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Designing a novel in vitro drug-release-testing method for liposomes prepared by pH-gradient method

Koji Nakamura, Keisuke Yoshino*, Keiko Yamashita, Hiroaki Kasukawa

Terumo Corporation, R&D Center, 1500 Inokuchi, Nakai-machi, Ashigarakami-gun, Kanagawa 259-0151, 4 Japan

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ABSTRACT

In order to evaluate the drug-release behavior from pH-gradient liposomal formulation, a simple release-testing method without using biological components was newly designed on the basis of inversed ammonia gradient principle. Various factors influencing drug-release (releasing factor) were examined. As a result, releasing factor's concentration, pH, osmolarity in test fluid, and releasing factor's structure were found to be the critical factors to be optimized. Various vincristine-loaded liposomes with different lipid compositions or with different lipid/cholesterol ratio were tested for drug-release behavior and successfully obtained drug-release profiles reflecting differences in the physicochemical properties of individual liposomes. Furthermore, since the comparative release study of vincristine-loaded liposomes and doxorubicin-loaded liposomes could reproduce the phenomena as other researchers recently reported, a possibility was suggested for the proposed method to estimate the physicochemical status of drug inside of liposomes. Proof of concept study concluded, as a whole, that the novel release-testing method would be useful for a formulation study and also useful as a tool for the quality assurance or quality control in the manufacturing of pH-gradient liposomal products.

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1. Introduction

Currently, studies on drug delivery systems have great attention to achieve the selective delivery and distribution of drugs to the target disease sites for increasing therapeutic activity and/or reducing drug-related toxicities. Various types of particulate systems such as liposomes, emulsions, lipid microspheres, and polymeric nanoparticles have been extensively studied in the past few decades and recognized as effective drug delivery systems (Nishioka and Yoshino, 2001; Müller et al., 2000; Fenske and Cullis, 2008; Torchilin, 2005; Samad et al., 2007). Of those, liposomal delivery system may be the most progressed formulation technology, and many products are widely used in clinics or are being evaluated in clinical trials. Doxil®, doxorubicin-loaded PEGylated liposome, is one of the most sophisticated liposomal products, which has been approved in more than 80 countries due to its highly improved therapeutic effect (Mayer et al., 1990; Haran et al., 1993; Vaage et al., 1993; Alberts and Garcia, 1997; Campos et al., 2001; Gabizon, 2001; Johnston and Kaye, 2001; Gabizon et al., 2003).

The manufacturing of this product includes a pH-gradient method to achieve high drug loading efficiency. Although the precise mechanism is not well understood, the therapeutic activity of liposomal formulation should be brought about by drug-release from regionally localized liposomes rather than the encapsulated form. In the case where the rate of liposome accumulation is rapid, the drug-release could be the dominating factor controlling biological activity. Therefore, to perform an optimization study of liposomal formulation and quality assurance of the final product, setting up of appropriate evaluation methods is quite important, not only for deliverability to the target site but also for the drug-release characteristics thereafter. In this sense, the evaluations for particle-size distribution, surface charge, thermodynamic stability, biostability, and drug-release property are essential.

However, the testing methods for some of those characteristics have not yet been well established and are still to be discussed. Especially, drug-release-testing is one of the most unharmonized and challenging tests even though a modified 2-compartment method and dialysis method are sometimes applied in literature. In general, the release of a drug from liposomes is influenced by an external environment and physical and chemical characteristics of a liposomal preparation; so, it should preferably be evaluated under a biomimetic environment to achieve in vivo/in vitro correlation. Especially, in case of pH-gradient liposomes, ordinary release-testing method using phosphate-buffered saline cannot be applied because the drug-release is not dominated by simple diffusion but by pH-gradient-related diffusion (Yamauchi et al., 2007; Krishna et al., 2001; Charrois and Allen, 2004a). In order to evaluate the drug-release characteristics of liposomal

* Corresponding author. Tel.: +81 465 81 4187; fax: +81 465 81 4114.
E-mail address: Keisuke_Yoshino@terumo.co.jp (K. Yoshino).

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formulations at a laboratory level, blood serum and plasma are often used as the test fluids in place of phosphate-buffered saline. However, this is not appropriate for routine use for the purpose of quality control or quality assurance of liposomal products because maintaining the standards of quality of biological components becomes a difficult issue. On the basis of the above reason, a simple method without the use of any biological components is required to evaluate the release profiles of pH-gradient liposomes.

The main purpose of our study is to establish the in vitro testing method for direct evaluation of the drug-release characteristics of liposomal preparations, without using biological components, thereby enabling accurate and highly reproducible test results to confirm the drug-release from liposomal preparations on a lot-to-lot basis.

In the present study, we attempt to propose a novel release-testing method for pH-gradient liposomes on the basis of the inversed ammonia gradient principle, which has been discussed in previous reports (Maurer et al., 1998; Zhigaltsev et al., 2006). The parameters affecting drug-release from pH-gradient liposomes are examined. Doxorubicin and vincristine are used as model liposomal drugs, and the mechanism of the drug-release from pH-gradient liposomes is discussed.

2. Materials and methods

2.1. Materials

Hydrogenated soybean lecithin (HSPC) was purchased from Lipoid GmbH (Ludwigshafen, Germany), cholesterol (Chol) was purchased from Solvay Pharmaceuticals (Nieuweweg, Netherlands), and dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), and distearoylphosphatidylethanolamine-PEG (M_w: 5000) (PEG_{5000}-DSPE) were purchased from NOF Corporation (Tokyo, Japan). Doxorubicin hydrochloride (DXR) was purchased from Boryung Pharmaceuticals Co., Ltd. (Seoul, Korea). Vincristine sulfate (VCR) was purchased from Changzhou Leo Chemical (Jiangsu, China). Other chemicals were purchased from Kanto Chemicals Co., Inc. All the other chemicals used were of analytical grade.

2.2. Preparation of PEGylated liposomes for the drug loading

HSPC (DSPC, DPPC, or DMPC) and Chol were dissolved in ethanol (1 ml) with heating at around 70 °C. The inner aqueous solution at pH 2.0 (9 ml), 250 mM citrate buffer, and sucrose were added to control the osmotic pressure at 500 mOsm and heated at around 70 °C, and this solution was added to the lipid dissolved ethanol solution, yielding the crude liposomes. The crude liposomes were subjected to extrusion for 2 times with a 0.2-μm membrane filter, and 10 times with a 0.1-μm membrane filter, to control the size around 100 nm.

Additionally, a PEG_{5000}-DSPE solution at a concentration of 40 mg/ml was added to obtained liposomes to be 0.75 mol% PEG_{5000}-DSPE of total lipid amounts and heated and incubated at 65 °C for 30 min to obtain PEG-modified liposomes (PEGylated liposomes). To incorporate the drug into PEGylated liposomes, pH-gradient method was selected in this study. To create the pH-gradient between the outside and inside of the liposomes, 10% sucrose/10 mM histidine solution (pH 6.5) was used as the outer aqueous solution. Afterwards, the PEGylated liposomes were applied on Sepharose 4 fast flow gel column (GE Healthcare, UK) to exchange outer aqueous solution for 10% sucrose/10 mM histidine (pH 6.5) to generate the pH-gradient.

2.3. Drug loading into PEGylated liposomes for the drug loading

In this study, vincristine (VCR) and doxorubicin (DXR) were used as model drugs. The drugs were dissolved in water at a concentration of 10 mg/ml. The drug solution and PEGylated liposomes were mixed to the given weight ratio and heated for 30 min at 60 °C. The unencapsulated drug was removed by Sepharose 4 fast flow gel column.

Finally, the suspension was filtered with a 0.2-μm membrane (Minisart Plus, Sartorius, Goettingen, Germany) to obtain drug-loaded PEGylated liposomes. The drug in the liposomes was determined by following the methods reported previously.

2.4. Analysis of DXR concentration

The total and unencapsulated DXR concentration in the preparation was determined by the high-performance liquid chromatography (HPLC). Briefly, 100 μl of DXR liposomes was added to 2 ml of methanol and completely dissolved. Separately, DXR was dissolved in methanol and made solutions with different concentrations by dilution with methanol. Unencapsulated DXR in liposomes was separated from the DXR liposomes by ultracentrifugation at 100,000 × *g* and 10 °C for 180 min after the DXR liposomes were diluted 10 times with saline. The supernatant was collected and used as the sample for measurement. Separately, DXR was dissolved in water and made into solutions with different concentrations. The analysis for DXR was performed by following HPLC condition.

HPLC condition was as follows: column; INTERSIL ODS-2 (4.6 × 250 mm, 5 μm); measuring wavelength, 254 nm; column temperature, 40 °C; mobile phase, phosphate solution containing sodium lauryl sulfate/acetonitrile = 1/1; flow rate, approximately 1.0 ml/min.

2.5. Analysis of VCR concentration

The total and unencapsulated VCR concentration in the preparation was determined by HPLC method. Briefly, 100 μl of VCR liposomes was added to 2 ml of methanol and completely dissolved. Separately, VCR was dissolved in methanol and made into solutions with different concentrations. Unencapsulated VCR in liposomes was separated from the VCR liposomes by ultracentrifugation at 100,000 × *g* and 10 °C for 180 min after the VCR liposomes were diluted 10 times with saline. Supernatant was collected and used as the sample for measurement. Separately, VCR was dissolved in water and made into solutions with different concentrations. On above prepared solutions, the analysis for VCR was performed by following HPLC condition.

HPLC condition was as follows: column; INTERSIL C8 (4.6 × 250 mm); measuring wavelength, 298 nm; column temperature, 40 °C; mobile phase, phosphate solution/diethylamine/methanol = 59/1/140; flow rate, approximately 1.0 ml/min.

2.6. The drug-release test from liposomes

The release medium consisted of phosphate buffer, NaCl for maintaining osmolarity, and ammonium acetate for triggering the drug-release. The determined pH of release media was adjusted with 0.1 M HCl or NaOH. Liposomes (100 μl) were added to 1 ml of the release media warmed at 37 °C and put into the water bath at 37 °C. As a function of time, the sample was taken from water bath and 300 μl of it added to 3 ml of pH 3.0 phosphate buffer solutions. Finally, the released drug was separated from the drug-loaded liposomes by ultracentrifugation at 100,000 × *g* and 10 °C for 180 min

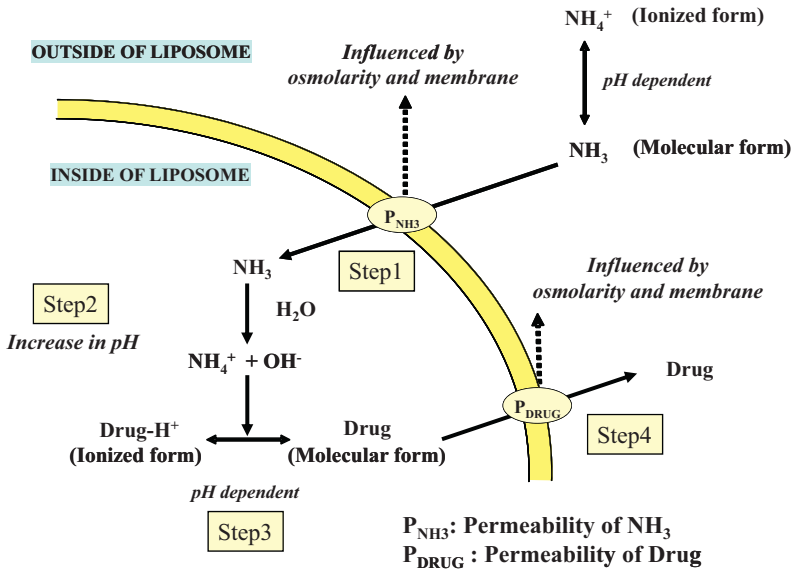

Fig. 1. Estimated drug-release mechanism from pH-gradient liposomes.

and determined the concentration of the released drug by following the analytical method of drug described elsewhere.

3. Results and discussion

3.1. Basic concept to achieve the drug-release from pH-gradient liposomes

The drug loading by pH-gradient method into a liposome is generally thought to be applicable to those drugs that can be dissociated in aqueous medium. When the pH-gradient is formed between the inner and outer sides of liposomes, a free form of drug molecule in the outer aqueous phase permeates the liposome membrane to be entrapped internally in an ionized form. In the general pH-gradient method, ammonia or ammonium-related substance is used to generate pH-gradient for loading of basic drugs. Thus, our strategy for drug-release-testing is on the basis of the principle that the drug-release process runs in the completely opposite way against the drug-loading process. The overall process of the drug-release for liposomal basic drug is illustrated in Fig. 1.

The most distinctive feature of the proposed method is that in the pH-gradient method, the drug is loaded into the inner aqueous phase of liposomes, whereas in this method, the ammonia molecule moves into the inner aqueous phase instead of the drug, resulting in a rise of inner pH to trigger drug-release. In general, in pH-gradient method, ammonia is used to generate a pH gradient for drug loading. However, in this method, ammonia is used to eliminate the pH gradient between the inner and outer aqueous phases to cause drug-release. So, it is an "inversed ammonia-gradient method." Although the principle has been known from previous works (Maurer et al., 1998; Zhigaltsev et al., 2006), the release-testing method has not been validated yet.

To achieve this process, a specific factor influencing drug-release (releasing factor) is required to be transferred into the inner aqueous phase of the liposomes (Step 1, Fig. 1), and it makes the inside pH increase (Step 2). Then it makes the drug form convert from the ionized form to the molecular form (Step 3) and makes the drug-release outside (Step 4). To verify the each steps proposed above, ammonium or low-molecular-weight ammonia-derivative compounds were used as releasing factors and various studies were conducted. For this experiment, various types of pH-gradient PEGylated liposomes of VCR and DXR were used (VCR liposomes and DXR liposomes). Lipid composition and drug concentration of each liposome are listed in Table 1.

3.2. Influence of the ammonium concentration on the drug-release from pH-gradient liposomes

As the first step, we examined the influence of ammonium concentration added in the test fluid on the drug-release behavior using VCR liposomes (LIP1). For this experiment, ammonium acetate was used as the donor of ammonium, which generates ammonia molecules in the test fluid according to the following equilibrium equations: $AcNH_4 \leftrightarrow Ac^- + NH_4^+$, and $NH_4^+ + OH^- \leftrightarrow NH_3 + H_2O$. As shown in Fig. 2, the drug-release was clearly accelerated in response to the ammonium concentration, as expected. It is notable that only less drug-release was observed at lower concentrations (5 mM, 10 mM, and 25 mM). This phenomenon seems reasonable when the ionic strength was considered. Namely, to attain drug-release, the inner pH had to be increased until the drug form was converted from the ionized form to molecular form as shown in Fig. 1. At lower ammonium concentration, however, enough amount of ammonia could not be supplied inside to increase the inner pH. From the results of this study, it was proved that the ammonium

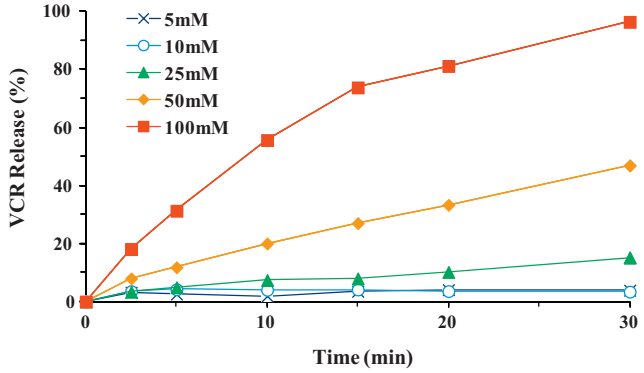

Fig. 2. Vincristine sulfate (VCR) release kinetics from VCR liposomes in release media with different ammonium ion concentrations generated by ammonium acetate, with the final osmolarity adjusted to 300 mOsm by NaCl. The observed pH was 7.0. Ammonium ion concentrations: ×, 5 mM; ○, 10 mM; ◊, 25 mM; △, 50 mM; □, 100 mM.

Table 1
Lot number and composition of liposomal formulations tested.

Lot No.	Lipid	Lipid:Chol (molar ratio)	Drug	Drug conc. (mg/ml)	Fig. #
LIP1	HSPC	54:46	VCR	1.23	Figs. 2, 3 and 6
LIP2	HSPC	54:46	VCR	1.37	Fig. 4
LIP3	HSPC	54:46	VCR	0.96	Fig. 5(A)
LIP4	HSPC	65:35	VCR	0.94	Fig. 5(A)
LIP5	DSPC	54:46	VCR	1.10	Fig. 5(B)
LIP6	DPPC	54:46	VCR	0.93	Fig. 5(B)
LIP7	DMPC	54:46	VCR	0.79	Fig. 5(B)
LIP8	HSPC	54:46	DXR	0.81	Fig. 6

concentration is an important factor to trigger drug-release, and also it was found that a minimum concentration of ammonia in the release media is required to attain sufficient drug-release.

To investigate the drug-release mechanism from pH-gradient liposomes in more detail, the drug-release studies were performed with varying pH and osmolarity of release media under a fixed ammonium concentration (50 mM) using VCR liposomes (LIP1).

As seen in Fig. 3(A), enhanced drug-release was observed under high pH conditions (pH 7.0 and 8.0), whereas drug-release was not observed at low pH (pH 4.0, 5.0, and 6.0). It is considered that the amount of ammonium, which cannot diffuse into liposomal membrane, increased at low pH, consequently, the inner pH did not increase to enough level for the drug-release to be triggered. As seen in Fig. 3(B), the drug-release was accelerated almost in a direct proportion to decreasing osmolarity. It is well known that drug permeability through the cellular and mucosal membranes is enhanced by increased osmolarity due to the solvent-drag effect. It is assumed that the enhanced drug-release is due to an increase in the accumulated amount of ammonia into liposomes induced by the solvent-drag effect caused by osmolarity difference between the inside and the outside of liposomes.

From the results above, it is considered that the observed enhanced drug-release is strongly related not only to the ammonia concentration but also to the pH and osmolarity of the release media. This would be the evidence to prove the drug-release mechanism that we estimated in Fig. 1.

3.3. *Influence of the structure of releasing factor on drug-release from pH-gradient liposomes*

Specific factors, such as ammonia, act as a trigger of drug-release. This effect is brought about from the distinctive physicochemical property of ammonia molecules, that is, high permeability of liposomal membrane and increased pH in the inner aqueous phase by protonization. To find an alternative releasing factors candidate and to make the role of releasing factors clearer, 3 low-molecular-weight amino compounds, diethylamine, 2-aminoethanol, and ethylenediamine were examined for enhancing effects on drug-release from pH-gradient liposomes. The key parameters of these amino compounds, their chemical structure, molecular weight (M_w), logarithmic acid dissociation constant (pK_a), and logarithmic octanol/water partition coefficient (Log P) are summarized in Table 2, together with those of ammonia. Fig. 4 shows the comparison of drug-release behavior from VCR pH-gradient liposome (LIP2) with various releasing factors. As was seen, ammonia and diethylamine showed the fastest drug-release, meaning that both compounds had a high potency of causing the

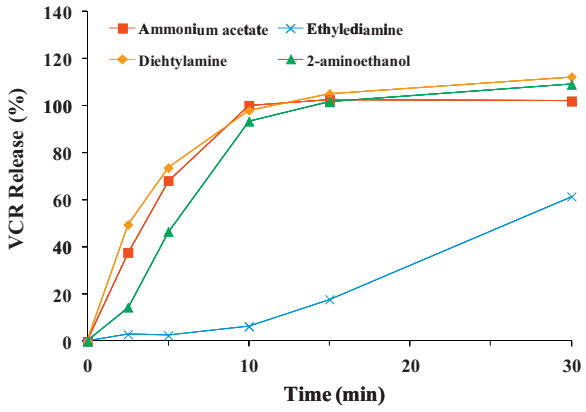

Fig. 4. Change in release kinetics of vincristine sulfate (VCR) from liposomes in the presence of 50 mM of various amino factors influencing drug-release into the release media based on phosphate buffer. pH was adjusted to 7.4 by 1 M HCl and the osmolarity was adjusted to about 250 mOsm. □, Ammonium acetate; × ethylenediamine; ◊, diethylamine; △, 2-aminoethanol.

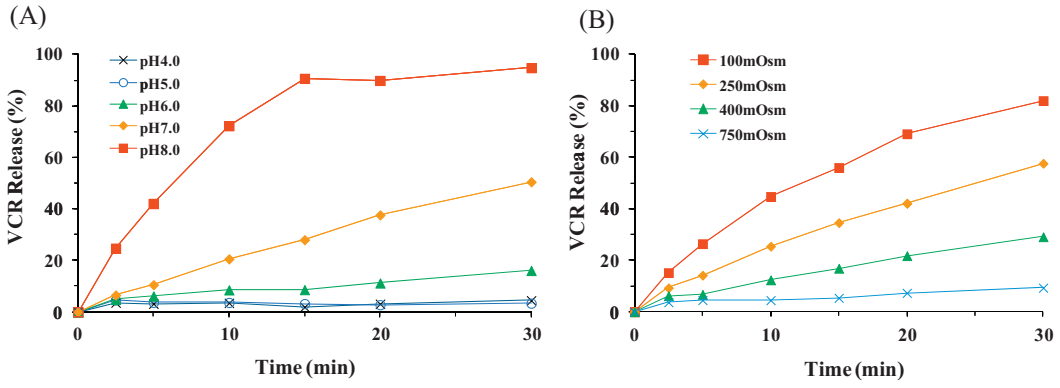

Fig. 3. (A) Vincristine sulfate (VCR) release kinetics from VCR liposomes in solutions with different pH, 50 mM ammonium ion concentration generated by ammonium acetate, and the final osmolarity adjusted to 300 mOsm by NaCl. pH was adjusted by 1 M HCl or NaOH. pH: ×, 4.0; ◯, 5.0; ◊, 6.0; △, 7.0; □, 8.0. (B) Vincristine sulfate (VCR) release kinetics from VCR liposomes in release media with different osmolarity, 50 mM ammonium ion concentration generated by ammonium acetate, and the final osmolarity was adjusted by NaCl. The observed pH was 7.0. Osmolarity: □, 100 mOsm; ◊, 250 mOsm; △, 400 mOsm; × 750 mOsm.

Table 2
Comparison of properties of various ammonia-related releasing factors.

Name	Ammonia	Diethylamine	2-Aminoethanol	Ethylenediamine
Molecular Structure	NH_3	$CH_3CH_2NHCH_2CH_3$	$NH_2CH_2CH_2OH$	$NH_2CH_2CH_2NH_2$
Release[a]	Fast	Fast	Medium	Slow
M_w	17.0	73.1	61.1	60.1
pK_a	9.3	10.9	9.5	10.7, 7.6
Log P[b]	N/A	0.58	−1.31	−2.04

[a] Rating is based on the results shown in Fig. 4.
[b] Log P values are quoted from ACD/Labs' ACD/PhysChem Suite.

drug-release. The release behavior of both liposomes was almost the same though the M_w of diethylamine is 3 times higher than that of ammonia. However, although the M_w of 2-aminoethanol, diethylamine, and ethylenediamine were the same, the drug release was quite different among them, with an observed ranking order of release rate of diethylamine > 2-aminoethanol > ethylenediamine. Meanwhile, the drug releases were found to be accelerated with higher Log P (diethylamine) and reduced with lower Log P values (2-aminoethanol and ethylenediamine). From the above results, we considered that the drug release profile would be affected rather by Log P, a parameter indicating the hydrophobicity of the releasing factors.

These drug-release experiments did not always provide enough information clearly elucidating the role of releasing factors for triggering drug-release, but it is suggested that membrane permeability would be the dominant factor for triggering drug-release. From this point, low molecular weight and simple amino compounds like ammonia could be feasible for practical use as an alternative candidate for releasing factors.

Through the preliminary dissolution studies above, it was confirmed that the hypothesis for the drug-release mechanism shown in Fig. 1 is reasonable. Also, it was found that, at least pH, osmolarity, and releasing factor's structure and its concentration in the testing fluid are important factors to influence the drug-release behavior. Considering the results of the preliminary studies above, the following conditions were set up for the next study:

1. Release media of pH 7.0: phosphate buffer, including 50 mM ammonium acetate and NaCl to adjust the osmolarity to 300 mOsm.
2. Sample preparation temperature, 37 °C ± 1 °C.
3. Sample preparation for analysis: after taking the release sample and diluting the pH 3.0 phosphate buffer by 10 times to stop the release reaction, the sample is subjected to ultracentrifugation to collect only the free drug.

3.4. Evaluation of release behavior of various pH-gradient liposome preparations

Liposomes are mainly composed of phospholipids and cholesterol; their physicochemical property is mostly explained by the phase-transition temperature of the phospholipid and the release behavior is similar to its parameter. The phase-transition temperature is measured by differential scanning calorimetry (DSC) of pure phospholipid. When cholesterol was included into the phospholipid, its transition temperature shifted. At more than 20% of cholesterol, the phase-transition temperature completely diminished (Genz et al., 1986). Therefore, the precise transition temperature of liposomes, including cholesterol, is unknown. However, even small changes in the physicochemical property could cause a huge impact on drug-release from liposomes and pharmacokinetics. Specifically, the drug-leakage rates play an important role in the therapeutic activity and toxicity of liposomal drug formulations (Charrois and Allen, 2004b). So, to keep constant the quality of the liposomal product by assuring adequate release kinetics, a release-testing method for evaluating differences in the physicochemical property caused by lipid compositions or variations of lipid quality is required.

Thus, to examine the applicability of the proposed testing method, drug-release studies were performed using various model liposomal preparations, each of which differs in lipid composition and drug species. The results are compared in Fig. 5. In the comparison of release behavior of 2 model liposomes (LIP3 and LIP4), it was clearly found that drug-release from LIP3 was much faster than from LIP4. Considering the fact that both liposomes are only different in cholesterol content (Table 1), the observed difference should be related to rigidity of the liposomal membrane. Actually, lower cholesterol content is known to give higher glass transition temperature (T_g) resulting in a more rigid membrane. The result of release study is thought to well reflect this generally recognized fact.

Fig. 6 shows the comparison of release behaviors of VCR from 3 liposomal formulations (LIP5, LIP6, and LIP7), each of which were of prepared using different types of lipids at an identical content, DSPC for LIP5, DPPC for LIP6, and DMPC for LIP7, respectively. As was shown, the drug-release from the liposomes became faster in the order of LIP7, LIP6, and LIP5. Considering the fact that T_gs of the lipids are in the order of DSPC > DPPC > DMPC in accordance with the chain length of the fatty acid, the observed difference in release rate is thought to well reflect the order of T_g of the lipid applied. Thus, all the data in Fig. 6 strongly imply that the drug-release is accelerated when the permeability of the lipid membrane is high, and the proposed release-testing method herewith can successfully distinguish the difference in drug-release characteristics.

The recent report demonstrated that doxorubicin-loaded PEGylated liposomes, prepared by varying the phosphatidylcholine species showed a different in vivo behavior and that clearance from the blood could be correlated with T_g of phosphatidylcholine (Egg PC > DPPC > HSPC), reflecting increased membrane permeability

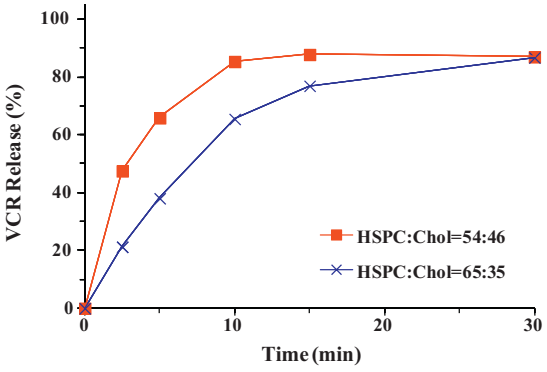

Fig. 5. Release kinetics of vincristine sulfate (VCR) from liposomes in release media, 50 mM ammonium ion concentration generated by ammonium acetate, and the final osmolarity was adjusted to 300 mOsm by NaCl. The pH was adjusted to 7.0 by 1 M HCl. □, hydrogenated soybean lecithin (HSPC): cholesterol (Chol) = 54:46; × HSPC:Chol = 65:35.

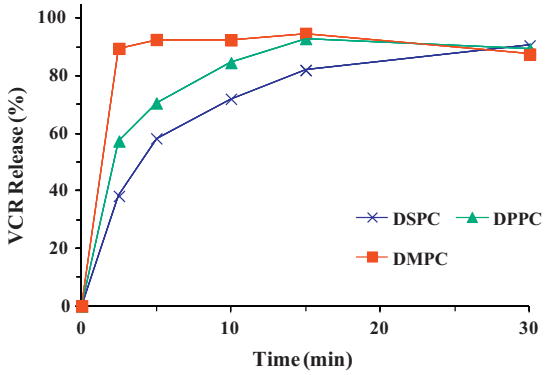

Fig. 6. Release kinetics of vincristine sulfate (VCR) from liposomes in release media, 50 mM ammonium ion concentration generated by ammonium acetate, and the final osmolarity was adjusted to 300 mOsm by NaCl. The pH was adjusted to 7.0 by 1 M HCl. □, Dimyristoylphosphatidylcholine (DMPC); △, dipalmitoylphosphatidylcholine (DPPC); × distearoylphosphatidylcholine (DSPC).

(Gabizon et al., 1993). This must be a typical example suggesting that the physicochemical property of liposomes largely influences its pharmacokinetic behavior in vivo. However, as described above, it was quite difficult to estimate the phase transition temperature of liposomes due to coexisting cholesterol and/or other additives. So far, in formulation studies of pH-gradient liposomes, it has been difficult to evaluate the difference in physicochemical properties of liposomal candidate preparations using various types of phospholipids and cholesterol at different ratios. However, the release-testing method proposed here enable not only to evaluate comprehensively the physicochemical properties but also to estimate its biological behavior without conducting biological evaluations.

3.5. Influence of drug structure on the drug-release from pH-gradient liposomes

To examine the effect of drug species on the release kinetics, drug-release studies were performed using 2 models of liposomal preparations of VCR and DXR (LIP1 and LIP8), both of which had the same lipid composition. The release profiles obtained are compared in Fig. 7. As was seen, DXR liposomes did not release the drug for over 30 min, whereas VCR liposomes provided relatively faster release profile. It was reported that the DXR liposomes provided almost no release when lipids of high transition temperatures were used (Charrois and Allen, 2004b; Maurer-Spurej et al., 1999; Li et al., 2000). On the other hand, VCR liposomes released the drug content in a relatively sustained manner (Zhigaltsev et al., 2005). This apparent difference could be due to the difference in permeability of the drug through the liposomal membrane and/or the difference in the physicochemical state of the entrapped drug in the liposomal interior. In fact, many researchers argued this point and suggested the possibility that DXR could get deposited inside the liposomes by forming a citric salt with citrate, which is used as a buffering agent of the interior aqueous phase, and this precipitation makes the DXR release less (Lasic et al., 1992, 1995; Li et al., 1998). However, VCR could not make a rigid citric salt between citrate and the ionized form of VCR, thereby yielding a faster release (Johnston et al., 2006). To discuss both differences in more detail, the pharmacokinetics study was conducted using DXR and VCR liposomes (Fig. S1). As it was seen, the percent dose of DXR liposomes after injection was higher than that of VCR liposomes in all periods. This means that the drug release from VCR liposomes is faster than from DXR liposomes in vivo, as well as in vitro. Since the phenomena shown in Fig. 7 could well be consistent with the data shown in Fig. S1 and also in other reports (Zhigaltsev et al., 2005; Lasic et al., 1992, 1995; Li et al., 1998; Johnston et al., 2006), the proposed drug-release-testing method could be an effective means to estimate the pharmacokinetics of liposomes in vivo without using any biological components.

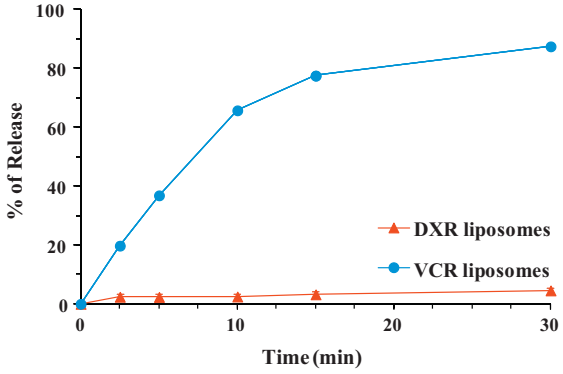

Fig. 7. Release kinetics comparisons between vincristine sulfate (VCR) liposomes (○) and doxorubicin hydrochloride (DXR) liposomes (△) in release media, 50 mM ammonium ion concentration generated by ammonium acetate, and the final osmolarity was adjusted to 300 mOsm by NaCl. The pH was adjusted to 7.0 by 1 M HCl.

As mentioned earlier, drug permeation through the membrane and/or the physicochemical status of the drug precipitated in the internal phase still remain as possible reasons for drug retention. Zhigaltsev et al. demonstrated that the drug release profiles of liposomes of vincristine and vinorelbine were clearly different in a 50% FBS–saline solution, despite no differences in their Cryo-EM micrographs and their similarity in chemical structure. From those facts, they finally concluded that the observed differences in retention of both vinca drugs could be related to the lipophilicity of the drug, where the more hydrophobic drugs were released more rapidly (Zhigaltsev et al., 2005). To confirm their findings, we conducted small-scale preliminary drug release studies for both vinca alkaloid-loaded liposomes using our new testing method, and consequently, an analogous behavior was observed (data not shown). This behavior suggests that the observed difference would mainly arise from the difference in partition coefficients influencing drug permeability. Also, we presume that the release of drugs, which form rigid complex inside the liposomes, has 2 limitation steps: drug dissolution in the interior compartment of the liposomes and drug permeation through the liposomal membrane. Even though optimization of various factors is required for product-by-product examination, proof of concept of a novel release-testing method was successfully achieved Even though optimization of various factors is required for product-by-product examination; proof of concept of a novel release-testing method was successfully achieved.

4. Conclusions

We constructed a prototype of a novel release-testing method for pH-gradient liposomes on the basis of inversed ammonia-gradient principle. Through the present studies, it was demonstrated that the proposed method would be applicable as a simple and reproducible release-testing method. Also, it was confirmed that the release profiles obtained well reflected the differences in various formulation factors, such as drug species, lipid composition, cholesterol content, pH, osmotic pressure, and else. As a conclusion, it can be said that the proposed novel release-testing method would be useful for a formulation study and as a tool for the quality assurance or the quality control in the manufacturing of pH-gradient liposomal products.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpharm.2012.04.011.

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