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Research article

Collagen microparticles: carriers for glucocorticosteroids

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Abstract

Collagen microparticles were evaluated as a carrier system for glucocorticoids, and their physicochemical characteristics were determined. The particles were prepared by emulsifying and cross-linking native collagen. Particles in a size range of about 10 μm were obtained. The particle charge was dependent on the pH. A positive charge resulted when the surrounding medium had a pH below 4.5 and a negative charge with a pH above 4.5. This charge determined the magnitude of the interaction with dissolved charged drugs. The positively charged drug, prednylidene diethylaminoacetate, bound significantly to the particles above pH 4.5, whereas the negatively charged prednisolone sodium phosphate was bound below this pH. Adsorption of uncharged lipophilic drugs such as hydrocortisone was largely independent of the pH. The adsorption isotherm for this drug was determined and found to follow a Langmuir adsorption isotherm. The release and stability of the microparticle system was tested with hydrocortisone only, because of its pH-independent binding properties to the particles. The liberation of this drug was not influenced by the pH of the release medium. Binding to the particles did not effect the stability of hydrocortisone. The results of this study demonstrate that collagen microparticles can be successfully used as a carrier system for lipophilic steroids. © 1998 Elsevier Science B.V.

Keywords: Microparticles; Collagen; Glucocorticoids; Surface charge; Drug adsorption; Solid state stability; Drug release

1. Introduction

Glucocorticoids are widely used for the treatment of a number of diseases of non-endocrine origin. Owing to their strong anti-inflammatory actions, they have been used for rheumatism, asthma, inflammatory bowel diseases, as well as for the treatment of dermatitis and allergies. The prime obstacle for conventional administration is the dose-dependent systemic side-effects. Selective delivery of drugs to specific target sites would reduce the necessary dose of a given drug while still achieving an effective local concentration. As a result, the therapeutic efficacy would be increased.

The use of particulate drug carriers is a possible strategy to achieve site-specific drug delivery, and to decrease undesirable interactions at other body sites. In recent years, microparticles have emerged as one of the most promising controlled release dosage forms. Several microparticle-based products for parenteral use are commercially available (for example, Enantone®, Decapeptyl Depot®, Pravidel®). These products have the advantage of being injectable as well as allowing the incorporation of sensitive drugs. When employed as an oral delivery system, the polymer matrix will protect labile drugs from degradation in the stomach, and the active principle is released by polymer erosion or degradation in the intestine. Furthermore, it was shown, that microparticles exhibit a tendency to accumulate in inflamed tissues of the body [1,2].

Crohn's disease and ulcerative colitis are the major

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forms of chronic inflammatory bowel diseases that may also affect any other parts of the gastrointestinal tract and multiple extraintestinal organs. Since the pathogenesis of these diseases remain unknown, the aim of the conventional therapy is to suppress the inflammatory symptoms. Corticosteroid agents are routinely used for medical intervention. To improve the therapeutic efficacy of these drugs, in the present study microparticles were investigated as potential mucoadhesive carriers for glucocorticosteroids employed in the treatment for these diseases. Such a carrier system could be an alternative to current commercial formulations for treating inflammatory bowel diseases.

Collagen is an interesting natural material for the preparation of microparticles. Collagen is frequently used as a wound healing material [3], and it is known to be highly biocompatible [4]. Collagen has been used in the preparation of injectable hydrogel matrices [5], biocompatible implants (Zyderm® Collagen), carrier systems for ocular applications [6], and flexible fleeces for the treatment of wounds (Opragen®).

The objective of this investigation was to study collagen microparticles as carriers for steroidal anti-inflammatory drugs. The parameters for drug loading were estimated for hydrocortisone, prednisolone sodium phosphate, and prednyliden diethylaminoacetate. The adsorption isotherms as well as the drug release and stability were investigated with hydrocortisone only, since this drug showed a high degree of binding as well as pH-independent binding to the particles.

2. Materials and methods

2.1. Chemicals and solvents

Native collagen from calf skin was provided in the form of an aqueous dispersion (0.75% w/v) by Lohmann Therapie Systeme (Andernach, Germany). Hydrocortisone, prednisolone phosphate, prednylidene diethylaminoacetate, acetonitrile, hydrochloric acid, sodium chloride, sodium dihydrogenphosphate, sodium monohydrogenphosphate, glutaraldehyde solution (25% v/v), and sodium hydroxide were obtained from Merck (Darmstadt, Germany), sorbitan monolaurate 20 (Span® 20) from ICI Specialty Chemicals (Essen, Germany). Ethanol 96%, 2-propanol and hydrogen peroxide solution (30% v/v) were supplied by Aug. Hedinger (Stuttgart, Germany). Liquid paraffin was purchased from Vaseline Fabrik Wasserfuhr (Bonn, Germany). All chemicals and solvents were of analytical or HPLC grade.

2.2. Preparation of collagen microparticles

Collagen microparticles were prepared according to a method previously described [7]. Briefly, 85 g native collagen dispersion (0.75% w/v) was emulsified in 250 g of liquid paraffin containing 10 g sorbitan monolaurate 20 (Span® 20) using a rotor/stator type homogenizer (Ultraturrax, IKA-WERK, Staufen, Germany) at 6000 rpm for 10 min. For cross-linking, 3 g glutaraldehyde solution was added to the emulsion with a blade stirrer RW 18 (IKA-WERK, Staufen, Germany) at 300 rpm. After 12 min, the cross-linking reaction was interrupted by the addition of 4 g hydrogen peroxide solution and the mixture was stirred for another 15 min. The emulsion was then diluted with 50 ml 2-propanol and centrifuged for 5 min at 1500 rpm (Beckman GPR Centrifuge, Beckman, Paolo Alto, CA, USA). The obtained sediment was suspended in a mixture of 100 ml water and 100 ml 2-propanol and again centrifuged. This purification procedure was repeated for a second time. The collagen microparticles were then purified by centrifugation (5 min at 1500 rpm) and washed twice with water.

2.3. Microparticle size

The microparticle size was measured by centrifugal sedimentation analysis using a centrifugal automatic particle analyzer (CAPA-500, Horiba, Kyoto, Japan). A rotational frequency of 500 rpm was applied. The amount of particles sedimented was determined by measuring the transmission at 530 nm. The lyophilized microparticles were suspended in 200 parts (w/v) of water before analysis.

2.4. Surface charge of collagen microparticles

The surface charge of collagen microparticles was determined by measuring the electrophoretic mobility. A Lazer Zee Meter™ Model 501 (Penkem, Bedford Hills, NY) was used. To investigate the pH dependency of the zeta potential, the measurements were performed in sodium chloride solutions (0.09% w/v) ranging in pH from 2 to 10. The pH was adjusted using appropriate amounts of hydrochloric acid and sodium hydroxide solution, respectively. The concentrations of the employed suspensions were always 1% (w/v). The measured values were corrected to a standard reference temperature of 20°C.

2.5. Binding experiments

2.5.1. Basic binding experiments

In general, the drug was adsorbed onto the surface of previously formed microparticles. Therefore, an aqueous microparticle suspension was incubated with a drug solution in micro test tubes (Brand, Wertheim, Germany). The mixture was allowed to equilibrate for 3 h at 20°C by shaking with a thermomixer (Model 5436, Eppendorf, Hamburg, Germany). After adsorption, the suspensions were centrifuged at $2500 \times g$ (Centrifuge 5417, Eppendorf, Hamburg, Germany) for 30 min. Unbound drug was measured in the supernatant and the amount of adsorbed drug was calculated. The drug content of the supernatant was determined either by UV-spectrophotometry, or in the case of hydrocortisone, by HPLC.

2.5.2. pH-dependency of drug adsorption

The adsorption experiments were performed at various pH values. The drugs hydrocortisone, prednisolone phosphate, and prednylidene diethylaminoacetate were studied. The pH was adjusted to 2, 4.5, or 6.5 using appropriate amounts of hydrochloric acid. Ethanolic (250 μl) (20% v/v) drug solution (20 mg/ml) was added to 1.0 ml of the microparticle suspension (about 4 mg particles per ml).

2.5.3. Determination of the adsorption isotherm of hydrocortisone microspheres

Ethanolic solutions with different amounts of hydrocortisone (1–6 mg/ml) were added to a 1% (w/v) aqueous microparticle suspension. The composition of the adsorption media was maintained constant (20% ethanol, 80% water), and the pH was adjusted with hydrochloric acid to 4.5. The microparticles were centrifuged after incubation in these media for 3 h using a Centrifuge 5417 (Eppendorf, Hamburg, Germany) at $2500 \times g$ for 30 min. After centrifugation, the drug-loaded microparticles were lyophilized (Lyovac GT2, Leybold Heraeus, Hürth, Germany), and the loading efficiency was determined as described below (HPLC assay, Section 2.6).

2.6. Stability of lyophilized hydrocortisone microspheres

Lyophilized microparticles with an initial drug content of 3% (w/w) hydrocortisone, and lyophilized pure hydrocortisone were stored at 20, 30, and 40°C. A sample of each batch was extracted at intervals of 4 weeks and the drug content was determined as described below (HPLC assay, Section 2.8.2).

2.7. In vitro drug release of hydrocortisone microspheres

The desorption of hydrocortisone from collagen microparticles was studied using a diffusion cell system (Fischer, Frankfurt, Germany). The system consisted of a donor chamber, an acceptor chamber, and a polycarbonate membrane (Millicell®-PCF, 4 μm pore size, Millipore, Bedford, MA), which was fixed between the half-cells. Either 7 ml of a 0.2 M phosphate buffer solution (pH 5, 6, 7 or 8), or 7 ml of a hydrochloric acid solution (pH 2) were placed into the donor and the acceptor cell. The microspheres were introduced into the donor chamber together with the respective medium. The employed microparticle concentration was 0.56 mg/ml containing 58.1 μg/ml hydrocortisone. As a control, a simple aqueous drug solution (55.0 μg/ml) was used to determine the maximal diffusion rate of hydrocortisone across the polycarbonate membrane. The experiments were carried out at 37°C under continuous stirring. After 0, 15, 30, 45, 60, 120 and 240 min, samples of 0.5 ml were taken from the receiver chamber and replaced by an equal volume of drug-free solution. The samples were diluted with water and assayed by HPLC.

2.8. Analytical methods

2.8.1. High performance liquid chromatography

The concentration of hydrocortisone was measured using an isocratic HPLC method. The HPLC system consisted of a Waters® 600 Multisolvent Delivery System (Millipore, Milford, MA), an automatic sample injection module WISP® Model 712 (Millipore, Milford, MA), a Degaser ERC-3512 (Erma, Tokyo, Japan), a UV-VIS Detector Model L4250 (Merck, Darmstadt, Germany), a Kontron PC-Integration unit (Kontron, Neufahrn, Germany), a guard column (4×4 mm i.d.) and an analytical column (125×4 mm i.d.), both prepacked with 5 μm RP-18 100 Superspher® (Merck, Darmstadt, Germany). The mobile phase consisted of acetonitrile and water (1:1), and was used at a flow rate of 1.25 ml/min. Hydrocortisone was detected by measuring the absorption at 250 nm.

2.8.2. Sample preparation for the HPLC assay

The drug content of the microparticles was measured after their enzymatic degradation. Lyophilized microparticles (between 4.0 and 8.0 mg) were accurately weighed and incubated with 1.5 ml of a pepsin solution (0.7 mg/ml in 0.01 N HCl) for 30 min at 37°C in micro test tubes (Brand, Wertheim, Germany) using a Thermomixer (Model 5436, Eppendorf, Hamburg, Germany). The microparticles were completely disintegrated and dissolved using this procedure. The obtained mixture (100 μl) was then diluted with water to 1.0 ml, and 50 μl of that dilution was injected in the HPLC system.

2.8.3. UV-spectrophotometry

For the basic binding experiments (230) prednisolone phosphate, prednylidene diethylaminoacetate, and hydrocortisone were analyzed by UV-spectroscopy. The samples were diluted with appropriate amounts of water to keep the UV-absorption in the range of Beer–

Lambert's law. The measurements were performed at 250 nm using a Hitachi U-3000 (Hitachi, Tokyo, Japan), spectrophotometer.

3. Results and discussion

3.1. Preparation

Collagen microparticles were prepared by emulsifying and cross-linking the native collagen [7]. The flow diagram for the preparation process is shown in Fig. 1. In the first step, a water-in-oil emulsion was made using an Ultraturrax. The resulting aqueous collagen droplets were cross-linked by glutaraldehyde. The mechanism of the cross-linking reaction is not clearly defined and has created controversy in the literature [8]. It was proposed that glutaraldehyde exists in form of an aldol condensate in aqueous systems. This α-β unsaturated dimer can react with the amino and hydroxyl groups of proteins. The reaction leads to the covalent cross-linkage of the collagen fibrils. The process of cross-linking was stopped by the addition of hydrogen peroxide. Hydrogen peroxide oxidizes the dialdehyde to the corresponding acid, which is not able to react with the functional groups of the collagen. In the final step, the obtained microparticles were purified by washing and centrifugation procedures.

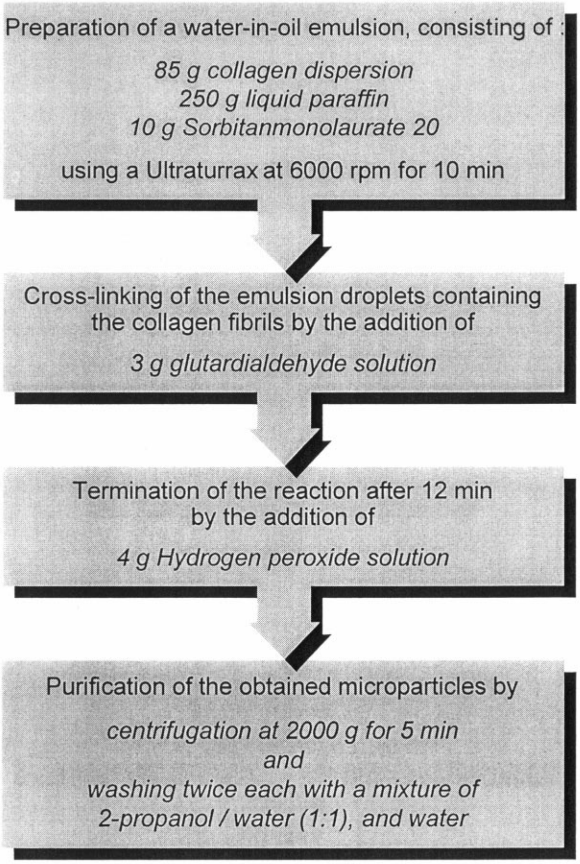

Fig. 1. Schematic diagram of the preparation of collagen microparticles.

The particles made by this process have a mean particle size of 10 μm. They can be produced in yields of about 60% relative to the total amount of collagen employed. The remaining 40% was not cross-linked by glutaraldehyde within the cross-linking time of 12 min and was removed during purification. In order to increase the yields, it was necessary to prolong the cross-linking time. However, the prolongation of reaction time also leads to an increase in particle size (about 40 μm). For this reason, the optimal cross-linking time for both, acceptable sizes as well as yields, was 12 min.

3.2. Surface charge of collagen microparticles

The surface charge of particles has a substantial influence on the stability of suspensions, on the interaction of microparticles with charged substances, as well as on the adherence of drug delivery systems onto biological surfaces. In our case, the reason for measuring the surface charge was to characterize the interaction of collagen microparticles with dissolved drugs present in the surrounding medium. The surface charge of collagen microparticles was determined by measuring their electrophoretic mobility. Fig. 2 shows the zeta potential of collagen microparticles as a function of pH ranging from pH 2 to 8. At pH 4.5 (P_0; point of zero charge) the particles are nearly uncharged. Below this pH the charge was positive and above this pH it was negative. The isoelectric point P_I, of collagen is about 9.4 [9]. Consequently, the P_0 of collagen microparticles

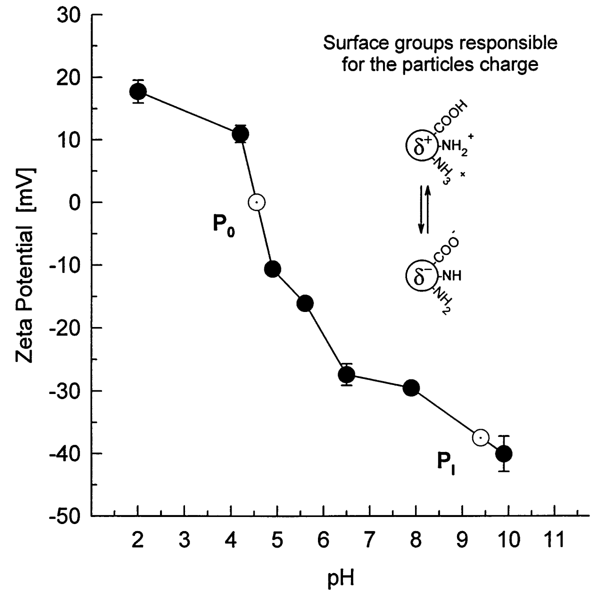

Fig. 2. Zeta potential of collagen microparticles as a function of pH (mean ± S.D.; $n = 10$); P_0 = point of zero charge of collagen microparticles; P_I = isoelectric point of collagen.

was shifted to a lower pH (4.5). Due to the reaction of the cross-linking agent, glutaraldehyde, with the amino groups of collagen, the number of protonizable amino groups was reduced. In addition, if the dialdehyde was bound to only one functional group of the collagen fibrils, the unbound aldehyde group could be oxidized by hydrogen peroxide to the corresponding acid. Consequently, new carboxylate groups would be introduced into the collagen molecules. Both reactions would lead to the alteration of the surface charge distribution of the collagen microparticles (reduction of positive charge) resulting in a lower pH for P_0.

3.3. Drug adsorption

The influence of pH on the drug adsorption onto collagen microparticles is shown in Fig. 3. The surface groups of microparticles, and the functional groups of drugs are also presented in the figure. The adsorption of the charged drugs was strongly influenced by the pH of the loading media, and the pH dependence of adsorption correlated well to the corresponding zeta potential of the particles. As expected, the charged drugs bound well to the oppositely charged microspheres surfaces. The adsorption of the uncharged drug, hydrocortisone, was largely independent of pH, although at pH 4.5 (pH $= P_0$) the highest amount of hydrocortisone was adsorbed to the particles. Binding of this drug was mainly governed by hydrophobic interactions with the surface of the collagen microparticles. In order to establish the adsorption isotherm of hydrocortisone, lyophilized hydrocortisone microparticles were degradated by pepsin prior to analysis. A typical chromatogram after particle digestion by pepsin is shown in Fig. 4. The binding of hydrocortisone onto the collagen microparticles was found to follow a Langmuir type of adsorption (Fig. 5, linearized Fig. 6).

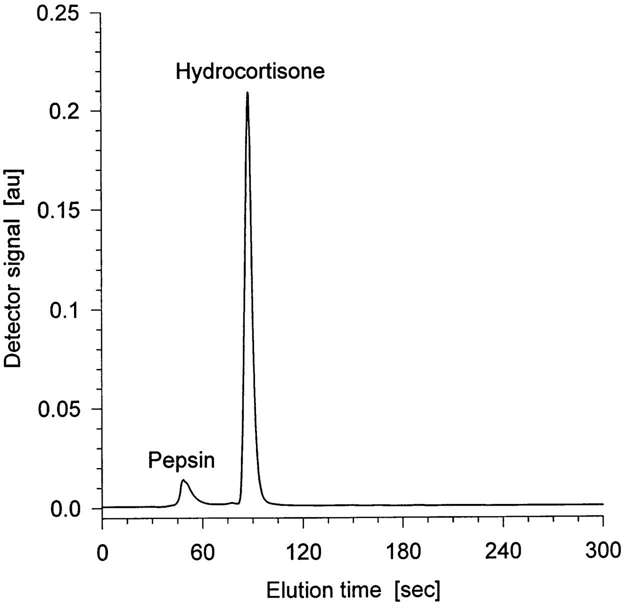

Fig. 4. Typical reversed-phase HPLC chromatogram of the separation of hydrocortisone from pepsin.

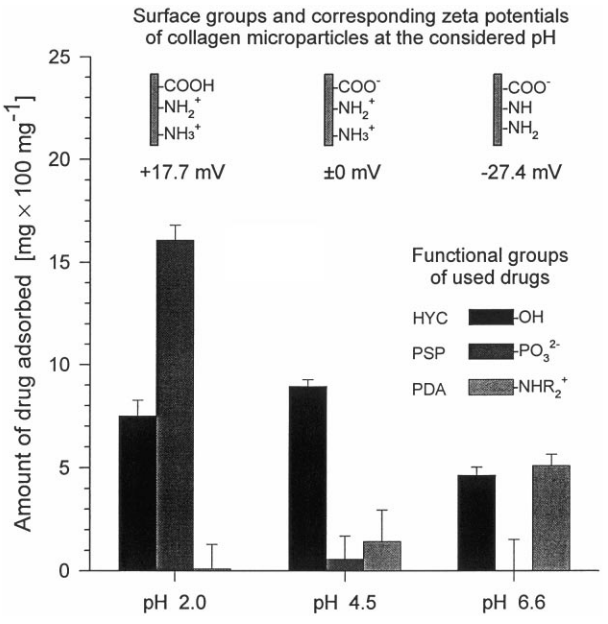

Fig. 3. Influence of pH on the adsorption of hydrocortisone (HYC), prednisolone sodium phosphate (PSP), and prednyliden diethylaminoacetate (PDA) onto the surface of collagen microparticles (mean ± S.D.; $n = 3$).

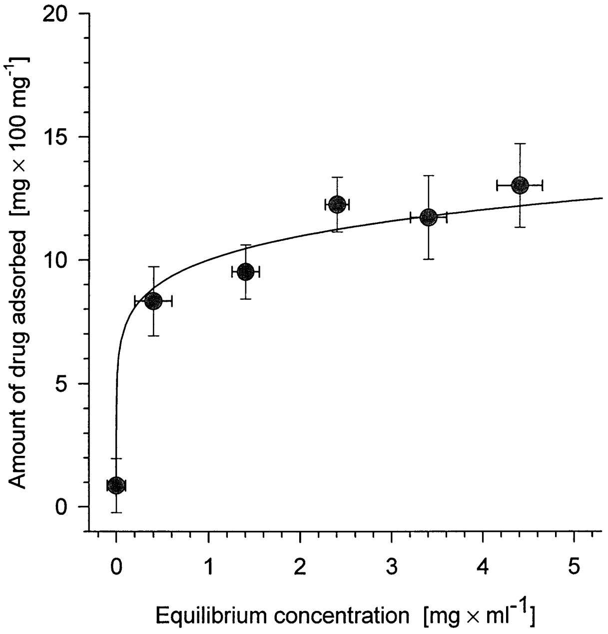

Fig. 5. Adsorption isotherm of hydrocortison onto collagen microspheres (mean ± S.D.; $n = 3$).

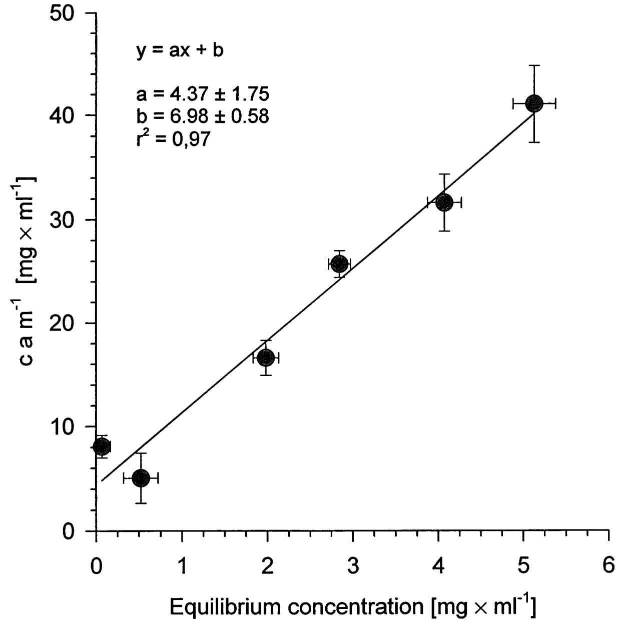

Fig. 6. Langmuir plot corresponding to the isotherm shown in Fig. 5 (mean ± S.D.; $n = 3$).

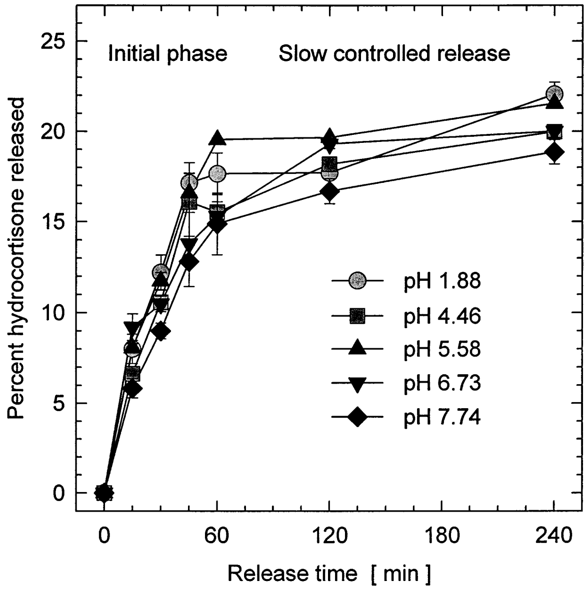

Fig. 7. Effect of pH on the release of hydrocortisone from collagen microparticles (mean ± S.D.; $n = 3$).

3.4. Drug release

As shown in Fig. 7, the release of hydrocortisone was not influenced by the pH of the release medium. About 20% of the adsorbed drug was released within 4 h. The release kinetics were characterized by an initial period of rapid release during the first 60 min followed by a nearly constant slow release rate. During the initial release period, the kinetics could be described by the square root law [$M(t) = kt^{1/2}$] [10,11]. It can be assumed, that initially the unbound portion of drug in the microparticles suspension diffused through the polycarbonate membrane. After this stage, desorption of microsphere-bound drug becomes the limiting route for the drug transport across this membrane. During the second phase, it was not possible to fit the obtained data unambiguously to any simple known release model as shown by the correlation coefficients for the different models in Table 1.

3.5. Stability of lyophilized hydrocortisone microspheres

The results of the long term stability of hydrocortisone in its pure form as well as adsorbed to collagen microparticles is given in Table 2. The obtained data demonstrate that binding to the collagen microparticles had no significant influence on the stability of hydrocortisone, and that the formulation was stable for 24 weeks.

3.6. Conclusion

Crohn's disease affects all parts of the alimentary tract, but mainly the lower illeum and the upper colon (cecum and ascending colon). Consequently, the administered medicine has to pass through the upper GI tract before the

Table 1
Correlation coefficients for statistical fit of Fig. 1 data to different model release systems

Correlation coefficient for the release of first 60 min					
Release equation	pH 1.88	pH 4.46	pH 5.58	pH 6.73	pH 7.74
Zero order	0.925	0.917	0.968	0.865	0.972
First order	0.930	0.919	0.973	0.873	0.975
Square root	0.983	0.960	0.983	0.985	0.979
Cubic root	0.924	0.866	0.992	0.969	0.988
Correlation coefficient for the release after 60 min					
Release equation	pH 1.88	pH 4.46	pH 5.58	pH 6.73	pH 7.74
Zero order	0.899	0.912	0.924	0.704	0.981
First order	0.899	0.914	0.924	0.706	0.982
Square root	0.838	0.957	0.869	0.784	0.998
Cubic root	0.899	0.912	0.924	0.704	0.981

Table 2
Thermal stability of microparticle preparations

Storage time (weeks)	Lyophilized hydrocortisone			Hydrocortisone adsorbed to microspheres		
	20°C (mean ± S.D.)	30°C (mean ± S.D.)	40°C (mean ± S.D.)	20°C (mean ± S.D.)	30°C (mean ± S.D.)	40°C (mean ± S.D.)
0	100	100	100	100	100	100
4	100.1 ± 0.5	101.0 ± 0.2	100.1 ± 0.5	96.3 ± 0.4	100.0 ± 0.8	99.3 ± 0.7
8	99.2 ± 0.6	99.8 ± 1.0	96.5 ± 0.4	99.7 ± 0.9	98.3 ± 0.2	96.3 ± 0.6
12	99.4 ± 0.3	99.1 ± 0.8	100.2 ± 1.0	98.0 ± 0.5	99.3 ± 0.8	96.3 ± 0.1
16	101.1 ± 0.5	98.1 ± 0.9	97.8 ± 0.2	96.7 ± 0.9	99.7 ± 0.6	99.3 ± 0.2
20	97.5 ± 0.6	97.7 ± 0.8	96.8 ± 0.4	99.7 ± 0.2	99.7 ± 1.0	96.0 ± 0.1
24	96.2 ± 0.4	99.7 ± 0.2	99.0 ± 0.5	96.3 ± 0.4	96.7 ± 1.0	96.0 ± 0.2

drug can interact with the inflamed tissue. The microspheres can be degraded by pepsin as shown in the analytical section, but the amount of this enzyme in the stomach is low in the fasted state. In this study, it was shown that collagen microparticles can be used as a carrier system for lipophilic steroids, and that the release of hydrocortisone was not influenced by the pH (between 2.0 and 7.8). For this reason, collagen microparticles loaded with a lipophilic uncharged steroid represent a promising formulation for the treatment of inflammatory bowel diseases.

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