Preparation of Gelatin Nanocapsules and Their Pharmaceutical Characterization

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Abstract: A production process is described that yields nanocapsules instead of nanoparticles. The process allows the encapsulation of lipophilic drugs such as triamcinolone acetonide with an *O/W* emulsion system. The capsular structure of the products was confirmed by transmission and scanning electron microscopy. The nanocapsules displayed a mean size of 141 ± 47 nm to 523 ± 340 nm (mean ± S.D.) and a drug content of 7 % to 15.4 % w/w. Drug release experiments showed that the encapsulated triamcinolone acetonide was released more slowly than micronized drug crystals. A more pronounced retarded release could be achieved by raising the pH during the hardening reaction or by encapsulating cholesterol into the nanocapsules.

Gelatin nanoparticles as a colloidal drug delivery system have attracted much recent interest because of their low antigenicity (1), their accumulation in possible target organs after intravenous administration (2) and their biodegradability (3). Surprisingly only a few reports exist concerning their drug carrying capacity, drug release, size distribution and interior structure (3–5). Furthermore, the reported production procedures are only applicable to hydrophilic drugs (5, 6), which additionally have to be stable against higher temperatures (6).

The present investigation describes a procedure that allows the production of nanocapsules instead of nanoparticles and compares their features with nanoparticles produced according to a method published by Oppenheim (7). In order to alter the drug release, the influence of cholesterol and of a higher pH during the hardening reaction between gelatin and glutardialdehyde was investigated.

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Materials and Methods

Materials

Triamcinolone acetonide was supplied from Heyden, West Germany. ^{3}[H]-Triamcinolone acetonide was purchased from New England Nuclear, USA with a specific activity of 32.8 Ci/mmol. Swine skin gelatin, type II was obtained from Sigma USA.

All other reagents were of reagent grade quality and obtained from Merck, West Germany.

Methods

Preparation of Nanocapsules and Nanoparticles

To 40 ml of gelatin solution (0.5 %, w/w) 2 ml chloroform was added and the mixture emulsified with an ultrasonic device (Branson Sonifier Cell Disrupter B 15, Branson Schallkraft, West Germany). The drug (triamcinolone acetonide 20 mg mixed with trace amounts of ^{3}[H]-triamcinolone acetonide) was dissolved in chloroform before dispersing it into the gelatin solution. After 30 min sonification of the gelatin – chloroform mixture, 16 ml of sodium sulfate solution (20 %, w/w) and 1 of isopropanol were poured into the emulsion to induce desolvation of gelatin. The formed capsule walls were hardened by addition of 0.8 ml glutardialdehyde solution (25 %, w/w) for another 10 min with continuous sonification. The resulting suspension of nanocapsules was poured into 10 ml of sodium metabisulfate solution (12 %, w/w) and stirred with a magnetic bar to stop the hardening reaction of the aldehyde (30 min). The nanocapsules were centrifuged (19 500 x g, 35 min) and washed three times with water. After the last washing the capsules were redispersed in water and the suspension was freeze-dried (freeze-drier GT 2, Heraeus, West Germany) for about 2 days at a maximum pressure of 1–2 bar.

Nanoparticles were produced according to Oppenheim (7) from an aqueous drug solution of 26.4 µg/ml.

Size Distribution

The size distribution of the nanocapsules was determined by scanning electron microscopy (SEM S 450, Hitachi, Deshi, West Germany). A small amount of dispersed spheres was nebulized, captured onto aluminium foil, dried overnight and coated with gold. For each distribution 500 to 1000 capsules were measured from photographs with the aid of an automatic picture analyzer (MOP AM 03, Kontron, West Germany). The specimen preparation for transmission electron microscopy (EM 9, Zeiss, West Germany) followed standard methods (8).

Electron Diffraction Pattern

The electron diffraction pattern of nanocapsules was directly observed using a transmission electron microscope (TEM) operated in the electron diffraction mode. After specimen preparation according to standard procedures for TEM (8), samples were introduced into the microscope and examined with a focused electron beam. With this experimental design it is possible on the one hand to detect crystalline structures and on the other hand to correlate the observed diffraction pattern with the normal electron microscopic picture of the specimen.

Drug Content and in vitro Drug Release Experiments

The determination of drug content was performed by liquid scintillation counting (Packard Tricarb 300, Packard Instruments, USA).

All drug release experiments were carried out maintaining sink conditions. The nanocapsules (0.5 mg) were dispersed in 10 ml phosphate buffer (pH 7.35, 37°C). The suspension was incubated under mild agitation, and at various time intervals 1 ml samples were taken, filtered (0.1 µm nucleopore membranes, Nucleopore Corp., USA) and the amount of released drug was determined by scintillation counting of 500 µl filtrate. The obtained dpm values were corrected for quenching by subtracting the dpm value measured for 500 µl phosphate buffer.

To examine the drug release over extended periods of time a dialysis procedure was employed with an apparatus described by Sounder and Ellenbogen

(9). Suspended nanocapsules (1 ml) were filled into dialysis bags which were placed into rotating centrifuge tubes (37°C, phosphate buffer, pH 7.35, 25 rpm). After defined time intervals the centrifuge tubes were changed and the amount of released drug was determined by liquid scintillation counting.

Results and Discussion

After freeze-drying a spongy mass was recovered with a yield of 27.3 % ± 22 % (rel. S. D.; n = 3) related to the amount of gelatin employed. The spheres were dispersible in water with the aid of an ultrasonic device. The resulting suspension displayed the Tyndall effect.

The stability of triamcinolone acetonide during the sonication was checked by TLC on silica gel (cyclohexane : ethyl acetate : water 25 : 75 : 1) (10); no degradation products were detectable.

Transmission electron microscopy of sectioned spheres revealed a capsular structure as can be seen in Fig. 1, in contrast to nanoparticles produced according to Oppenheim (7) which had a homogeneous gelatin matrix without visible capsule wall (data not shown). Fig. 1 also shows that the spheres consist of a dark nucleus surrounded by a lighter ring, followed by the darker capsule wall. The dark nucleus of the nanocapsules consisted of crystalline triamcinolone acetonide which was identified by its electron diffraction pattern (Fig. 2). Reference examinations confirmed that the observed diffraction patterns were not caused by crystalline impurities resulting from incomplete washing of the nanocapsules. Furthermore, the encapsulation of the drug containing chloroform phase could be deduced from holes in the nanocapsule walls that were observed during the development of the production procedure (Fig. 3). These holes were due to the rapid evaporation of encapsulated chloroform which destroyed the capsule wall. By restricting the applied pressure to 1–2 bar during freeze-drying, these holes were avoided. Although we did not check whether there is any remaining chloroform in the nanocapsule preparation, it is reasonable to assume, that under the applied drying conditions (2 days, 1–2 bar) the chloroform has entirely evaporated.

Scanning electron microscopic (SEM) pictures showed spherical capsules for all preparations (Fig. 4). On the basis of such pictures the size distributions of three separately prepared charges were determined to examine the size variations (Fig. 5). The described procedure allows the production of nanocapsules with a log normal size distribution and a size below 300 nm. These nanocapsules have therefore a mean size comparable to the nanoparticles described in the literature (100–500 nm) (Table I) (11, 12).

The drug release experiments revealed that nanocapsules and nanoparticles released the drug more slowly than micronized crystalline triamcinolone acetonide (mean crystal diameter 1.9 μm) (Fig. 6 and 7).

In order to sustain the drug release to a greater extent the described production process seemed to allow two variations: 1) alteration of the capsule wall structure and 2) encapsulation of an additive. Widder (6) reported that longer hardening times diminished the drug release from microspheres. This effect is due to a higher crosslinking of the proteineous matrix which restricts the capability of water to diffuse into the matrix. Because the time intervals used by Widder (6) were too long to be applicable in the described process, the pH was raised during the hardening, leading to a similar effect. Blauer (13) showed

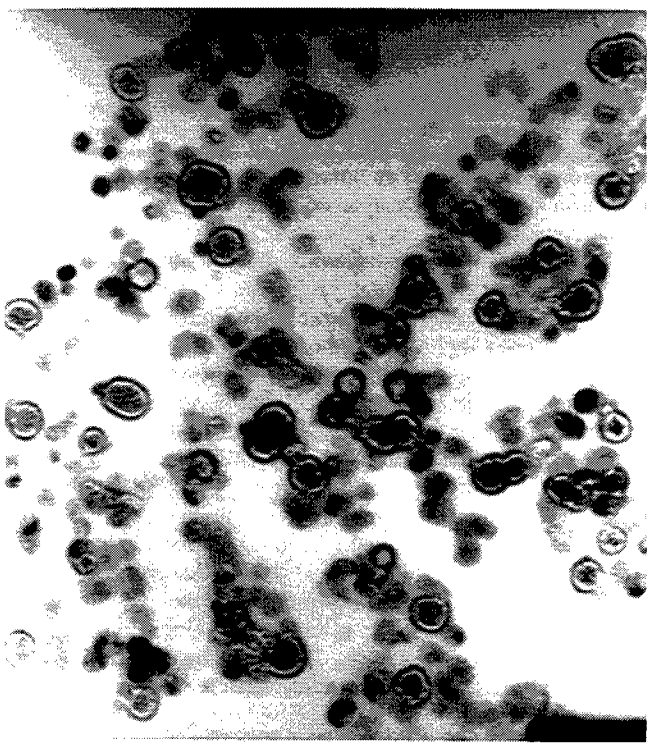

Fig. 1 TEM picture of sectioned nanocapsules showing a nucleus surrounded by a distinct capsule wall (40000 x).

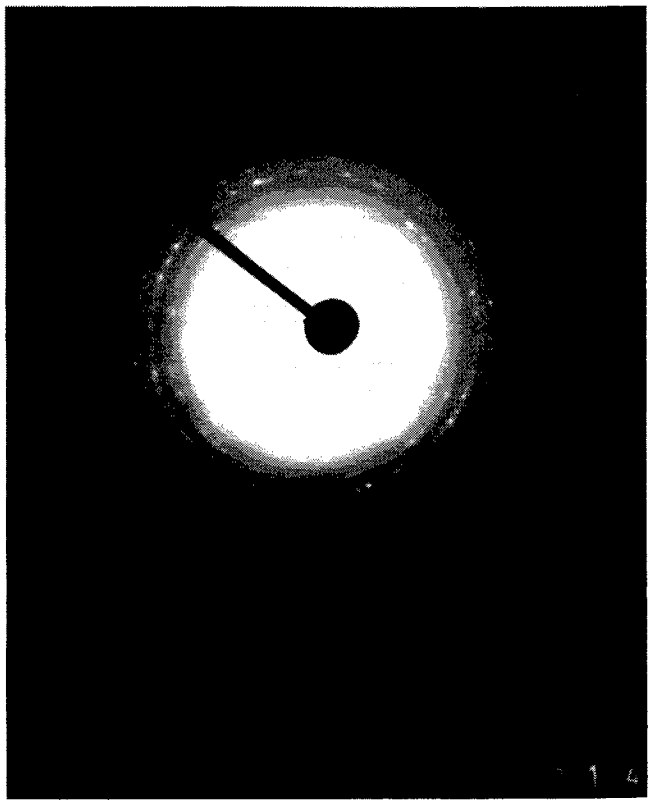

Fig. 2 Electron diffraction pattern of a capsule nucleus indicating crystalline triamcinolone acetonide.

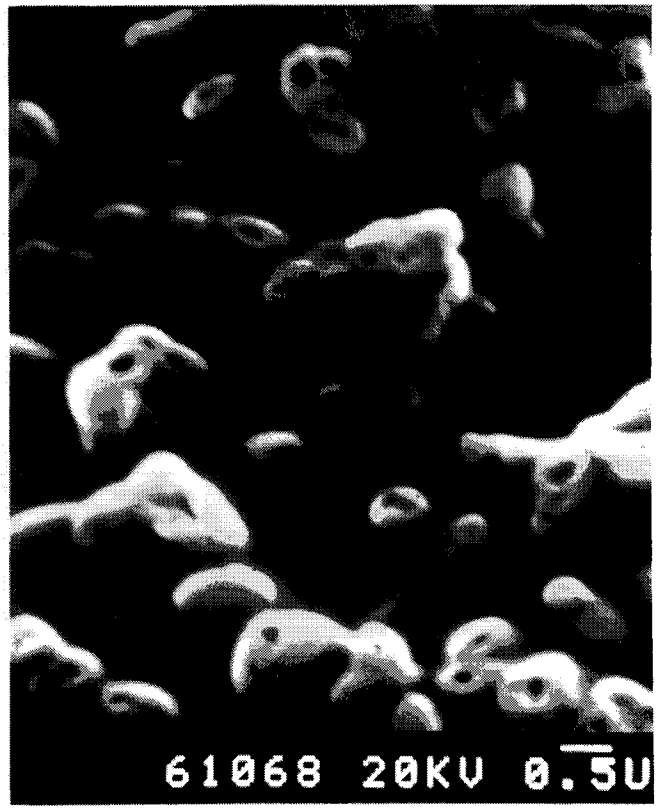

Fig. 3 SEM picture of gelatin nanocapsules with holes in their walls caused by evaporated chloroform, bar represents 500 nm (15000 x).

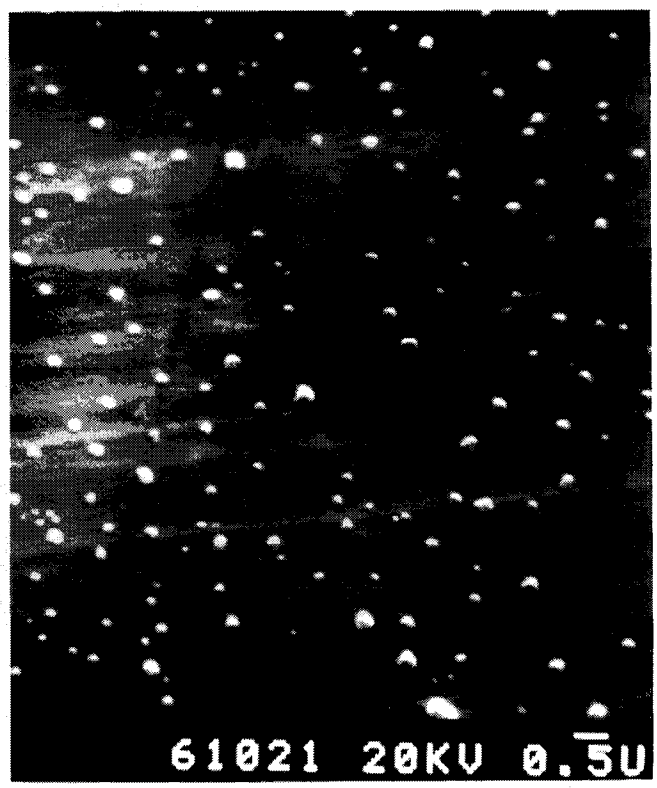

Fig. 4 SEM picture of gelatin nanocapsules, bar represents 500 nm (10000 x).

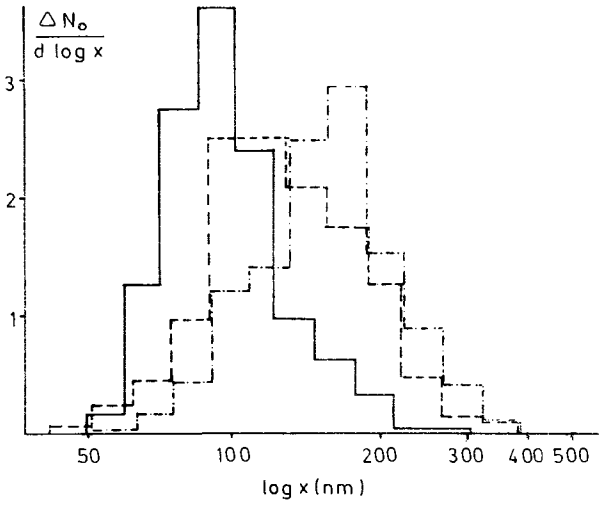

Fig. 5 Size distributions of three separate nanocapsule batches prepared at pH 5.8.

	n	$\bar{x}$ ± S.D. [nm]
– – –	743	140 ± 54
–·–·–	589	181 ± 68
——	561	103 ± 34

Table I. Drug Content, Encapsulation Efficiency and Mean Diameter of Prepared Nanocapsule/Nanoparticle Batches, n = 3

Nanocapsule batch	Drug content % w/w ± rel. S.D.	Encapsulation efficiency[1] % w/w ± rel. S.D.	Mean diameter[2] [nm] ± S.D.
pH 5.8	15.4 ± 9.8	47.9 ± 15.5	141 ± 47
pH 10	7.7 ± 3.8	45.9 ± 4.5	523 ± 340
Cholesterol	7 ± 4.1	38.7 ± 25	144 ± 90
Nanoparticles (7)	0.0012 ± 7.1	0.068 ± 31.6	208 ± 66

(1) encapsulation efficiency = $\frac{\text{mg of drug bound by total amount of nanospheres}}{\text{total amount of applied drug mg}} \times 100$

(2) n = 500 – 1000 measured particles

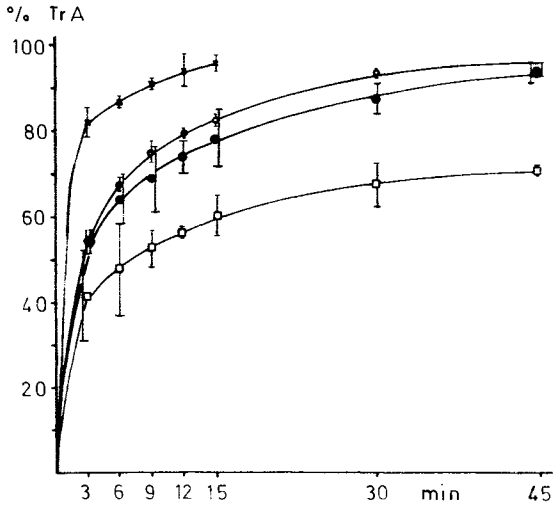

Fig. 6 Kinetics of drug release from nanocapsules determined by membrane filtration (mean of 3 experiments ± S.D.).
x—x free drug
●—● nanocapsules hardened at pH 5.8
○—○ nanocapsules hardened at pH 10
□—□ nanocapsules with cholesterol

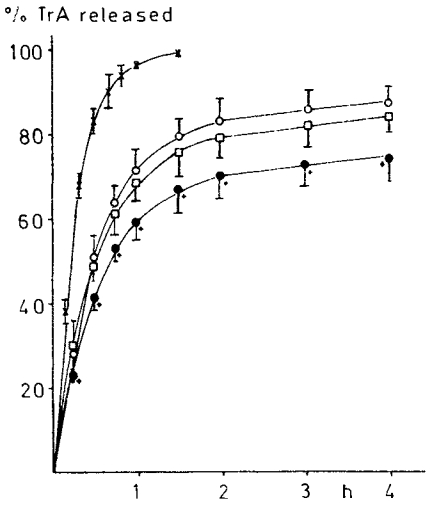

Fig. 7 Drug release of nanoparticles (7) and the influence of the crosslinking pH during hardening on drug release of nanocapsules without surface adhering drug, determined by dialysis (mean of 9 experiments, ± S. D., + $p < 0.02$ Student's t-test).
x—x free drug
□—□ nanoparticles (7)
●—● nanocapsules pH 5.8
○—○ nanocapsules pH 10

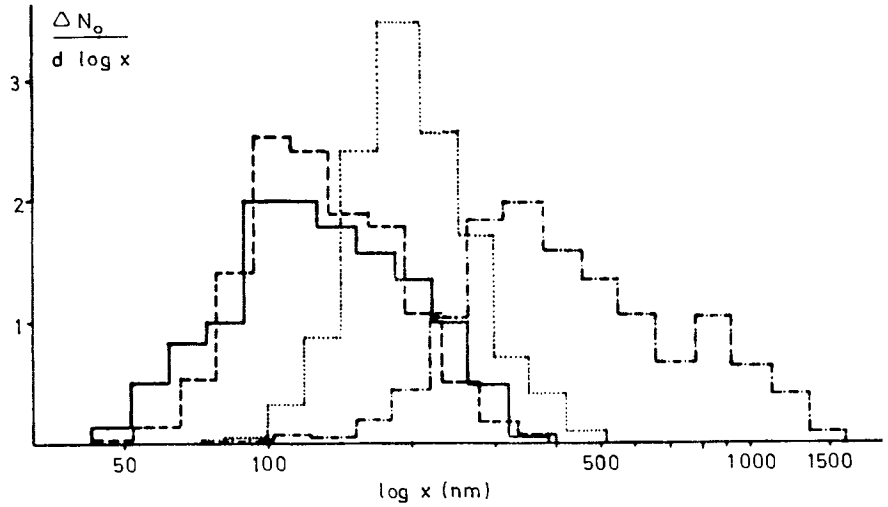

Fig. 8 Size distribution of nanoparticles (7) and the influence of crosslinking pH and encapsulated cholesterol on the size distribution of nanocapsules.
..... nanoparticles (7)
– – – nanocapsules pH 5.8
–·–·– nanocapsules pH 10
—— nanocapsules with cholesterol

an enhanced reaction rate by raising the pH during the reaction between glutardialdehyde and gelatin. Therefore, at pH 10 a higher crosslinked capsule wall will result than at pH 5.8, if the hardening time is the same.

As it can be seen in Fig. 6, there is no difference in drug release between nanocapsules hardened at pH 5.8 or pH 10 probably because of the surface adhering drug which dissolves with a similar velocity in both cases.

To eliminate this influence the nanocapsules were washed with absolute ethanol to remove the adsorbed drug. After washing, the drug content of the nanocapsules hardened at pH 5.8 was 0.12 % ± 4.2 % w/w (rel. S. D.; n = 3) and 0.088 % ± 10 % w/w (rel. S. D.; n = 3) for the nanocapsules hardened at pH 10. Nevertheless, this drug content is about ten times higher than for the nanoparticles produced according to Oppenheim (7) (Table I). Looking at the drug release which was determined by the dialysis procedure, a slight but significant retardation ($p < 0.02$, Student's t-test) between both nanocapsule preparations could be observed (Fig. 7). As discussed above this effect is caused by a denser network of the gelatin wall, resulting in a slower drug release.

The nanoparticles release the triamicinolone acetonide with a comparable velocity as the nanocapsules hardened at pH 5.8 (Fig. 7). The influence of an additive on the drug release is shown in Fig. 6. The encapsulation of cholesterol leads to a more sustained release (Fig. 6), because cholesterol makes it more difficult for water to dissolve the drug embedded in the lipophilic additive. This result is in accordance with Lippolds findings (14), who showed that drug release from matrices was slowed by lipophilic substances because of a lower water permeability of the matrices. To get some information about the mechanism of drug release nanocapsules were examined by SEM after completing drug release. After 4 hours of drug release the suspension of the nanocapsules in the dialysis bags was appropriately diluted with water and prepared for SEM analysis. These examinations showed that the nanocapsules remained intact. Therefore, 60–80 % of the drug diffused out of the capsules (Fig. 7) and was not released by degradation of the wall material. This result is in accordance with Widder (6)

who found that the degradation of a proteinaceous matrix was much slower than the drug release. The remaining drug amount (20 to 40 %) (Fig. 7) diffuses out of the nanocapsules very slowly because of the diminishing concentration gradient over the capsule wall. After 2 hours of drug release no excess crystalline triamcinolone acetonide remains in the capsules and therefore the release rate declines (15).

Figure 8 and Table I show the effects of the production variations with regards to drug content, size distribution, mean size and encapsulation efficiency. For the nanocapsules hardened at pH 10 the size distribution was shifted to greater diameters (Fig. 8). This effect is probably due to thicker capsule walls because of an enhanced coacervation of gelatin at higher pH values leading to a higher amount of deposited wall material (16).

With regard to the encapsulation efficiency no influence can be observed by changing the pH or encapsulating cholesterol (Table I). The lower drug content for the pH 10 and cholesterol nanocapsule batches in comparison with the pH 5.8 nanocapsules reflects the fact that a constant amount of encapsulated drug became surrounded by a higher amount of wall material. Moreover, Table I shows that the nanocapsules having a similar mean diameter as the nanoparticles possess a much higher drug content than the nanoparticles.

In conclusion the present investigation demonstrates that the described production process yielded nanocapsules with a high drug content in comparison with nanoparticles. Furthermore, it is possible to encapsulate lipophilic drugs, whereas the methods described by Widder (6), Oppenheim (7) and Yoshioka (5) are only suitable for hydrophilic substances. Moreover, these nanocapsules can be used as a drug delivery system to direct lipophilic drugs ot organs of the RES, because of their accumulation in liver, spleen and kidney after intravenous application (2).

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Effect of Salicylate on the Uptake of Cefmetazole into Brain of Mice

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Abstract: Cefmetazole distribution into mice cerebral cortex was minimal when the drug was administered alone. However, the co-administration of salicylate or diethyl maleate enhanced cefmetazole uptake into the cerebral cortex, while it decreased the level of reduced nonprotein sulfhydryls in cerebral cortex. The enhanced cerebral uptake of cefmetazole was suppressed by the simultaneous administration of cysteamine with a concomitant recovery of the reduced nonprotein sulfhydryl concentration in cerebral cortex.

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Rat colonic membrane permeability against polar compounds was previously shown to correlate with reduced nonprotein sulfhydryls levels in intestinal tissue (1); moreover, it has been suggested that the integrity of brain tissue may be controlled by glutathione (2). Therefore, agents such as diethyl maleate (1) and salicylate (3) that affect sulfhydryl levels and increase the colonic membrane permeability, may also change the uptake of polar compounds into brain tissue through the blood brain barrier.

In the present study, the effect of either diethyl maleate or salicylate on the distribution of cefmetazole into brain tissue of mice was examined.

Materials and Methods

Materials. Sodium cefmetazole was supplied from Sankyo Co. Ltd. (Tokyo, Japan). Sodium salicylate was obtained from Nakarai Chemicals Co. Ltd. (Kyoto, Japan). Diethyl maleate and cysteamine were obtained from Sigma Co. Ltd. (Mo. USA). Other reagents used were of analytical grade.

Animals. Male, d,d-strain mice, 45 to 50 g, were used; experiments were carried out at 10 a.m.

Uptake study of drugs into mice cerebral cortex. At 30 min after *i.p.* injection of 1 ml/kg saline solution containing cefmetazole with or without diethyl maleate or sodium salicylate, blood samples from the aorta and cerebral cortex tissues were collected. Blood samples were centrifuged to obtain plasma for the drug assay. Cerebral cortex was gently rinsed with saline immediately, after excision and weighed. Homogenized cerebral cortex was employed for the assay of drugs and reduced nonprotein sulfhydryls. Pretreatment with diethyl