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Development of Specific Organ-Targeting Drug Delivery System I:

Physico-pharmaceutical characteristics of thermally denatured albumin microspheres containing cytarabine

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Abstract □ In attempt to develop a drug delivery system using serum albumin microspheres, bovine serum albumin microspheres containing antitumor agent, cytarabine, were prepared. The shape, surface characteristics, size distribution, behavior of *in vitro* distribution, drug release behavior, and degradation of albumin microspheres in animal liver tissue homogenate and proteolytic enzyme were investigated.

The shape of albumin microspheres was spherical and the surface was smooth and compact. The size distribution of the albumin microspheres was affected by dispersion forces during emulsification and albumin concentration. Distribution of albumin microspheres after intravenous administration in rabbit was achieved immediately. *In vitro*, albumin microsphere matrix was so hard that it retained most of cytarabine except initial burst during the first 10 minutes, and the level of drug release during the initial burst was affected by heating temperature, drug/albumin concentration ratio and size distribution. After drug release test, the morphology of albumin microspheres was not changed. Albumin microsphere matrix was degraded by the rabbit liver tissue homogenate and proteolytic enzyme. The degree of degradation was affected by heating temperature.

Keywords □ Bovine serum albumin, Drug delivery system, Cytarabine, Cottonseed oil, Size distribution, Surface characteristics of albumin microspheres, Preparation of albumin microspheres, Drug release from albumin microspheres, Distribution of albumin microspheres after I.V. administration in rabbit.

It is desirable to deliver the drug to its specific target organ and control the release of the drug in order to obtain the higher therapeutic index and the lower adverse effect. Cancer therapy, for example, demands that antitumor drugs with profound systemic toxicity should be delivered to target tissue with minimal interaction with normal tissue. One approach is to alter the distribution in the body and pharmacokinetics by incorporating drugs into particulate carriers, which theoretically can be concentrated at the target tissue.

Albumin microspheres, composed of a denatured serum albumin matrix, have been studied at a drug delivery system. Radiologists utilize the phagocytic activity of the reticuloendothelial system to achieve specificity in the delivery of radiolabelled albumin and sulfur colloids[1-3]. In 1974, Kramer suggested the possibility that albumin microspheres could be utilized as the prominant drug carriers n the treatment of cancer and fungal or bacterial infestations of the reticuloendothelial system and in the delivery of immunosuppressive agents[4,5]. He suggested that intravenous administration of the microspheres might result in preferential uptake in such tissues as liver, spleen, and bone marrow due to phagocytosis with a possible reduction in required total doses and thereby less systemic side effects. Sugibayashi *et al.* studied

the drug-carrier property of albumin microspheres in chemotherapy. They investigated the preparation, distribution and antitumor effects of microsphere entrapped 5-fluorouracil and adriamycin[6-9]. Widder *et al.* reported the preparation of intravascularly administrable magnetic albumin microspheres containing ultrafine magnetic particles, magnetite and antitumor drugs[10-12].

This paper describes the preparation and physico-pharmaceutical properties of bovine serum albumin microspheres containing antitumor agent, cytarabine as a marked compound.

EXPERIMENTAL METHODS

Preparation of bovine serum albumin microspheres

Bovine serum albumin microspheres containing cytarabine were prepared by a modification of a method of Scheffel *et al.*[3].

1) One ml of 25 (10, 30, or 40) w/v% bovine serum albumin[1] (BSA) solution containing cytarabine[2] was added to 100 ml of cottonseed oil in 100 ml Erlenmeyer flask with stirring and emulsified for 15 minutes by a motor-driven glass stirrer. If necessary, the resultant emulsion was homogenized by a probe type ultrasonicator[3].

2) Another 100 ml of cottonseed oil were heated to 105°C (155°C, or 175°C) in a 300 ml three-necked flask under continuous stirring with a motor-driven glass stirrer (approximately 2000 r.p.m.).

3) The homogenate was then added dropwise into the pre-heated cottonseed oil, the temperature adjusted to 100~105°C (150~155°C, o 170~175°C) for 10 minutes, and stirring maintained for 10 minutes.

4) The suspension was cooled to room temperature.

5) The microspheres were washed free of oi by adding anhydrous ether, centrifuged fo 15 minutes at 4000 r.p.m. and decanted th supernatant.

6) After the fourth washing, the resultin microspheres were dried in a vacuum desiccator

Determination of morphology and size distribution

The shape and the surface characteristics o the microspheres were observed with photomicroscopy[4] and scanning electron microcsopy[5].

The dry product was suspended in phosphat buffered saline solution, and observed with photomicroscopy. The dry product was coated b gold with an ion-coater (250~300Å, Ion Sputtering), and observed with scanning electro microscopy. Then, calculations were made o the mean diameter, size distribution, and standard deviation.

Determination of in vivo distribution of albumin microspheres after I.V. administration i Rabbit

The 99m Tc-labelled microspheres were obtained as a reduction of albumin microsphere (prepared by sonication method) with $NaTcO_4$ and $SnCl_2$. A 1-ml dose containing 0.2 m microspheres equivalent to 0.9 mCi radioactivity is used in this experiment. AfterI. V. administration of a dose, the rabbit was scanne

1. Bovine serum albumin (BSA), fraction V (Sigma Co.) were used, the molecular weight of BSA wa assumed to be 69,000.
2. Cytarabine was supplied by Choong Wae Pharm. Co. and used without further purification.
3. Ultrasonicator, Cleaning Equipment Co.
4. Photomicroscope, Vickers Instruments.
5. Scanning electron micrpscope, S.E.M. JEOL JSM=35.
6. $NaTcO_4$, Institut National Des Radioelements, Belgium.

with scintillation-camera[7]. Immediately following dosing, images of the whole body were recorded.

Determination of Drug Release

pH 7.4 phosphate buffered saline (PBS) solution was used as release medium. Ten ml (or 20 mg) of dry product were added to 100 ml of PBS and agitated by magnetic stirrer at constant speed. At periodic intervals, 5 ml volumes were removed, filtered through a millipore membrane,[8] and assayed spectrophotometrically[9] at 274 nm.

Determination of matrix degradation in the animal liver tissue homogenate

Tissue homogenate was prepared by following method[13)]. The liver of male albino rabbit was removed, washed with ice-cold normal saline solution and weighed. All subsequent procedures were done at 4°C. The liver was homogenized in 9 volumes of 0.01 M phosphate buffer, pH 6.0, in a glass homogenizer. The homogenate was centrifuged[10] at 5000 r.p.m. for 10 minutes and the supernatant was used within 30 minnutes.

Then 10 mg of albumin microspheres were incubated in 10 ml of liver homogenate supernatant, or 0.01 M phosphate buffer, pH 6.0, at 37±1°C for about 48 hours. To prevent the degradation of albumin microspheres by microorganisms, broad spectrum antibiotic cefazolin was added to the all incubation mediums (0.2 w/v%). After 48 hours incubation, albumin microspheres were filtered through a millipore membrane and the morphology was observed with photomicroscopy and scanning electron microscopy.

Determination of matrix degradation by proteolytic enzyme

Ten mg of albumin microspheres was added to 100 ml of the PBS solution (pH 7.8) and ultrasonicated for 10 min. to produce a homogeneous suspension of microspheres. Then, 50 mg of protease[11] was added and incubated with slowly stirring by magnetic bar at 37±2°C. At periodic time intervals, 5 ml of the solution containing albumin microspheres was removed and its turbidity was measured at 500nm by UV spectrophotometer[12].

Determination of total amount of cytarabine entrapped in albumin microspheres

After the complete digestion of microspheres by protease, 6 μl aliquots of the solution were injected into the chromatographic system. Analyses were performed on a C-18 column (filled with Spherisorb ODS, 5 μm) using 20% MeOH at a constant flow rate of 1.0 ml/min. as the mobile phase. Absorbance of the efluent from the column at 270 nm was monitored. Peak heights were used for quantitation of the assay.

RESULTS AND DISCUSSION

Physicochemical properties

The shape and the surface characteristics of the microspheres were observed with photomicroscopy and scanning electron microscopy.

As shown in Figure 1, the geometry of albumin microspheres was spherical and the surface was smooth and compact. It is probably due to the preparation procedure in which the

7. Gamma-camera 420, Ohio-Nuclear Inc.
8. Millipore filter, GS WP 02500 25ea. GS 0.22u.
9. UV spectrometer, Pye-Unicam SP-1750 type.
10. Centrifuge, Beckman automatic high speed refrigerated centrifuge.
11. Protease was supplied by Dong-A Pharm. Co.
12. UV spectrophotometer, Hitachi model 638-50.

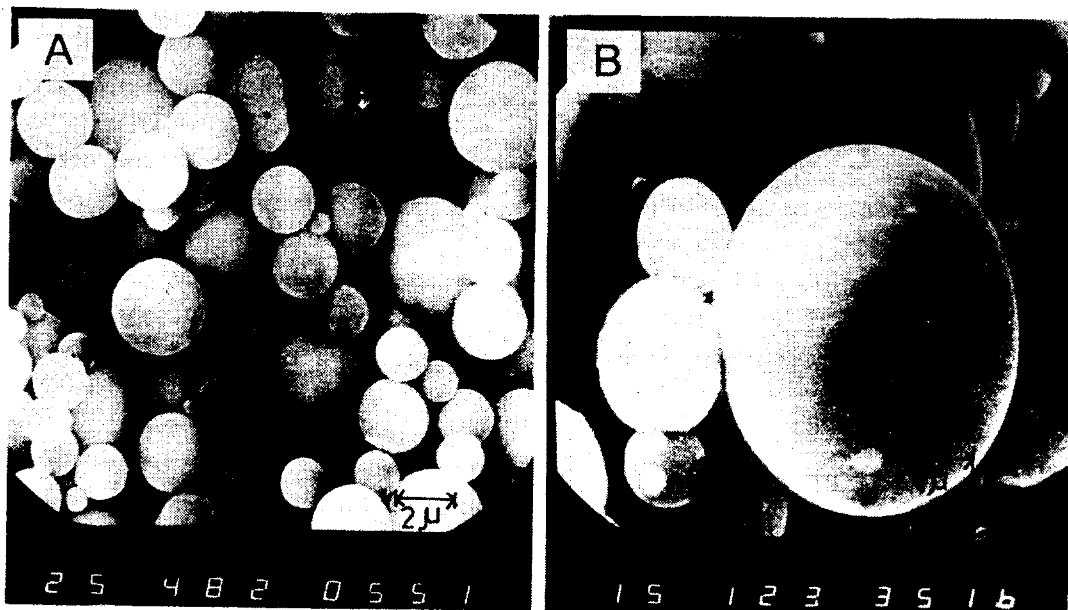

Fig. 1: Scanning electron micrograph of bovine serum albumin microspheres.
A: ×4,800, B: ×12,000.

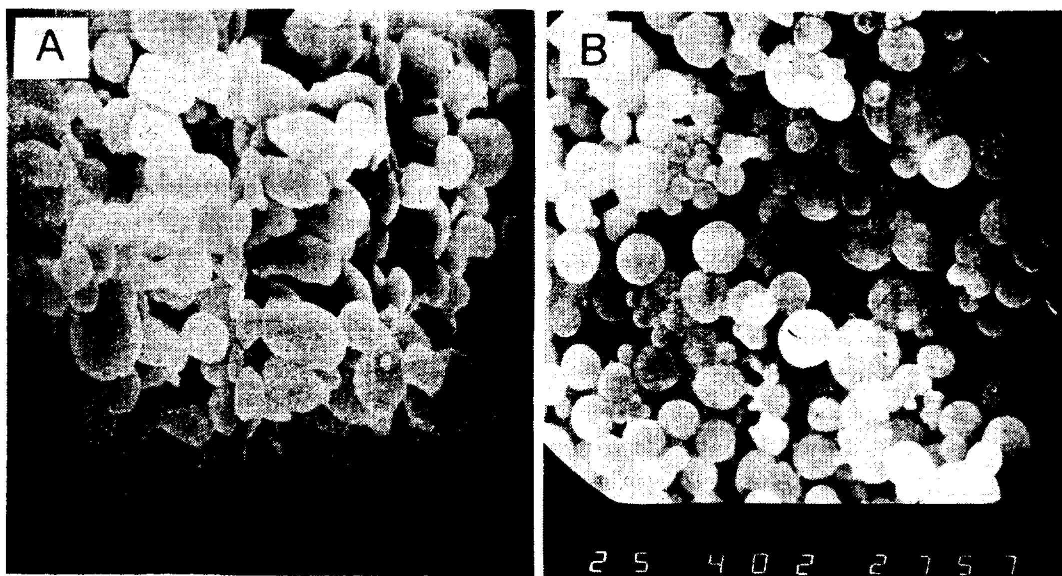

Fig. 2: Effect of the heating temperature on the geometry of bovine serum albumin microspheres. Albumin microspheres prepared at 100~105°C (A) and at 170~175°C (B).
All products were prepared with 25 w/v% BSA, the stirring speed during the emulsification process was 2500 rpm.

ıner albumin phase of w/o emulsion was imıediately solidified at high temperature with onstant stirring.

Because the albumin droplets formed during he emulsification process were small and the emperature of the oil was so high, water vaporation from the albumin droplets and .enaturation of albumin might occur as soon as he albumin droplets were added to the oil. \s the hardening of albumin matrix was perormed with constant stirring at high speed, riction of oil with the albumin microspheres night result spherical geometry of microspheres vith smooth and compact surface.

The spherical geometry of albumin microspheres were affected by heating temperature .s shown in Figure 2. At given condition, low emperature (100~105°C) resulted a less smooth pherical geometry and little bit irregular shape of albumin microspheres than high heating temperature (170~175°C) did. The albumin concentration also affected the geometry of microspheres. Low concentration of albumin microspheres at given condition is shown in Figure 3.

Albumin microspheres retained their original size when suspended in absolute ethanol or diethyl ether, while when suspended in distilled water, aggregation and swelling occured. It was observed that the degree of aggregation and swelling decreased as the temperature of the hardening process increased. The swelling of albumin microspheres may be due to the hydration of albumin matrix.

The swelling properties probably affect the drug release from albumin microspheres *in vivo.* To prepare intravascular injectable drug carrier, aggregation of albumin microspheres must be prevented. The drying method may also affect the albumin microspheres.

Size Distribution

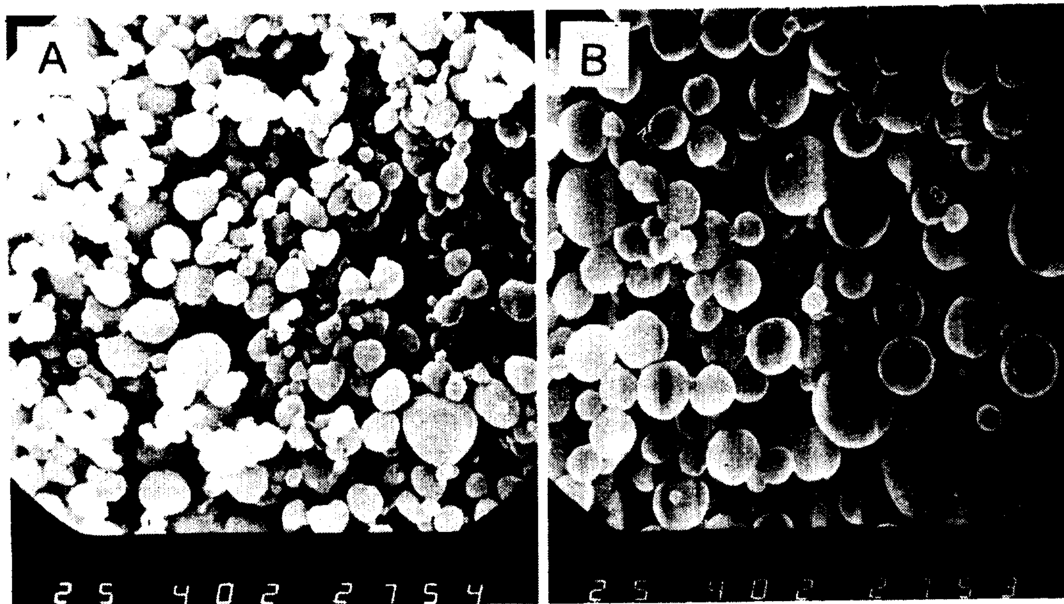

Fig. 3: Effect of albumin concentration on the geometry of bovine serum albumin microspheres. Albumin microspheres prepared with 10 w/v% BSA (A) and with 40 w/v% BSA (B). All products were prepared at 150~155°C, the stirring speed during the emulsification process was 2500 rpm.

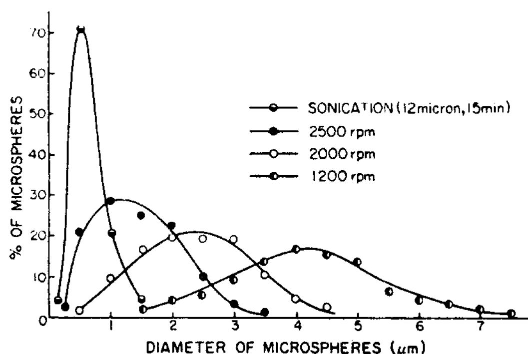

Fig. 4: Effect of dispersion energy on the size distribution of microspheres.
All products were prepared at 150～155°C using 25 w/v% BSA solution.

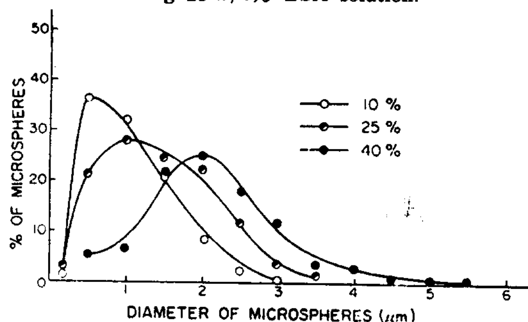

Fig. 5: **Effect of albumin concentration on the size distribution of microspheres.**
The stirring speed during the emulsification process was 2500 rpm and the heating temperature was 150～155°C.

The size distribution of albumin microspheres was strongly affected by the mechanical agitation in the process of emulsification and albumin concentration.

Figure 4 and table I show the effect of dispersion energy on the size distribution of microspheres. The size distribution curve clearly became narrower and sharper with incrasing dispersion energy.

Figure 5 and table II show the effect of albumin concentration on the size distribution of microspheres. The size distribution curve of albumin microspheres became broader and mean diameter of albumin microspheres became larger

Table I: Bovine serum albumin microsphere* size versus dispersion energy

Dispersion Energy	Diameter, um measured by SEM	
	mean	S.D.
1200 rpm	4.2	1.669
2000 rpm	2.3	0.863
2500 rpm	1.7	0.708
Ultrasonication (12 micron, 15 min.)	0.6	0.085

* All products were prepared at 150～155°C usin 25 w/v% BSA solution.

Table II: Bovine serum albumin microsphere* size versus albumin concentration.

Albumin Concentration	Diameter, um measured by SEM	
	mean	S.D.
10 w/v%	1.0	0.283
25 w/v%	1.2	0.708
40 w/v%	2.2	1.319

* The stirring speed during the emulsification pr cess was 2500 rpm and the heating temperatu was 150～155°C.

with increasing albumin concentration. Increas in the relative viscosity of the albumin solutio due to the increase of albumin concentratio may lead to prevent subdivision of albumi droplets into smaller sizes.

These results indicate that size distributio of the microspheres depends mainly on siz distribution of the droplets of albumin solutio dispersed in cottonseed oil during the proces of emulsification. Albumin microspheres of thes size range are suitable to intravascular injection

Determination of in vivo distribution of micro spheres

Whole body scanning showed that almost al of the radioactivity was localized in organ with reticuloendothelial system, especially i liver. Figure 6 shows the scan obtained fron

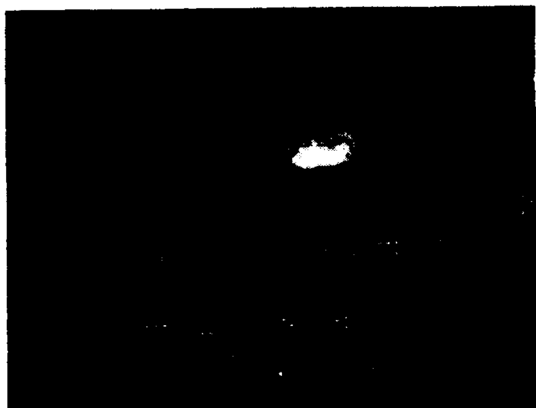

Fig. 6: Determination of *in vivo* distribution of albumin microspheres.

rabbit immediately following administration and at 20 minutes.

Table III summarized quantitative distribution of microspheres in several organs with determination of the radioactivity. Albumin microspheres were rapidly cleared from the circulation. Retention of microspheres in the liver appears to be due to phagocytosis of the microspheres by cells of the reticuloendothelial ststem.

Drug release behavior

Cytarabine is relatively stable to temperature used in the process of hardening. After cytarabine powder was heated at 175°C for 20 minutes, the weight changes of the powder, UV spectrum of cytarabine in the phosphate buffered saline (pH 7.4), and absorbance of the cytarabine solution at 274 nm were determined. No evidence of degradation of cytarabine by heating was found in the results.

The distribution of cytarabine between aqueous phase and oil phase was determined. After aqueous cytarabine solution was mixed with equal volume of cottonseed oil for 3 hours by magnetic stirrer, absorbance of the cytarabine in aqueous phase were determined at 274 nm.

Table III: Distribution of microspheres in the liver, lung, spleen, bone marrow, and kidney after intravenous injection of ^{99m}Tc-labeled microspheres.

Time	Organ	Total Radioactivity	
		dpm	% of total counts
immediately	Liver	113,960	80.10
	Spleen	5,717	4.02
	Lung	8,142	5.72
	Bone Marrow	430	0.30
	Kidney	1,810	1.27
	Others	12,221	8.59
after 20 minutes	Liver	103,578	69.09
	Spleen	5,225	3.49
	Lung	7,406	4.94
	Bone Marrow	542	0.36
	Kidney	1,660	1.11
	Others	31,543	21.01

The amount of cytarabine distributed in the oil phase was negligible. Cytarabine is slightly soluble in ether which was used as washing solvent. So the loss of cytarabine in the process of preparation may be negligible.

In vitro, cytarabine was released during the first 10 minutes and was hardly released thereafter. The level of drug release during the initial burst was affected by heating temperature, drug/albumin concentration ratio, and size distribution. Figure 7 shows the effect of the heating temperature on the release of cytarabine from albumin microspheres. The level of drug release decreased as heating temperature increased.

It is thought that an increase in heating temperature leads the hardness of albumin matrix to increase, which probably results decreased swelling and drug release. The initial burst is probably resulted from the release of cytarabine loosely held on the surface of albu-

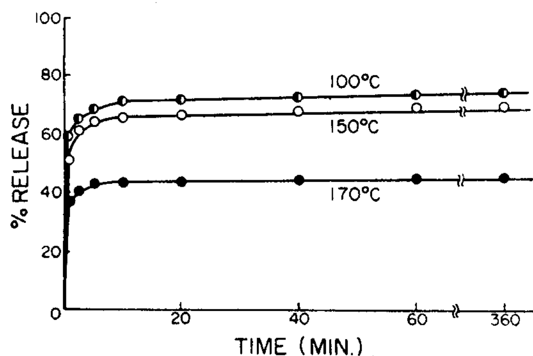

Fig. 7: Effect of the heating temperature on the release of cytarabine from albumin microspheres.
All products were prepared with 25 w/v % BSA solution. The stirring speed during the emulsification process was 2500 rpm, drug/albumin concentration was 0.2/1.0.

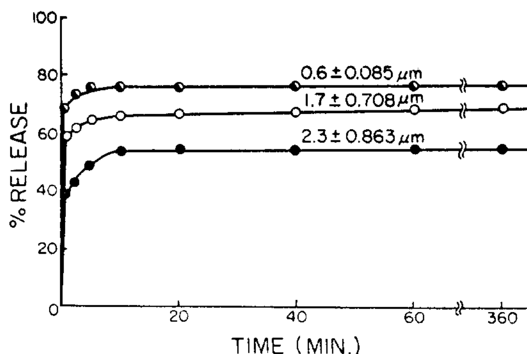

Fig. 8: Effect of the size distribution of microspheres on the release of cytarabine from albumin microspheres.
All products were prepared with 25 w/v % BSA solution. The temperature was 150~155°C, and the drug/albumin concentration was 0.2/1.0.

min microspheres.

Figure 8 shows the effect of microsphere size on the release of cytarabine from albumin microspheres. As the microsphere size decreases, the release of drug increases. An increase of total surface area due to the reduction of albumin microsphere size may increase the drug

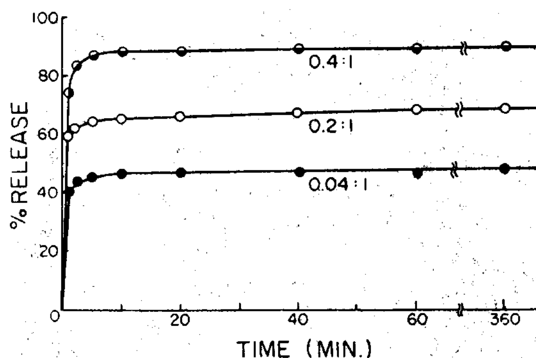

Fig. 9: Effect of the drug/albumin concentration ratio on the release of cytarabine from albumin microspheres.
All products were prepared with 25 w/v % BSA solution. The speed during the emulsification process was 2500 rpm, and the heating temperature was 150~155°C.

release.

Drug content per mg of microspheres can be controlled by varying the drug concentration in the aqueous albumin solution. Figure 9 shows that the release of cytarabine from micro-

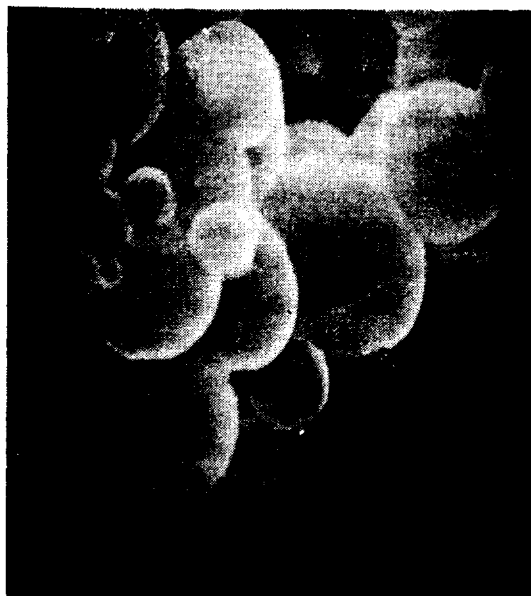

Fig. 10: Scanning electron micrograph of albumin microspheres after release test.

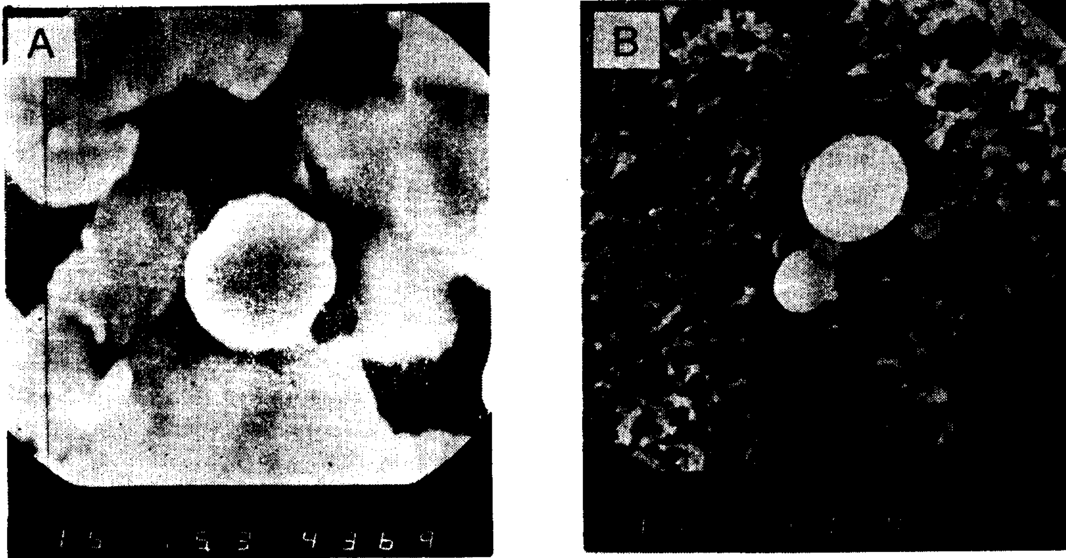

Fig. 11: Matrix degradation of albumin microspheres by the animal liver tissue homogenate.
A: Albumin microspheres after incubation for 48 hours in the liver tissue homogenate at 37 ± 1°C, B: Albumin microsphers after incubation for 48 hours in the pH 6.0 phosphate buffer solution at 37 ± 1°C.

spheres entrapping various amounts of the drug is increased. After drug release test, the morphology of albumin microsphere was not changed as shown in Figure 10.

Drug may be held on the surface and in the inner space of albumin microsphere matrix. The initial burst may be resulted from the release of surface drug, and the next slow release may be resulted from the diffusion of the drug in the inner space of albumin microspheres.

Determination of matrix degradation in the animal liver tissue homogenate and proteolytic enzyme

In this study, albumin microsphere matrix was so hard that it retained most of cytarabine except initial burst. 68~70 % of drug retained in the inner space of albumin matrix in our preperation. After 48 hours incubation of albumin microspheres in animal liver tissue homogenate, albumin microsphere matrix was partly digested as shown in Figure 11.

Figure 12 showes the effect of heating temperature on the degradation of albumin microsphere matrix in proteolytic enzyme solution. The degree of degradation was affected by the heating temperature. It was increased as heating

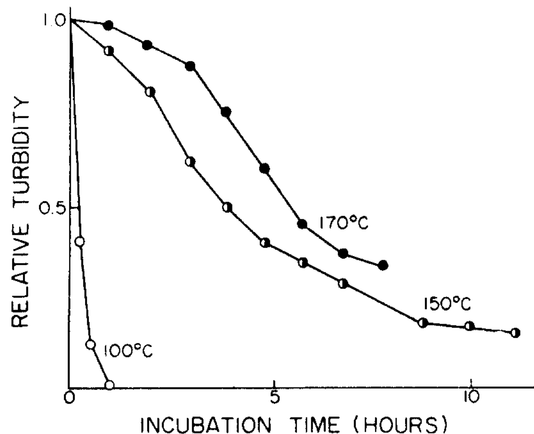

Fig. 12: Effect of the heating temperature on the degradation of albumin matrix in proteolytic enzyme solution.

temperature decreased.

As shown in current results, the drug release behavior and the matrix degradation were influenced by the conditions of preperations such as stirring speed, stirring time, heating temperature, heating time, albumin concentration, etc. Albumin microspheres with different physico-chemical properties may be prepared by controlling the condition of preparation. Specially controlled albumin microspheres seems to be an ideal drug delivery system which retains drug in the blood stream until microspheres are phagocytized by the specific target site and biodegraded by intrinsic proteolytic enzymes. And, drug can be localized in specific target site and continuously released.

ACKNOWLEDGEMENT

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