticles was studied in double-distilled water, in HCl solution (0·1 N) at pH 1 and in phosphate buffer solutions at pH 6·8 and 7·4. In particular, by use of the method proposed by Robert et al (1987) we observed that microparticle diameter increased until equilibrium was reached. Equilibrium swelling ratio Q was determined from the relationship:

$$Q = d_{eq}/d_0$$

where d_{eq} and d_0 are, respectively, the diameters of swollen and dry microparticles.

Stability of PHEA microparticles in the presence of enzymes

Samples of PHEA microparticles (50 mg) were incubated in a water bath at 37 ± 0·1°C with continuous stirring (100 rev min^{-1}) with 2 mL HCl solution (0·01 N) at pH 2 containing pepsin or phosphate buffer solution at pH 8 (K_2HPO_4, KH_2PO_4) containing α-chymotrypsin. Enzyme solutions were prepared immediately before the experiment. The concentrations of pepsin and α-chymotrypsin ranged from 0·01 to 1·0 mg mL^{-1}. Each experiment was performed in triplicate. At timed intervals (2, 4, 8, 24 h) the samples were centrifuged at 8000 rev min^{-1} for 10 min at 15°C and the supernatant was separated. The solid precipitate was washed with double-distilled water (2 × 5 mL) with continuous stirring at 50°C for 5 h to extract any soluble polymer chain entrapped in the crosslinked structure of the network. Finally, the PHEA microparticles were washed twice with acetone and centrifuged at 8000 rev min^{-1} for 10 min at 15°C. The solid residue was then separated, dried and weighed.

In-vitro release studies

Samples of PHEA microparticles (10 mg) containing diflunisal were dispersed in flasks containing HCl (0·1 N; pH 1) and maintained at 37 ± 0·1°C in a water bath for 2 h with magnetic stirring (100 rev min^{-1}). After this time, 0·2 M tribasic sodium phosphate solution was added to adjust the pH to 6·8, according to the method reported in USP XXII (drug-release test, method A for enteric-coated particles). Sink conditions were maintained throughout the experiment. Then, at suitable time intervals, samples were filtered and analysed by UV spectrophotometry at 251 nm to determine the amount of drug released. All experiments were performed in triplicate and results were in agreement within ±3% error.

Laboratory animals

Male Sprague-Dawley rats, 120–150 g, (Charles River, Italy) used for biocompatibility, gastric ulceration and bioavailability studies, were fasted for 24 h before treatment; water was withheld 6 h before the experiments. Throughout the experiment rats were housed in cages with wire-mesh floors.

Biocompatibility of PHEA microparticles

To evaluate damage to the gastrointestinal tract by PHEA microparticles, these were suspended in distilled water (1·2 g kg^{-1} (20 mL)$^{-1}$) and administered orally to groups of four rats. Control rats received distilled water (20 mL kg^{-1}). Rats were killed with an overdose of sodium pentobarbitone 24 h after treatment (and did not receive food or water during this period). Stomach and duodenum were removed, inverted, washed in ice-cold saline and all lesions on the mucosa layer, irrespective of size, were counted by visual examination under × 5 magnification. Samples of each examined area were processed for histological analysis by a standard technique (Luna 1968). Tissues were fixed in phosphate-buffered 10% formalin (4% formaldehyde) solution for at least 24 h before production of 5-μm haematoxylin- and eosin-stained sections for morphological evaluation. The prepared specimens were evaluated through a Leitz light-microscope with a photomicrographic system.

Gastric ulceration assay

Free diflunisal (180 mg kg^{-1}) or diflunisal-loaded PHEA microparticles (1078 mg kg^{-1}, corresponding to 180 mg

kg^{-1} of the free drug) were freshly prepared as aqueous suspensions with 0·5% (w/v) methylcellulose and administered orally in a volume of 10 mL kg^{-1} to two groups of seven rats. Six hours after the treatment the rats were killed with an overdose of sodium pentobarbitone (100 mg kg^{-1}, i.p.); their stomachs were removed, opened along the greater curvature, inverted, washed in cold saline and the lesions on the gastric mucosa were evaluated visually under ×5 magnification. A numeric score was given to stomachs as follows: 3, necrotic ulcers; 2, haemorrhage or non-necrotic lesions; 1, erythema; 0, no observable damage. The ulcerogenic index (UI) was obtained by calculating the mean score (±s.e.m.) for each group. Significance differences were calculated according to Student's *t*-test.

Bioavailability studies

Rats were treated orally with an aqueous suspension of free diflunisal (100 mg kg^{-1}) or PHEA microparticles containing diflunisal (599 mg kg^{-1} corresponding to 100 mg kg^{-1} diflunisal). Groups of three animals were anaesthetized with sodium pentobarbitone (35 mg kg^{-1}, i.p.) 0·5, 1, 2, 4, 6 and 24 h after administration of the sample and blood (4 mL) was withdrawn through a catheter inserted into the jugular vein. Rats were then killed immediately with sodium pentobarbitone (40 mg kg^{-1}, i.v.). Blood was collected into heparinized tubes, centrifuged (6000 rev min^{-1} for 10 min at 4°C) and plasma kept at −70°C until further processing. After thawing, plasma (400 μL) was withdrawn and added to methanol (1·6 mL) for deproteinization. After immediate mixing and centrifugation at 4°C for 5 min at 8000 rev min^{-1}, the clear supernatant (10 μL) was analysed by HPLC. A retention time appropriate for determination of diflunisal was found when the reversed-phase C_{18} column was eluted at room temperature with 70:30 (v/v) CH_3OH–0·1% (v/v) H_3PO_4. The flow-rate was 1·5 mL min^{-1} and the eluate was monitored at 254 nm. Quantification of diflunisal was performed by use of a calibration curve; the straight-line equation (in the concentration range 1–30 μg mL^{-1}) was $y = 12{\cdot}273x - 5{\cdot}5378$ (y = peak area × 10^{-4}; x = diflunisal concentration, mg mL^{-1}) with a coefficient of linear regression of 0·99829.

Each experiment was performed in triplicate. Preliminary experiments showed no interfering peaks in the blank plasma chromatogram. The method used enabled plasma diflunisal recovery of 98·8%.

Results and Discussion

We have shown elsewhere that gamma irradiation of aqueous solutions of α,β-poly[*N*-(2-hydroxyethyl)-DL-aspartamide] (PHEA) gave rise to network structures which were shaped as microparticles; we proposed the use of these as new hydrogels (Spadaro et al 1996).

PHEA hydrogel was characterized by swelling tests in dilute HCl solution at pH 1 and in phosphate buffer solutions at pH 6·8 and pH 7·4 and the results were compared with those obtained in double-distilled water. It was found that microparticle diameter quickly increased, reaching a plateau in about 120 s in all the media investigated. Equilibrium swelling ratios Q are reported in Table 1.

Table 1. Equilibrium swelling ratios of PHEA microparticles.

Penetrant medium	Equilibrium swelling ratio (±s.d.)
Double-distilled water	1·370±0·014
Hydrochloric acid, pH 1·0	1·160±0·017
Phosphate buffer, pH 6·8	1·325±0·049
Phosphate buffer, pH 7·4	1·340±0·032

The data obtained indicated high affinity of PHEA hydrogel for the aqueous media investigated. This result was highly significant because the biocompatibility of hydrogels is attributed, among other things, to their high water content (Peppas & Mikos 1986). The extent of swelling varied slightly in media with a pH value close to neutrality; at pH 1 it was lower than in other media. This behaviour could be attributed to the formation of new ionizable groups during irradiation, even if in amounts not detectable by FTIR analysis.

In-vitro enzymatic digestion experiments were performed by incubating PHEA microparticles in the presence of different concentrations of pepsin or α-chymotrypsin for 24 h. No weight decrease of these samples was measured, thus evidencing the lack of degradation in these media. In addition, although the starting water-soluble macromolecule (PHEA) had been shown to be biocompatible (Antoni et al 1979; Giammona et al 1992, 1995), we evaluated the biocompatibility of PHEA microparticles after oral administration to rats because cross-linking gamma irradiation might change the structure and properties of the polymer. As far as was apparent from histological analysis (Fig. 1), PHEA microparticles caused no gross lesion of the stomach and duodenum 24h after their oral administration. Histological results obtained from control rats were similar to those found in the treated animals. In particular, no atrophic or disorderly arranged and vacuolated cells were detected.

To prepare PHEA microparticles containing the selected drug (diflunisal), drug loading was performed after gamma irradiation. The amount of drug loaded was 16·7% w/w. Fig. 2 shows a typical particle size distribution of PHEA microparticles loaded with diflunisal. It is apparent that particle-size distribution is asymmetric; most particle diameters are within the range 50–60 μm.

Microparticles containing diflunisal were also characterized by measurement of swelling under the same conditions as were used for the drug-free gel; swelling ratios (d_{eq}/d_0) of PHEA microparticles containing diflunisal were very similar to those of the drug-free microparticles. To obtain preliminary information about the potential use of diflunisal-loaded PHEA hydrogel, release studies were performed in simulated gastrointestinal fluid (pH 1 and 6·8). The diflunisal release profile is depicted in Fig. 3.

At pH 1 release was moderate because of the low solubility of diflunisal and low extent of swelling of PHEA microparticles in acidic medium. Increasing the pH to 6·8 resulted in a rapid increase in the rate of release of diflunisal, a plateau being reached within 10 h. Release was, therefore, bimodal, with a 'slow' step in the simulated gastric fluid followed by a 'quick' step in the simulated intestinal fluid. As a consequence of this behaviour, this system might ensure reduction of topic damage on gastric membranes, while maintaining the convenience of oral administration (Langman et al 1994).

Further, the small size of diflunisal-loaded PHEA microparticles could enable distribution to a wide area of the gas-

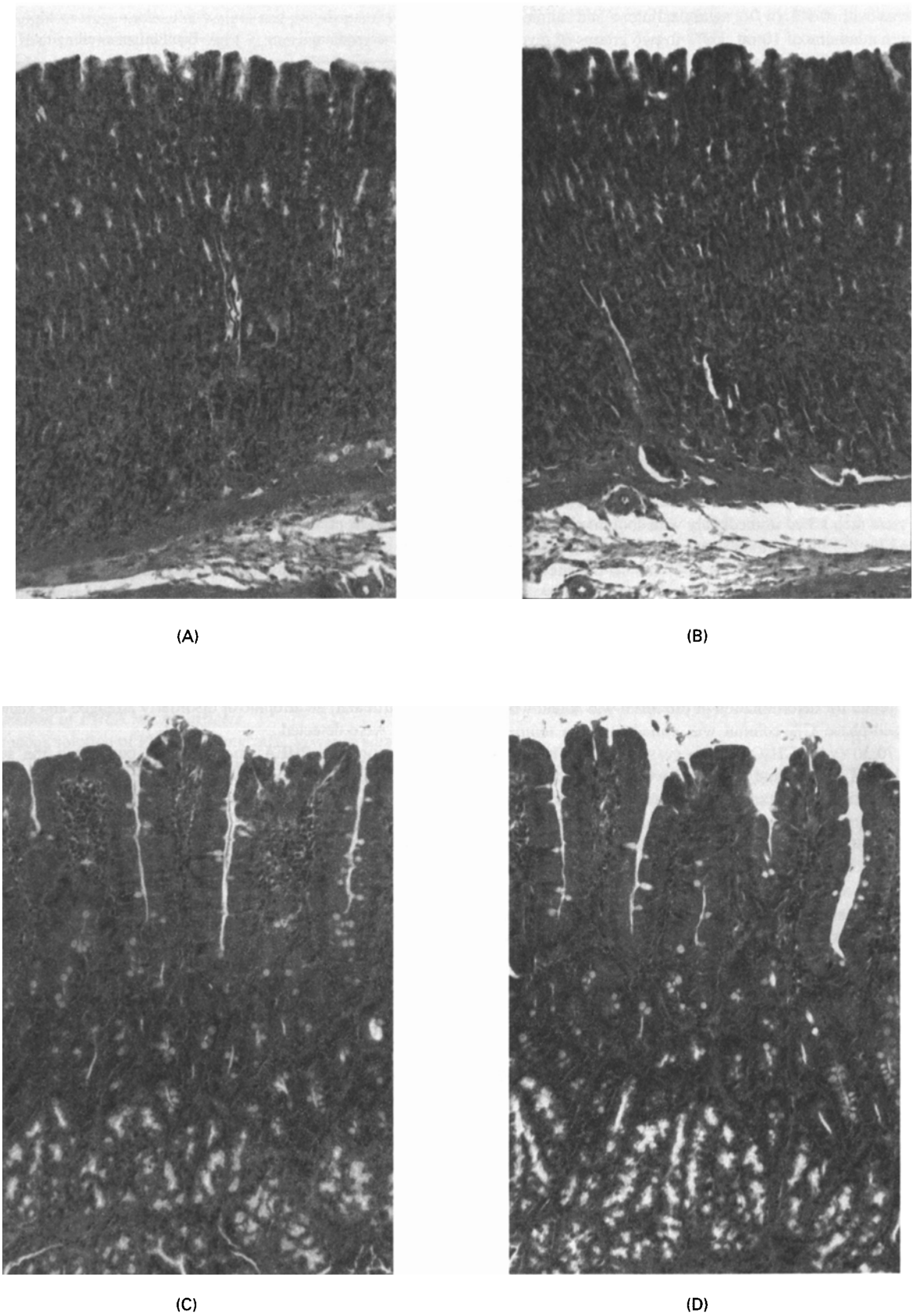

FIG. 1. Histological analysis of gastrointestinal samples taken from rats treated 24 h earlier with PHEA microparticles or with water alone, administered orally. A, stomach of rat treated with water; B, stomach of rat treated with PHEA microparticles; C, duodenum of rat treated with water; D, duodenum of rat treated with PHEA microparticles.

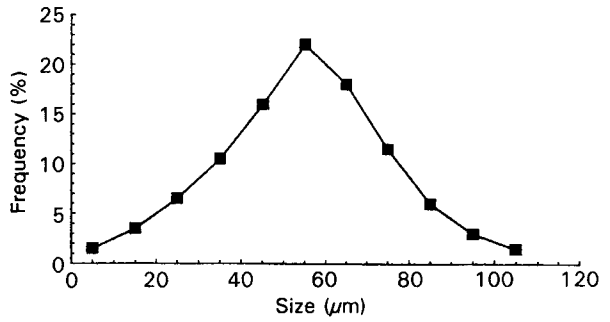

FIG. 2. Size-distribution profile of PHEA microparticles.

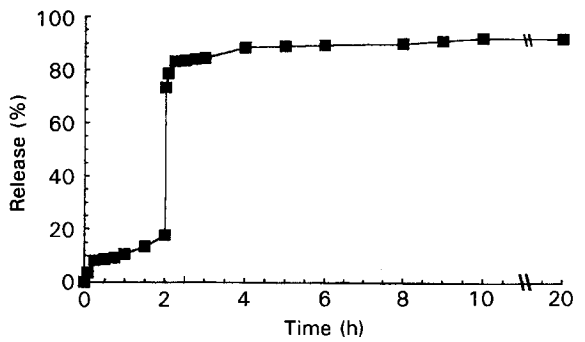

FIG. 3. Release profile of diflunisal from PHEA microparticles in hydrochloric acid (pH 1) between 0 and 2 h and in phosphate buffer solution (pH 6·8) between 2 and 20 h.

trointestinal surface thus avoiding localized regions of high concentration and improving oral tolerability.

Acute oral administration of free diflunisal (180 mg kg^{-1}) caused significant gastric damage, characterized by the appearance of gastric haemorrhage and, in some rats, a few necrotic ulcers. Treatment of the rats with diflunisal-loaded PHEA hydrogel resulted in a significant reduction of gastric lesions; gastric erythema was observed in only 3 of 7 animals (Table 2).

Finally, the oral bioavailability of diflunisal from PHEA microparticles was compared with that of the free drug. The results of these experiments are reported in Fig. 4. The suspension of free diflunisal resulted in a peak plasma concentration 30 min after administration; plasma concentration then decreased rapidly. PHEA microparticles resulted in higher plasma concentrations of diflunisal and these remained almost constant for 4–6 h. Consequently, the values of the area under

Table 2. Gastric lesions in rats after oral administration of diflunisal and of PHEA microparticles loaded with 16·7% (w/w) diflunisal.

Compound	No. of animals with lesions	Ulcerogenic index (mean ± s.e.m.)
Diflunisal	7/7	2·14 ± 0·26
PHEA microparticles containing diflunisal	3/7	0·42 ± 0·20*

*$P < 0.001$, significant compared with free diflunisal (Student's t-test).

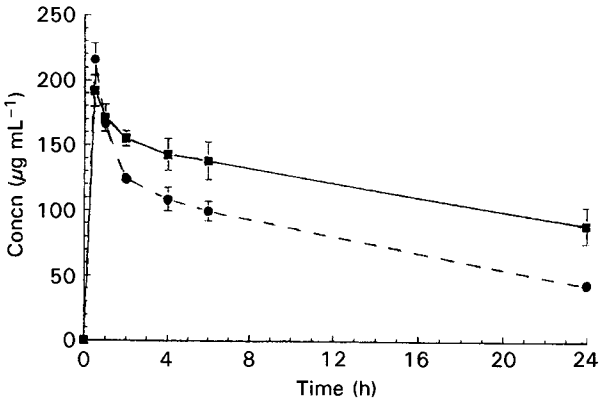

FIG. 4. Diflunisal plasma concentration–time curves after oral administration to rats of an aqueous suspension of PHEA microparticles containing diflunisal (■) and of an aqueous suspension of diflunisal (●).

plasma concentration–time curve (AUC) calculated for PHEA microparticles, 2929·04 μg mL^{-1} h^{-1}, was greater than that calculated for the suspension of free drug, 2026·82 μg mL^{-1} h^{-1}, equivalent to an increase of bioavailability of 44·51%.

Conclusions

Microparticles obtained by gamma irradiation of PHEA showed features that could be exploited in the field of drug delivery. PHEA hydrogel had high swelling ability in aqueous media, and did not undergo degradation when incubated with pepsin and α-chymotrypsin solutions. Tests performed on laboratory animals established that PHEA microparticles were inert after oral administration to rats, thus suggesting their potential use as an oral drug-delivery system. PHEA microparticles were able to incorporate small molecules such as diflunisal and to release most of the drug at pH 6·8, i.e. in simulated intestinal fluid. Finally, pharmacological tests showed that incorporation of diflunisal in PHEA microparticles resulted in increased gastric tolerance and oral bioavailability, thus offering many advantages over conventional systems.

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