International Journal of Pharmaceutics, 52 (1989) 101–108
Elsevier

IJP 01751

Evaluation of indomethacin nanocapsules for their physical stability and inhibitory activity on inflammation and platelet aggregation

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(Received 26 September 1988)
(Accepted 1 November 1988)

Key words: Indomethacin nanocapsule; Stability; Anti-inflammatory activity; Platelet aggregation; Isobutylcyanoacrylate; Transmission electron microscopy

Summary

Indomethacin nanocapsules were investigated for their physical stability and anti-inflammatory activity in the carrageenan paw oedema tests in rats and also for their inhibitory activity on platelet aggregation induced by ADP. From the physical stability view point, there was a marked difference for the extent of drug loss between the indomethacin nanocapsules which were stored for 12 months at ambient temperature when freeze-dried and in the form of suspension. The indomethacin content of the freeze-dried nanocapsules and in the suspension form decreased by 52.8% and by 17.5% respectively. Compared to free indomethacin the anti-inflammatory activity of indomethacin increased 10 times when it was prepared as nanocapsules and the inhibition of platelet aggregation also increased with nanocapsulated indomethacin. But empty nanocapsules also showed an inhibition in the platelet aggregation because of their isobutylcyanoacrylate content.

Introduction

In our previous study we have investigated the therapeutic activity of indomethacin liposomes and, according to the data, encapsulation of indomethacin into liposomes enhanced its therapeutic activity (Gürsoy et al., 1988).

On the other hand, nanocapsules are one of the other appropriate colloidal drug carrier systems due to their biodegradability and good stability (Kreuter, 1978). However, little is known about the therapeutic benefits of indomethacin-loaded nanocapsules. Indomethacin has side effects when used in large or repeated doses (Donnelly et al., 1967). Therefore it was of interest to see if the results obtained with liposome-encapsulated indomethacin were similar to those with indomethacin-loaded nanocapsules.

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The scope of the present investigation was to evaluate indomethacin nanocapsules for their physical stability and anti-inflammatory activity in rats and inhibitory effect on platelet aggregation in vitro relative to empty nanocapsules and free drug.

Materials and Methods

Preparation of the indomethacin nanocapsules

Indomethacin nanocapsules were prepared as previously described by Andrieu et al. (1986). Briefly, an ethanolic solution of indomethacin, isobutylcyanoacrylate monomer, EpiKuron 200, and benzyl-benzoate is added under magnetic stirring to an aqueous solution of Pluronic F 68. Nanocapsules are formed instantaneously by anionic interfacial polymerization of the monomer. Ethanol is removed under vacuum to obtain the following formula:

Indomethacin	0,04 g
(Sigma, 75015 Paris, France)	
Benzylbenzoate	2,0 ml
(Prolabo, 75011 Paris, France)	
EpiKuron 200	1,0 g
(Lucas Meyer, 62.2000 Hamburg, F.R.G.)	
Pluronic F68	1,0 g
(ICI, 92142 Clamart, France)	
Isobutylcyanoacrylate	0,5 ml
(Ethnor, 95580 Margency, France)	
Water	40 ml

The mean size of the nanocapsules as measured by a laser light scattering method (Nanosizer, Coultronics, Andilly, France) was 220 ± 50 nm. The initial indomethacin concentration was 1000 g/ml.

Stability tests

The physical stability of the indomethacin nanocapsules was measured by the extent to which encapsulated indomethacin was retained in the nanocapsules during storage in different conditions.

A known amount of nanocapsules suspension was placed into several vials and the vials were stored for a year at ambient temperature, at 4 °C in a desiccator, and at −30 °C and thereafter the samples were examined at 2 month intervals.

Investigation of the leaked indomethacin

A defined volume of nanocapsules suspension was centrifuged for 20 min at 20,000 rpm (Beckman Model J-21B Centrifuge). For the separation of leaked indomethacin from the bound one, supernatant was discarded and the centrifugation procedure was repeated, sedimented nanocapsules were dissolved in chloroform. Indomethacin content was assayed spectrophotometrically at 320 nm. Measurements were performed using the chloroform solutions of indomethacin nanocapsules as reference. Indomethacin lost from the nanocapsules was calculated with regard to the initial indomethacin contents of nanocapsules. The results were expressed in the percentage of the indomethacin lost from the nanocapsules.

On the other hand freeze-dried indomethacin nanocapsules were also investigated for their physical stability. For this purpose a certain amount of indomethacin nanocapsules suspension was placed into vials and after freeze-drying (Lyovac GT2, Leybold-Heraus) for 48 h at −10 °C the vials were sealed and maintained at ambient temperature, at 4 °C in a desiccator and at −30 °C. At 2 month intervals indomethacin nanocapsules were rehydrated to their original volume and centrifuged at 20,000 rpm to remove any leaked indomethacin, and the percent loss was calculated.

Preparation of platelet-rich plasma (PRP)

Venous blood was obtained from volunteers who had not received any medication known to affect platelet aggregation for at least one week. PRP was prepared by centrifuging the citrated blood sample at 1500 rpm for 12 min. The concentration of platelets in plasma was 150,000–350,000 mm^3.

Platelet aggregation test

Platelet aggregation was studied with the turbidimetric method of Born (1962) using an aggregometer (Model PAP-2A, Bio/Data Corporation, U.S.A.). Estimates were made from the recordings of light transmission.

Adenosine 5-diphosphate (ADP) (Agregatest, Diagnostica Stago, France) was used as an aggre-

gation agent and its concentration was adjusted in order to yield an aggregation curve for each PRP. The final concentration of ADP was between $1.1. \times 10^{-6}$ and 2.3×10^{-6} M. The inhibition (percent) of ADP induced platelet aggregation was calculated according to Kobayashi and Didisheim (1973). The maximum increase in light transmission after addition of ADP to control PRP was assigned the value of 100% and the inhibition percent was determined by comparing the value with those PRP to which empty nanocapsules or indomethacin nanocapsules had been added. Also aggregation studies were carried out on the PRP preincubated with the samples mentioned above for 30 min before addition of ADP.

Ten aggregation studies on different PRP's were undertaken for each indomethacin nanocapsules concentration.

Sample preparation for transmission electron microscopy (TEM)

The effect of indomethacin nanocapsules and empty nanocapsules on the morphology of aggregated platelets was investigated by TEM. The final plasmatic concentrations of indomethacin nanocapsules were 50 μg/ml and 10 μg/ml. The concentrations of empty nanocapsules were adjusted according to the indomethacin nanocapsules, by the same dilutions. TEM studies were carried out on PRP samples preincubated with 10 μg/ml indomethacin nanocapsules and empty nanocapsules for 30 min at 37 °C.

Nanocapsules were added to PRP samples and were aggregated by ADP and thereafter 1% glutaraldehyde in Millonig phosphate buffer (pH 7.4) was added (Hayat, 1970) and the samples were centrifuged at 3500 rpm for 15 min to yield the platelet pellet. First the platelet pellet was fixed with 3% glutaraldehyde in phosphate buffer for 30 min, then the pellet was washed with phosphate buffer and postfixation was carried out using 1% osmic acid in phosphate buffer for 1 h. After dehydration in a progressive acetone series, the specimens were embedded into Vestopal W, ultrathin sections were contrasted with uranyl acetate and lead citrate (Reynold's solution) and examined with TEM 9S2 (Zeiss).

Animal experiments

The study was carried out on male Wistar strain albino rats (120–180 g), housed in a well controlled environment with a 12 h light–dark cycle. The test procedure was conducted between 09.00 and 15.00 h during the light phase.

On the day of experiment, the rats were fasted for 18 h but had free access to water. Immediately before the i.p. injection of each compound, the basal volume of the right hind paw was measured by means of a mercury plethysmometer (Ugo Basile). Afterwards, the rats were injected with one of the following compounds at a volume of 0.1 ml/100 g b.wt.: (i) saline; (ii) empty nanocapsules (0.3 mg/kg); (iii) a suspension of free indomethacin (3 mg/kg, i.v.) indomethacin nanocapsules (0.3 mg/kg). The concentration of empty nanocapsules was adjusted according to the indomethacin concentration of indomethacin nanocapsules. Thirty minutes after the treatment, carrageenan (0.05 ml of 1% suspension in saline) was injected intraplantarly into the right hind paw of each rat to induce inflammation. Paw volumes up to the ankle joints were measured before and at hourly intervals for 6 h following carrageenan administration. The basal volume of each rat paw was taken as 100% and variations from this volume were given as percent difference.

Statistics

The data were analyzed for significance of difference by Student's t-test.

Results and Discussion

Physical stability

As shown in Table 1 when indomethacin nanocapsules were stored as suspension at ambient temperature the loss of indomethacin was 17.5% after 12 months. However, there was no loss of indomethacin from the nanocapsule suspensions maintained at 4 °C and at −30 °C for 12 months.

On the other hand, at ambient temperature there was a marked difference in the extent of drug loss between the indomethacin nanocapsules that were stored as suspension and those in

TABLE 1
Loss of indomethacin from nanocapsules

Periods of storage (months)	Suspension		Freeze-dried	
	Ambient temp.	4°C	Ambient temp.	4°C
2	0	0	8.5±3.9	0
4	3.0±2.1	0	26.0±2.8	0
6	4.0±3.5	0	50.5±4.7	4.16±2.4
8	8.0±1.9	0	53.2±4.0	7.0 ±2.2
10	13.2±2.9	0	54.0±5.0	9.5 ±3.5
12	17.5±2.7	0	52.8±2.8	9.3 ±5.2

Percent loss of indomethacin from nanocapsules during storage for a year at ambient temperature and at 4°C as suspension and in freeze-dried form. Means of 6 experiments ±S.D.

TABLE 2
Inhibition of platelet aggregation

μg/ml PRP	Inhibition (%)	
	Empty nanocapsules	Indomethacin nanocapsules
10.0	61.9±4.9	63.5±5.5
5.0	39.5±4.4	46.7±6.2
2.5	25.6±6.6	40.3±4.6

Percentage of the inhibition of platelet aggregation induced by ADP after the addition of empty nanocapsules and indomethacin nanocapsules to PRP. Both indomethacin nanocapsules and empty nanocapsules dilutions were carried out according to the concentration of indomethacin in indomethacin nanocapsules. Means of 10 experiments ±S.D.

freeze-dried form. After the rehydration of the freeze-dried indomethacin nanocapsules that were stored at ambient temperatures, losses of 8,5%, 26.0% and 50.5% indomethacin were found after 2, 4 and 6 months respectively. However, after 12 months percent loss of drug did not change. But when freeze-dried nanocapsules were stored at 4°C, the drug loss was only 9.3% at the end of 12 months.

However, there was no loss of indomethacin

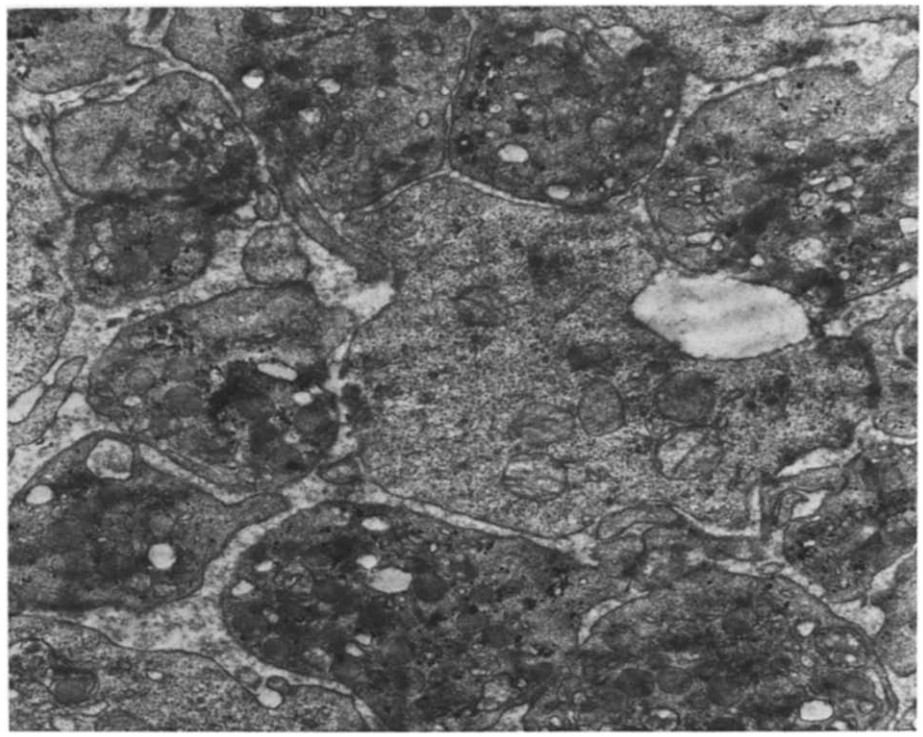

Fig. 1. Transmission electron micrographs of platelets after ADP-induced aggregation in the presence of empty nanocapsules (50 μg/ml) (A), and indomethacin nanocapsules (B) (50 μg/ml) ×9500.

from nanocapsules stored at −30 °C both as suspension or in freeze-dried form for a year.

It was of interest to observe that at the end of one year the percentage of indomethacin loss was high when nanocapsules were stored at ambient temperature in freeze-dried form. Since in this form the nanocapsules are free-flowing dry powder a good stability was expected. But these results directed us to suggest that some alterations may occur in the capsule wall structure during storage and when the freeze-dried nanocapsules were rehydrated, indomethacin was easily released from the nanocapsules.

Platelet aggregation and morphology

The ADP induced aggregation of platelets was investigated in PRP in the presence of indomethacin nanocapsules and empty nanocapsules at 50 μg/ml concentration. A 100% inhibition of the aggregation curve was observed in the in vitro studies. These results were confirmed by TEM. In the TEM micrographs the platelets treated with indomethacin nanocapsules and empty nanocapsules appeared intact, there was no aggregation and release (Fig. 1).

On the other hand decreasing the plasmatic concentration of indomethacin nanocapsules and empty nanocapsules to 10 μ/ml gave almost a similar inhibition percent of the maximum amplitude of the aggregation curve (Table 2). But TEM examinations of these samples showed some aggregated and released platelets with pseudopods (Fig. 2). However, incubation of PRP with indomethacin nanocapsules and empty nanocapsules in 10 μg/ml concentration for 30 min did not change the percent inhibition of aggregation curve in vitro. But their TEM studies showed different results. In the presence of empty nanocapsules platelets were partly aggregated and released (Fig. 3A). But with indomethacin nanocapsules platelets were aggregated though only some of them were released: in addition endotubular system

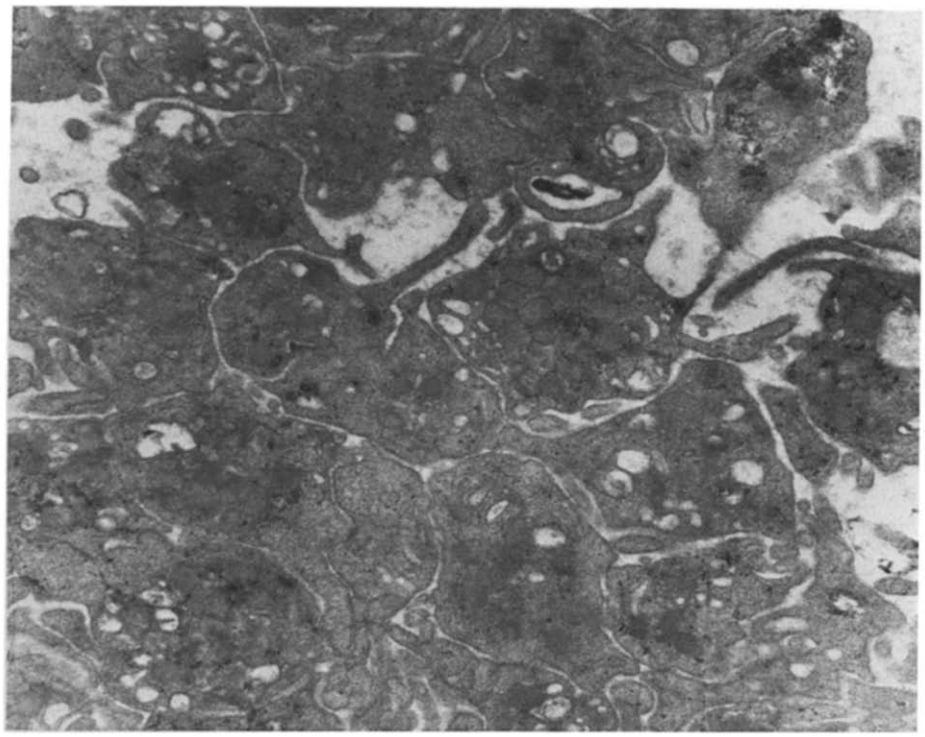

Fig. 2. Transmission electron micrographs of platelets after ADP-induced aggregation in the presence of empty nanocapsules (10 μg/ml) (A) and indomethacin nanocapsules (10 μg/ml) (B). ×6000.

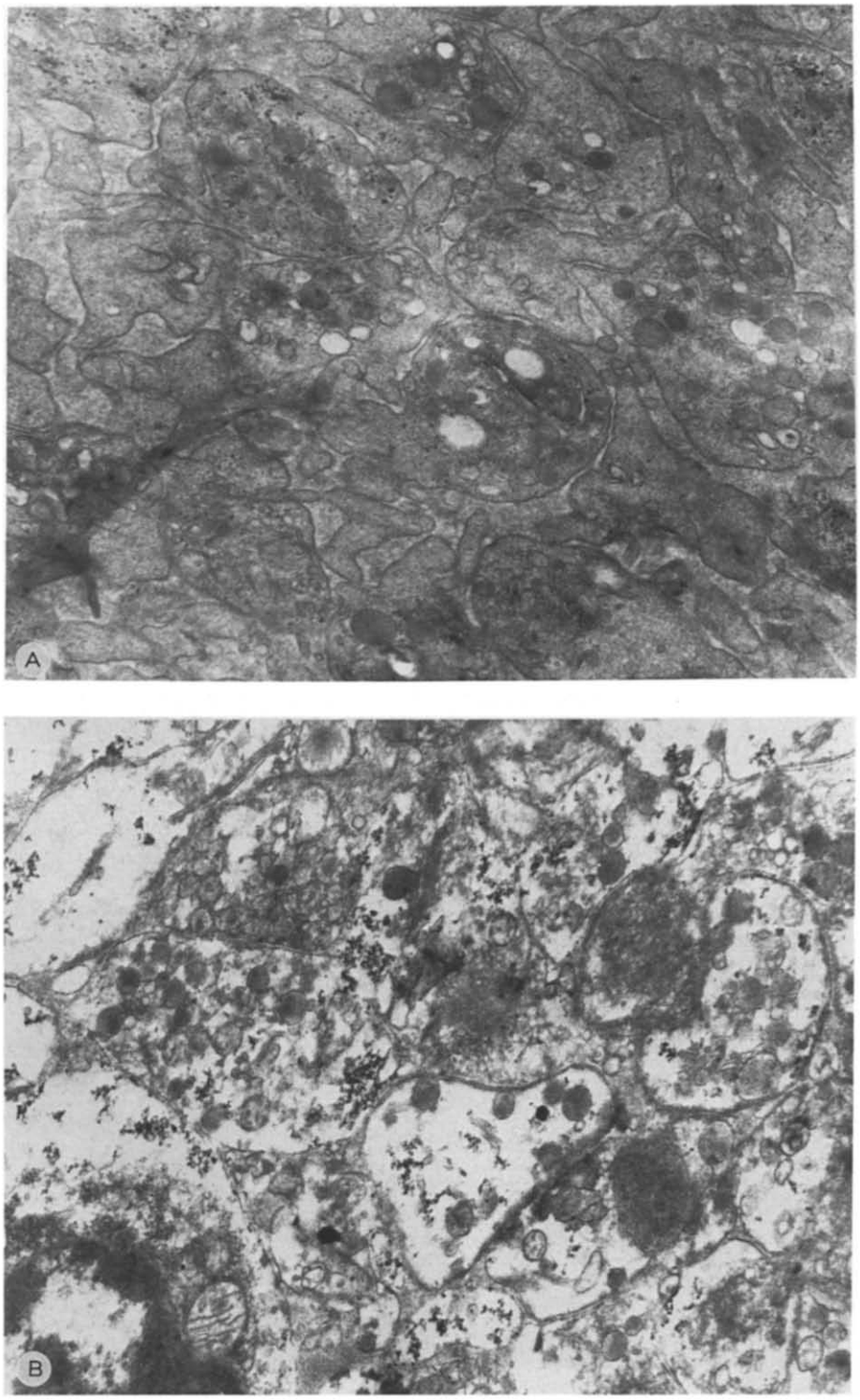

Fig. 3. A: transmission electron micrographs of platelets aggregated by ADP after 30 min incubation with empty nanocapsules (10 μg/ml) $\times$9500. B: transmission electron micrographs of platelets aggregated by ADP after 30 min incubation with indomethacin nanocapsules (10 μg/ml). $\times$9500.

damage, cytoplasmic matrix lysis and fragmentation of the platelet membrane were observed (Fig. 3B). Therefore after 30 min incubation the morphological difference was obvious between platelets which were treated by indomethacin nanocapsules and those that were treated by empty nanocapsules. Indomethacin had a toxic effect on platelets. As shown in Table 2 the inhibition of ADP-induced aggregation of platelets in the presence of indomethacin nanocapsules and empty nanocapsules was concentration dependent and a dose-related inhibition of aggregation was observed. Especially for empty nanocapsules the decrease of the concentration from 10 to 5 μg and to 2.5 μg yielded significant differences ($P < 0.001$) for the inhibition of ADP-induced aggregation of platelets. As the plasmatic concentration of empty nanocapsules decreased the percent inhibition of platelet aggregation was also decreased.

But the percent inhibition of platelet aggregation in the presence of indomethacin nanocapsules was significant ($P < 0.02$) at 5.0 μg/ml and 2.5 μg/ml. There was a significant difference ($P < 0.001$) for the percent inhibition of platelet aggregation in vitro, between the platelets which were treated by indomethacin nanocapsules and those treated by empty nanocapsules at 5.0 μg/ml and 2.5 μg/ml concentrations. The inhibition was high with indomethacin nanocapsules. However, the empty nanocapsules had an inhibitory effect on platelet aggregation induced by ADP. Taking into consideration that this effect might develop from the substances used to produce the nanocapsules, all these substances were examined in the final plasmatic concentrations that were used to produce nanocapsules.

Except isobutylcyanoacrylate the other substances had no inhibitory effect on platelet aggregation but isobutylcyanoacrylate had a 80% inhibition. Therefore it is suggested that inhibition of the platelet aggregation by empty nanocapsules was produced by isobutylcyanoacrylate.

On the other hand free indomethacin in 200 μg/ml plasmatic concentration can inhibit platelet aggregation and this inhibition is time-dependent. As the incubation time was prolonged the percentage of the inhibition increased. After 45 min incubation of the free indomethacin with PRP 50% inhibition of platelet aggregation was observed (Gürsoy et al., 1988). But with indomethacin nanocapsules even 2.5 μg/ml plasmatic concentration (without preincubation) 40.3% inhibition was observed. Although empty nanocapsules showed an inhibitory activity to a certain degree, indomethacin itself had also an inhibitory activity. Even such a small dose can produce an inhibition. This activity is the result of nanocapsulation of the drug.

Anti-inflammatory effect

A single dose of free indomethacin, 3 mg/kg, strongly inhibited carrageenan-induced oedema. The inhibition started at 1 h after carrageenan injection and lasted for 4 h. On the other hand, 0.3 mg/kg indomethacin-loaded nanocapsules also inhibited the oedema, but in a different manner. Its inhibitory activity started 4 h after carrageenan injection and lasted 2 h (Fig. 4).

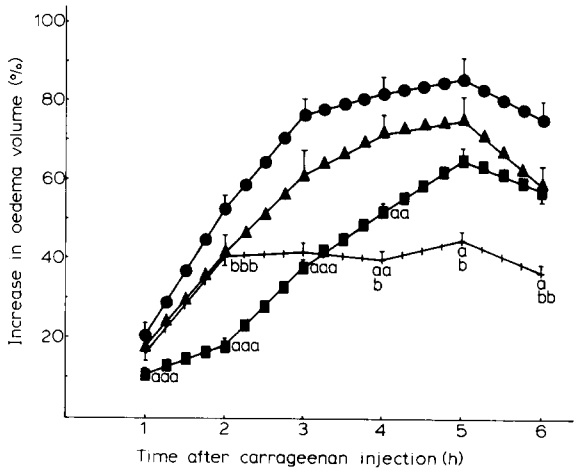

Fig. 4. Effects of indomethacin-loaded nanocapsules, empty nanocapsules and free indomethacin on the development of oedema after intraplantar injection of carrageenan. The test compounds were given i.p. 30 min before the carrageenan injection. The basal volume of each rat paw was taken as 100% and variations from this value were given as percent difference. Vertical bars denote S.E.M.; (●), saline (7); (▲), empty nanocapsules (9); (1) 3 mg/kg free indomethacin (15); (+) 0.3 mg/kg indomethacin-loaded nanocapsules (7). The number of rats used is given in parentheses. $^{a}P < 0.005$, $^{aa}P < 0.01$, $^{aaa}P < 0.001$ vs saline group; $^{b}P < 0.05$, $^{bb}P < 0.02$, $^{bbb}P < 0.001$ vs free indomethacin group.

As a conclusion, when indomethacin was nanocapsulated its anti-inflammatory activity increased 10 times, compared to free indomethacin. On the other hand empty nanocapsules showed anti-aggregating activity due to isobutylcyanoacrylate. But when indomethacin was incorporated in the nanocapsules its inhibitory activity increased. Even with a very small dose such as 2.5 μg/ml an inhibitory activity was observed.

When freeze-dried indomethacin nanocapsules were stored at ambient temperature for 6 months, their indomethacin contents decreased by 50%.

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