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RESEARCH PAPER

Liposomes Containing Drug and Cyclodextrin Prepared by the One-Step Spray-Drying Method

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ABSTRACT

The one-step spray-drying method was applied in the preparation of liposomes containing drug and cyclodextrin (CD). Spray-dried lecithin liposomes, entrapping metronidazole or verapamil alone or together with hydroxypropyl-β-cyclodextrin (HPβCD), were characterized for morphology, size distribution, and drug entrapment efficiency. The main factor influencing the liposomal size was the volume of aqueous medium used for hydration of the spray-dried product. No differences in size or entrapment between liposomes prepared by immediate hydration of dried powder or by hydration after 1 year of powder storage at 4°C were observed. All liposomes were tested for their serum stability. The most stable liposomes (still retaining about 10% of the originally entrapped drug even after 24 hr incubation with serum) were liposomes prepared by the direct spray-drying of the mixture of lipid, drug, and HPβCD.

INTRODUCTION

The spray-drying process is considered to be a single-step, fast procedure applied in the formulation and processing of biopharmaceuticals. Recently, Branchu et al. (1) reported the stabilizing effect of hydroxypropyl-β-cyclodextrin (HPβCD) in the spray-drying of protein pharmaceuticals. Moreover, cyclodextrins (CDs) represent good candidates for the dissolution rate enhancement of insoluble drugs. They serve as a carrier system for poorly soluble drugs (2). Among cyclodextrins, β-cyclodextrins form the most stable inclusion complexes for

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many drugs. Their relatively low aqueous solubility presents the main limit in their broader application. However, chemically modified β-cyclodextrins such as HPβCD overcame the solubility problems, having inclusion properties that differ from those of parent β-cyclodextrin (3). The behavior of CD in vivo is still connected with several problems (4); therefore, the incorporation of complexes into another carrier system, such as liposomes, would improve their properties. A novel drug delivery system, drugs-in-cyclodextrins-in-liposomes, was proposed (5) combining the advantages of both systems into one improved delivery system.

The methods applied in the drug-CD complex formation and liposome preparation are diverse. Previously, we prepared drug-CD complexes by either partial solubilization of the drug or the complete solubilization by means of spray-drying. Those complexes were entrapped in liposomes prepared by the different preparation methods. All procedures required preparation of complexes first, followed by incorporation of drug-CD complexes into liposomes (6–8). From an industrial point of view, those procedures are relatively expensive and time consuming. Since spray-drying is a very simple and industrially applicable method for the production of both CD complexes (6) and liposomes (9), we applied the direct spray-drying of a mixture of lipid, drug, and CD in the preparation of liposomes. The model drug substance was metronidazole, a drug known for its low solubility in both hydrophilic and lipophilic media, and verapamil hydrochloride, which served as a hydrophilic marker. All liposomes were characterized for their morphology, size distribution, and entrapment efficiencies, as well as their serum stability.

EXPERIMENTAL

Materials

Metronidazole and verapamil hydrochloride were a gift from Belupo (Koprivnica, Croatia). Lecithin was the product of Siegfried Handel (Zofingen, Germany). D(–)Mannitum (mannitol) and chloroform were purchased from Kemika (Zagreb, Croatia). HPβCD was a generous gift from Wacker Chemicals (Munich, Germany). Horse serum was kindly provided by Pliva-Animal Health Division (Zagreb, Croatia).

Methods

Preparation of Spray-Dried Liposomes

Empty liposomes were prepared by suspending lecithin (4.601 g) and mannitol (0.460 g; presieved) in chloroform (200 ml). The mixture was sonicated for 8 min (bath sonicator) and subjected to spray-drying on a Buchi 190M Mini Spray Dryer (Switzerland). The spray-drying conditions were as follows: inlet and outlet temperatures were 120°C and 80°C, respectively; airflow rate was 700 NI/hr; and the flow rate was 1000 ml/hr. The dried product was hydrated with different volumes of phosphate buffered saline (PBS; pH 7.4) by stirring for 45 min.

Liposomes containing HPβCD (in the absence of drug) were prepared by the addition of CD (9 g) to the initial mixture of lipid and mannitol. All other preparation steps were the same as for the empty liposomes.

Liposomes containing drug were prepared under exactly the same conditions; metronidazole (1.026 g) or verapamil chloride (7.66 g) was added to the initial mixture, bath sonicated, and subjected to spray-drying. The inlet and outlet temperatures were adjusted to be between 90°C–120°C and 65°C–80°C, respectively. Other spray-drying and hydration conditions were the same as stated above.

Liposomes containing both drug and CD were prepared by the same procedure. HPβCD (9 g) was added to the initial mixture (containing drug, lipid, and mannitol); the mixture was bath sonicated, spray-dried, and hydrated with PBS under the conditions stated above.

Control liposomes containing drug-CD complex were prepared in such a way that the spray-dried powder (lecithin and mannitol) was hydrated with PBS containing drug-CD complex (when appropriate; 10 ml), stirred for 45 min, and used as the other preparations.

All spray-dried powders were divided into two equal parts, one that was hydrated immediately after the spray-drying, and the second part, which was kept as a powder at 4°C for 1 year and then hydrated under the same conditions.

All liposomal suspensions were prepared by the hydration of spray-dried powder (containing 100 mg lipid) with PBS and kept in the refrigerator at 4°C prior to characterization and further experiments.

Preparation of Drug-Cyclodextrin Complex

Drug-HPβCD complexes were prepared by complete solubilization (7). Briefly, drug (ethanolic solution) was mixed with an aqueous solution of HPβCD (in the molar ratio of 1:1), and the solution bath was sonicated for 15 min. The clear solution was spray-dried, and the drug content in the complex was measured spectrophotometrically. Under these conditions, no spontaneous precipitation of the inclusion was observed.

The complexes were used for the hydration of spray-dried powder (lecithin and mannitol) in the preparation of liposomes containing drug-CD complex. Formation of inclusion complexes of the drug with HPβCD was confirmed by the solubilization of the drug in the presence of an aqueous solution of CD (6).

Vesicle Size Distribution

A microscopic imaging analysis technique for determination of liposomal size distributions was applied. The morphology and particle size distributions (based on the number of particles) were determined in an Olympus BH-2 microscope equipped with a computer-controlled image analysis system (Optomax V, Cambridge, UK). A representative sample of 10,000 vesicles was measured (10).

Entrapment Efficiency Determination

Entrapment efficiencies were determined to compare liposome encapsulation abilities as a function of chemical characteristics of the substances to be entrapped and the preparation method. Dialysis was applied to separate unentrapped drugs from liposomes. A sample of liposome suspension was placed in a tube (Spectra Por, MW cutoff 10,000) and extensively dialyzed against the buffer solution for 24 hr. The volume of buffer was adjusted so that the concentration of the drug was kept below the solubility of the drug. To confirm that the dialysis resulted in a complete separation of unentrapped drug, liposomal samples were subjected to gel chromatography on a Sepharose 4B CL column (Pharmacia). The drug concentration (both free and liposomally entrapped) was determined spectrophotometrically. Recovery of the drug was measured for all samples and was between 92.9% and 95.8% of the starting amount.

Serum Stability Study

Liposomes, free of unentrapped drug, were tested for their stability in the presence of horse serum (50%) at 37°C. Liposome suspension (300 μl) was incubated with serum (2 ml) and PBS (1.7 ml), both preheated to 37°C. Samples (0.5 ml) were taken at certain time intervals (0, 0.5, 1, 2, 4, and 24 hr) and dialyzed. The drug released from liposomes and the drug still present in liposomes were determined spectrophotometrically. Controls (serum free) contained 300 μl of liposomes in 3.7 ml PBS and were treated under the exact conditions.

RESULTS AND DISCUSSION

The recoveries of the mixture prepared by spray-drying were estimated based on the amount of lecithin in the product as determined by the Bartlett method (11). Kikuchi et al. (9) reported that, without the core material (such as mannitol), a large portion of the dried lipid mixture adhered to the wall of the drying chamber, suggesting that core material was necessary for successful preparation. In our case, spray-drying of lipid mixture without mannitol resulted in the adhesion of approximately 35% of the mixture to the wall. However, mannitol plays an important role in increasing the surface area of the lipid mixture, enabling successful hydration of spray-dried product.

Spray-dried product was very amorphous and easily hydrated regardless of the content. In all experiments, the hydration was easy, even for the samples kept for 1 year (at 4°C) in the form of a spray-dried powder prior to actual hydration. Lecithin used in our experiments was 98% pure, and in contrast to the original preparation method (9), we were able to recover lecithin with the same yield as for distearoyl phosphatidylcholine (DSPC) liposomes (data not shown). The crystallinity of lipids did not play a major role in our spray-drying procedure, when mannitol and especially HPβCD were present in the mixture. Recovery for the initial mixture was dependent on the conditions of the spray-drying and the content of the initial mixture. The highest recovery was observed for spray-dried liposomes containing both HPβCD and drug and was 84.5%. The lowest recovery was seen for liposomes containing metronidazole without HPβCD (71.0%). Those findings suggest that cyclodextrin increases the recovery of spray-dried product by preventing its adherence on the drying chamber.

Lecithin liposomes prepared by the one-step spray-drying method were of regular spherical shape regardless of the presence of the drug or CD. No morphological difference between empty liposomes and liposomes containing drug, CD, or both were observed. Control liposomes prepared by the hydration of spray-dried lipid powder with drug-CD complex were identical morphologically to other liposomes. The actual preparation method, spray-drying conditions, and, even more, the volume used for the hydration influenced the size of liposomes (Table 1).

Storage of spray-dried powder for 1 year prior to hydration (performed under the same conditions as for immediate hydration) did not influence the vesicle size (Table 1). Kikuchi et al. (9) reported the efficient hydration of spray-dried powders even after 3 years of refriger-

Table 1

Influence of Initial Hydration Volume V *on Liposomal Size*

V (ml)	Mean Diameter (nm) of Liposomes Containing					
	—	CD	Met.	Met. + CD	Ver.	Ver. + CD
1	469	493	475	518	546	528
	(472)	(489)	(469)	(524)	(532)	(521)
2	328	387	269	311	432	402
	(329)	(374)	(279)	(321)	(430)	(410)
3	322	352	323	319	419	390
	(328)	(362)	(315)	(309)	(412)	(399)
4	307	338	287	404	435	379
	(317)	(350)	(299)	(409)	(441)	(386)
5	291	272	292	369	384	327
	(290)	(284)	(300)	(370)	(369)	(330)
10	282	306	268	327	360	309
	(295)	(310)	(280)	(331)	(352)	(318)

Empty liposomes (—), liposomes containing HPβCD (CD), liposomes with metronidazole (Met.) or verapamil (Ver.), as well as liposomes containing both drug and HPβCD (Met. + CD; Ver. + CD) were prepared by the spray-drying procedure as described in text. Mean diameters were measured by an image analysis technique. The values in parentheses represent liposomes hydrated after 1 year of storage as spray-dried powder at 4°C.

ator storage. The main difference in their (9) and our procedure was that we applied the drug (and cyclodextrin) already in the initial mixture used in spray-drying and hydrated the spray-dried powder with buffer, whereas they hydrated the spray-dried lipid powder with the drug solution. Our aim was to simplify the procedure as much as possible; therefore, the ''one-pot'' conditions were applied. The original method (9) has the advantage that it is possible to use a solution of any hydrophilic drug to hydrate the spray-dried powder and to form liposomes. However, we believe that our approach enables the formation of liposomes with hydrophilic and lipophilic drugs, and especially with cyclodextrins, in a very simple preparation method. Spray-dried powder can be stored and hydrated prior to the actual application.

The main factor influencing the liposomal size was the hydration volume. The hydration with 10 ml buffer led to liposomes of fairly uniform size and a mean diameter of about 300 nm, whereas hydration with 1 ml (for example) produced liposomes with a mean diameter of about 500 nm (Table 1). Volumes larger than 10 ml (15, 20, 25 ml) did not further reduce the size (data not included). Further dilution of original liposomal suspension did not affect the liposomal size.

Liposomes containing metronidazole (in the absence of CD) were smaller than liposomes containing both metronidazole and HPβCD, whereas the opposite was observed for liposomes containing verapamil. Entrapment of hydrophilic drug resulted in the increase in liposomal size compared to liposomes containing lipophilic drug (Table 1). Control liposomes, prepared by hydration with drug-CD complex solution (10 ml) had a mean diameter of 364 nm for liposomes with metronidazole-HPβCD complex and 349 nm for verapamil-HPβCD complex, respectively.

By applying extrusion or a similar size-reducing method, it is possible to decrease the original size further; however, such a procedure would influence the original drug entrapment.

For all liposome preparations, the entrapment efficiencies were relatively high (Table 2). Entrapment efficiency for liposomes containing metronidazole was 46.7% of the metronidazole taken into the preparation (0.104 mg per milligram lipid). That represents a very high entrapment considering that metronidazole is a poorly soluble drug (in both hydrophilic and lipophilic media); in our previous work, we were able to entrap that amount of metronidazole only in liposomes prepared by the classical film method and with a mean diameter of about 900 nm (10). Liposomes of similar size entrapped a maximum up to 0.70 mg of metronidazole per milligram lipid (10,12). Entrapment efficiency for liposomes

Table 2

Entrapment of Drugs in Spray-Dried Liposomes

Liposomes Containing	Size (nm)	Entrapment Efficiency (%)	Drugs per Lipid Mass Ratio (mg/mg)
Metronidazole	268	46.7 ± 4.2	0.104
	(278)	(47.2 ± 6.0)	(0.105)
Metronidazole + CD[a]	327	36.41 ± 3.2	0.080
	(336)	(35.9 ± 5.0)	(0.080)
Metronidazole + CD[b]	394	23.9 ± 5.4	0.052
	(411)	(22.5 ± 5.1)	(0.050)
Verapamil	360	43.5 ± 5.0	0.724
	(371)	(44.8 ± 3.9)	(0.716)
Verapamil + CD[a]	309	41.1 ± 2.9	0.684
	(317)	(40.0 ± 3.5)	(0.666)
Verapamil + CD[b]	376	39.9 + 4.0	0.661
	(388)	(39.0 ± 3.7)	(0.649)

Liposomes were prepared as described in text. The volume used for hydration was 10 ml in all preparations. The values in the parentheses represent liposomes hydrated after 1 year of storage as spray-dried powder at 4°C. The values denote the mean ± SD of three preparations.

[a] Liposomes prepared by spray-drying of the initial mixture of lipid, mannitol, and HPβCD.

[b] Liposomes prepared by hydration of spray-dried powder (lecithin and mannitol) with drug-HPβCD complex.

containing verapamil was 43.5% of verapamil taken into the preparation. For liposomes containing metronidazole and HPβCD, higher entrapment of the drug was achieved when liposomes were prepared from the initial mixture already containing drug and HPβCD, than for liposomes prepared by the hydration of spray-dried powder with drug-HPβCD complex. In the latter case, it is evident that the complex is entrapped in the aqueous part of the vesicle, whereas in the first preparation procedure, the interaction and the exact position of drug and cyclodextrin in the vesicle remains unproven. Liposomes containing verapamil alone or verapamil and HPβCD (prepared by both procedures) entrapped a similar amount of the drug. To increase the entrapment of the drug further, we varied the initial ratio of drug to CD (1:1, 1:1.5, and 1:4), but it did not influence the entrapment efficiency significantly (data not shown).

A possible application of liposomes as a carrier system for drug and cyclodextrin in vivo requires substantial retention of drug by liposomes in the circulation (13). Results of serum stability studies (Table 3) indicate that the most stable liposomes are those containing both drug and cyclodextrin. Liposomes containing metronidazole released 55.3% of the drug in the first hour and 95.1% after 24 hr incubation, whereas liposomes containing both metronidazole and cyclodextrin released 37.3% and 91.1%, respectively. The release of verapamil from liposomes incubated in serum for 1 hr was 56.8% and 96.3% after 24 hr, whereas liposomes containing verapamil and HPβCD released 51.8% and 89.0%, respectively. The retention of entrapped verapamil in the presence of HPβCD was significantly higher compared to liposomes containing the drug alone. Under exactly the same incubation conditions, liposomes containing drug-CD complex retained 9.4% of metronidazole and 11.9% of verapamil (24-hr values), respectively.

Although the encapsulation of drug-CD complexes into liposomes can increase the entrapment of the lipophilic drug and reduce its release from the carrier (7), in the case of metronidazole, the entrapment was higher when the drug alone was present in the initial mixture used for spray-drying. However, the retention of entrapped drug in the presence of serum increased when cyclodextrin was present in the liposome. In the case of a hydrophilic drug such as verapamil, the entrapment was similar regardless of the presence of CD, but the retention increased with cyclodextrin present for both complexed and mixed drug.

Incorporation of lipophilic drugs into the lipid bilayer of liposomes is often limited in terms of drug-to-lipid

Table 3

Serum Stability (Retention of the Originally Entrapped Drug in Liposomes [%])

Liposomes Containing	Incubation Time (hr)				
	0.5	1	2	4	24
Metronidazole	49.8 ± 2.4	44.7 ± 2.9	37.3 ± 3.1	19.9 ± 4.0	4.9 ± 1.9
	(92.4 ± 2.8)	(87.9 ± 3.3)	(85.5 ± 4.6)	(75.4 ± 3.6)	(73.1 ± 2.1)
Metronidazole + CD	72.2 ± 1.7	62.7 ± 3.8	47.8 ± 5.0	27.0 ± 4.1	8.9 ± 3.2
	(96.0 ± 3.3)	(93.7 ± 4.2)	(88.2 ± 3.7)	(79.9 ± 5.1)	(78.2 ± 2.6)
Verapamil	50.2 ± 3.5	43.2 ± 4.2	27.3 ± 2.8	16.0 ± 3.4	3.7 ± 2.3
	(87.2 ± 3.5)	(78.2 ± 3.7)	(70.8 ± 4.7)	(63.1 ± 4.2)	(56.2 ± 3.3)
Verapamil + CD	68.4 ± 4.0	48.2 ± 3.2	40.4 ± 3.2	26.6 ± 2.7	11.0 ± 1.3
	(94.2 ± 1.4)	(88.1 ± 2.6)	(69.1 ± 4.3)	(59.9 ± 2.9)	(57.4 ± 3.1)

Liposomes were incubated in the presence or absence (values in parentheses) of serum at 37°C.

mass ratio, and drug choice can interfere with bilayer formation and its stability (14). Often, it is difficult or impossible to achieve a sustained-release effect in vivo for lipophilic drugs due to the rapid redistribution of the drug into other lipophilic compartments (such as lipoproteins). That is why the concept of drugs-in-cyclodextrins-in-liposomes presented improvement in both carrier systems by combining the advantages of the two systems into a single advantageous system. It enables the increase in drug-to-lipid mass ratio for lipophilic drugs since the complex is incorporated into an aqueous part of vesicle (5).

It is known that CD can remove the lipid components from cell membranes by forming inclusion complexes with the lipid (4). In that way, liposome bilayers could be destabilized and drug released in the presence of blood or even buffer (5). As in previous studies (7), the leakage of the entrapped drug was much less in the presence of PBS only. Data indicate that the presence of CD, even when not complexed with the drug, increases the stability of liposomes in the presence of serum.

HPβCD, unlike natural cyclodextrins, is surface active and has a high aqueous solubility and potential as an excipient for parenteral and oral routes. It was shown to have a stabilizing effect in the spray-drying of a model protein, enabling the retention of protein catalytic activity (1). In our case, it seemed to improve the recovery of spray-dried product and served as a serum stability enhancer, increasing the retention of originally entrapped drug (especially hydrophilic drug) in the presence of serum.

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