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Encapsulation of an antivasospastic drug, fasudil, into liposomes, and in vitro stability of the fasudil-loaded liposomes

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Abstract

The objectives of this work were to develop a liposomal fasudil, an antivasospastic drug, as a possible means to deliver the encapsulated drug to the brain, and to characterize the stability of the liposomal formulation in vitro. Transmembrane electrochemical gradients of H^+ or ammonium sulfate were created, and their effect on the uptake of fasudil into preformed hydrogenated soy phosphatidylcholine/cholesterol (HSPC/CHOL) liposomes were examined. Fasudil was successfully loaded into preformed liposomes in response to sulfate ion (SO_4^{2-}) and, in part, by H^+. Encapsulation levels approaching 100% could be achieved up to a drug to lipid ratio of 0.364 (mol/mol). A stability study of the fasudil-loaded liposomes was performed by storage at 4 °C in 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES)-buffer (pH 7.4) and by incubation in human cerebrospinal fluid (CSF) at 37 °C. The formulations were stable with respect to drug retention as well as size alteration, for the period studied. A leakage study clearly showed the sustained release properties of the fasudil-loaded liposomes in human CSF. We recently reported that the intrathecal administration of liposomal fasudil significantly decreased ischemia, with no obvious adverse effect in a rat model [Neurol. Med. Chir. 41 (2001) 109]. Taken together, efficient encapsulation of fasudil into preformed liposomes, their long-term stability at 4 °C and the sustained release characteristics in CSF indicate that fasudil-loaded liposomes could be potential candidates for further clinical evaluation.

Keywords: Liposomes; Drug encapsulation; Fasudil; Remote-loading method; Ammonium sulfate gradient

Abbreviations: CF, 5(6)-carboxyfluorescein; CHOL, cholesterol; CSF, cerebrospinal fluid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HSPC, hydrogenated soy phosphatidylcholine; PL, phospholipid; Tc, phase transition temperature.

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1. Introduction

Fasudil hydrochloride (1-(5-isoquinolinesulphonyl)-homopiperazine hydrochloride or HA 1077) (Fig. 1), a recently developed antivasospatic drug (Asano et al., 1987), is an inhibitor of protein kinases, myosis light chain kinase, cyclic AMP-dependent protein kinase (Asano et al., 1989) and protein kinase C (Seto et al., 1995). Fasudil improves cerebral haemodynamics (Satoh et al., 1991) and inhibits the production of superoxide anions in neutrophils (Arai et al., 1993). The administration of the drug via an intrathecal route would be expected to be more effective than via an intravenous route. However, intrathecal injection of fasudil is not viable route, because of side effects (Shibuya et al., 1992). A formulation of fasudil which would allow intrathecal injection and achieve good retention of large amounts of the compound for a therapeutic window would greatly increase the utility of this compound in clinical settings.

Liposomes have considerable potential as potent drug carriers. Recently, a liposome formulation of the anti-cancer drug, doxorubicin, has been approved for Kaposi's sarcoma or ovarian carcinoma (Martin, 1998). The encapsulation of drugs into liposomes alters their pharmacokinetics and biodistribution, resulting in an increase in therapeutic efficacy and/or a decreased toxicity (Allen et al., 1995; Allen and Stuart, 1988). Sustained release property is one of the benefits in the use of liposomes as drug carriers. We recently reported that a liposomal formulation of fasudil was beneficial in reducing side effect and increasing therapeutic efficacy of the drug in a rat acute ischemia model, probably due to the sustained release of the drug in cerebrospinal fluid (CSF) following intrathecal injection (Takanashi et al., 2001). However, the physicochemical properties of fasudil-encapsulated liposomes remained to be understood.

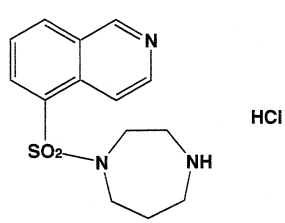

Fig. 1. Chemical structure of fasudil hydrochloride.

Therefore, our objectives in this study are to evaluate the stability of fasudil encapsulated liposomes and release kinetics of the encapsulated drug in vitro in order to develop more suitable fasudil-encapsulated liposomes, not only as possible means to deliver the encapsulated drug to the brain, but also to meet pharmaceutical demands such as large-scale production and long-term stability. In order to improve the encapsulation efficiency of fasudil, we employed remote (active) loading methods, in which drug molecules are loaded into preformed liposomes using potential differences across liposomal membranes such as a proton gradient (Mayer et al., 1993; Bolotin et al., 1994). The uptake behavior of fasudil in preformed liposomes in response to transmembrane electrochemical ion gradient was characterized. The stability study was performed with respect to drug retention and size change in the prepared formulations during storage at 4 °C. In addition, the kinetics of the leakage of liposomal contents (fasudil or fluorescent dye) in human CSF was examined.

2. Materials and methods

2.1. Materials

Hydrogenated soy phosphatidylcholine (HSPC) was a generous gift from Nippon Oil and Fat, Co (Kanagawa, Japan), and Fasudil hydrochloride was a generous gift from Asahi Chemical Industry, Co (Tokyo, Japan). Cholesterol (CHOL) was of analytical grade (Wako Pure Chem., Osaka, Japan). 5(6)-Carboxyfluorescein (CF) was purchased from Eastman Kodak Co (NY, USA). Human CSF was purchased from Sigma Chemical, Co (MO, USA). Sephadex G-50 and Sepharose CL-4B was purchased from Pharmacia Biotech (Uppsala, Sweden). All other chemicals were of analytical grade purity.

2.2. Preparation of liposomes

Liposomes were composed of HSPC:CHOL (2:1, molar ratio). Briefly, dried lipid film was hydrated in an optimal buffer at a phospholipid (PL) concentration of 20 mM. For the preparation of CF-containing liposomes, the dried film was hydrated in a CF solution (10 mM). The resulting preparations were then passed repeatedly, under pressure, through 400, 200, 100, and 80 nm pore size polycarbonate membranes (Costar/Nuclepore, MA, USA) using an extruder. For CF-containing liposomes, the prepared liposomes were separated from unencapsulated dye (CF) by passing the liposomes over a Sephadex G-50 column equilibrated with 25 mM HEPES and 140 mM NaCl (HEPES buffer, pH 7.4). The mean diameters of prepared liposomes were determined using a NICOMP 370 HPL submicron particle analyzer (Particle Sizing System, CA, USA) and were in the range of 130 ± 10 nm.

2.3. Transmembrane proton and ammonium sulfate gradients

A transmembrane proton gradient was created by preparing liposomes in 300 mM citrate buffer (pH 3.0) and passing them over a Sephadex G-50 column which had been equilibrated with the 10% (w/v) sucrose solution containing 25 mM Trizma base (Sigma, MO, USA) (pH 8.0). Other transmembrane proton gradients were formed by preparing liposomes in 250 mM ammonium sulfate (pH 3.0–8.0) and passing them over a Sephadex G-50 column which had been equilibrated with a 10% (w/v) sucrose solution (pH 3.0–8.0).

2.4. Loading of fasudil into liposomes

Fasudil hydrochloride was added to the preformed liposomes at various drug:PL ratios (w/w), followed by incubation at 65 °C to monitor the uptake of the drug. At various times up to 4 h, aliquots (300 μl) of the mixture were removed, and the liposomes were therein separated from the loaded drug over a Sephadex G-50 column equilibrated with HEPES buffer (pH 7.4). The concentration of fasudil hydrochloride was determined spectrophotometrically ($\lambda = 320$ nm) (Shimadzu UV-1600, Kyoto, Japan). The PL concentration in the liposome solution was determined using the Bartlett colorimetric assay (Bartlett, 1959). Encapsulation efficiencies were calculated as the drug:PL ratio, after separation of the free drug, divided by the drug:PL ratio before incubation.

2.5. In vitro stability assay

Fasudil-loaded liposomes (5 mM PL) were mixed with either human CSF or HEPES buffer (pH 7.4) at a ratio of 1:9 (v/v). The resulting suspension was incubated at either 37 or 4 °C. Aliquots (300–500 μl) were withdrawn at specified time points for the determination of fasudil retention and replaced with an equivalent volume of either fresh CSF or HEPES buffer (pH 7.4). The fasudil-loaded liposomes were separated from the leaked drug by means of a Sephadex G-50 or Sepharose CL-4B columns, which had been equilibrated with HEPES buffer (pH 7.4). The PL in the liposome fractions was determined as described above. The drug in the liposome fractions was quantified by high performance liquid chromatography (HPLC) (TOSOH, SC-8020, Tokyo, Japan), following extraction of the drug into methanol. Each sample (50 μl) was injected onto a YMC-Pack C8 column (250×46 mm) (YMC, NY, USA). The column was run using isocratic eluent conditions (30% acetonitrile in H_2O, 0.05% trichloro acid) and a flow rate of 1 ml/min. Fasudil eluted at 3.8 min as detected by its ultraviolet absorbance at $\lambda = 320$ nm using a UV detector (UV-8020, TOSOH, Tokyo, Japan). Fasudil was quantified by comparing the fasudil peak areas to a standard fasudil curve ($r^2 = 0.985$). The retention of the drug in the prepared liposomes was calculated by dividing the drug to PL ratio at indicated time point by the initial drug to PL ratio.

The leakage of CF from CF-loaded liposomes in human CSF was assessed by determining the fluorescence intensity of leaked CF according to a previously described method (Funato et al., 1992).

3. Results and discussion

3.1. Effect of transmembrane electron chemical ion gradients on uptake of fasudil into preformed liposomes

The use of the remote-loading technique results in higher drug encapsulation efficiencies, compared with a passive liposome entrapment technique, in which the drug is included in the hydration buffer during liposome preparation. Weak base like doxorubicin or vincristine, which coexists in aqueous solutions in neutral and charged forms have been successfully loaded into preformed liposomes via the use of a pH gradient or an ammonium sulfate gradient (Bolotin et al., 1994; Mayer et al., 1986). Therefore, we expected that fasudil would be loaded into preformed liposomes by remote-loading technique since this drug is also a weak base. The intent of the first set of experiments was to evaluate how efficiently fasudil was accumulated into HSPC/CHOL liposomes (2:1, mol/mol) in response to a proton gradient (ΔpH of 5.0). As shown in Fig. 2, fasudil was loaded into preformed liposomes, but the loading efficiency was low at 10 min (0.1198 μmol fasudil hydrochloride per μmol PL, corresponding to an encapsulation efficiency of 27.5%, as calculated from the percentage of available drug which is encapsulated into liposomes). Another proton gradient (ΔpH of 5.0) in combination with ammonium sulfate (250 mM) was tested for loading fasudil into preformed liposomes (Fig. 2). The drug was rapidly accumulated and the magnitude of the achieved uptake was 0.364 μmol fasudil hydrochloride per μmol PL corresponding to an encapsulation efficiency 83.5% at 10 min. The encapsulation efficiency was 3.3-fold higher than that in the presence of a proton gradient alone. Interestingly, the loading processes were separated into a fast phase for drug uptake and then a slower phase for the efflux of drug, once it was loaded. Lipophilic drugs are able to permeate bilayer membranes orders of magnitude faster in the neutral (uncharged) form than in the charged form. An unprotonated amphipathic base crosses the liposomal membrane and is then protonated inside the liposomes (H^+ rich). Thus, fasudil

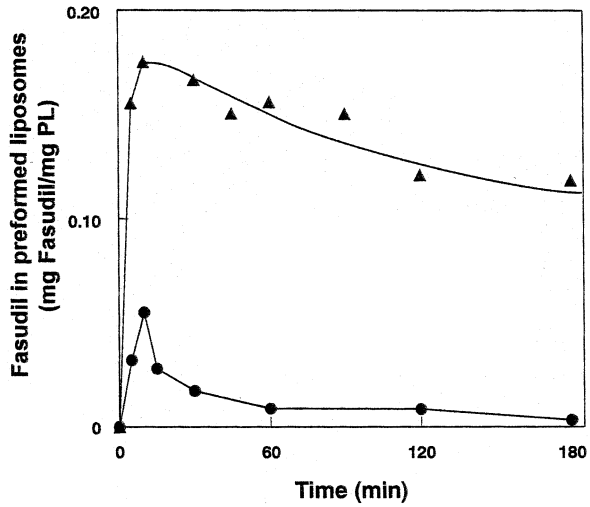

Fig. 2. Effect of differences in the internal buffer on fasudil uptake into preformed HSPC/CHOL liposomes. Liposomes were prepared in the presence of either 300 mM citric acid (pH 3.0) (●) or 250 mM ammonium sulfate (pH 3.0) (▲). The prepared liposomes were incubated in the presence of fasudil at 65 °C for the indicated periods after the external vesicle medium was exchanged with 10% (w/v) sucrose containing 25 mM Trizma base (pH 8.0). Fasudil, which was encapsulated in the preformed liposomes was determined as described in the Section 2.

molecules could be concentrated and trapped within the liposomes during incubation at higher temperature than the phase transition temperature (T_c) for HSPC (58 °C) (Uster et al., 1996). The efflux of highly protonated fasudil, once it was loaded during incubation, cannot be explained by simple or passive diffusion or by an increase in membrane permeability, since the efflux of doxorubicin, once loaded, was not observed under similar experimental conditions due to formation of water-insoluble, gel-like, complexes with SO_4^{2-} (Bolotin et al., 1994). It is known that some highly ionized compounds such as quaternary ammonium compounds form electrochemical neutral complexes with either anions or cations. It is generally assumed that these neutral ion-pair complexes cross the lipid membrane by passive diffusion (ion-pair transport), since the complex is soluble to water as well as lipid. This leads us to hypothesize that protonated fasudil molecules, inside the liposomes, form electrochemical neutral complexes with anions such as SO_4^{2-} and citric

acid, and the resulting complexes then begin to effuse by crossing the liposomal membrane during incubation at higher temperature than T_c for HSPC.

The issue of whether the transmembrane proton gradient acts as a driving force for fasudil influx and accumulates in the aqueous compartment of the liposomes, in combination with the ammonium sulfate gradient was further examined (Fig. 3A and B). Liposomes were prepared with varying pH gradients (as described in Section 2) in the presence of 250 mM ammonium sulfate, and drug uptake was subsequently determined following incubation for 30 min. Interestingly, no significant increase in the uptake of the drug in response to ΔpH was observed. This result clearly demonstrates that the transmembrane proton gradient is only part of the major driving force for the drug accumulation into the liposomes. Bolotin et al. (Bolotin et al., 1994) reported that efficient (more than 90%) and stable doxorubicin loading into preformed liposomes was obtained by using ammonium sulfate gradients ($(NH_4)_2SO_4$ in liposomes $> (NH_4)_2SO_4$ in outer medium). As shown in Fig. 4, increasing the ammonium sulfate concentration produced an increase in the efficiency of drug encapsulation. It should be noted that no liposomal size change and aqueous trapped volume changes were observed by alterations in the ammonium sulfate concentration as determined by light scattering and the use of a fluorescence dye (CF) as an aqueous marker (data not shown). These results clearly demonstrate that the ammonium sulfate gradient, rather than the transmembrane proton gradient, is the major driving force for the uptake of fasudil.

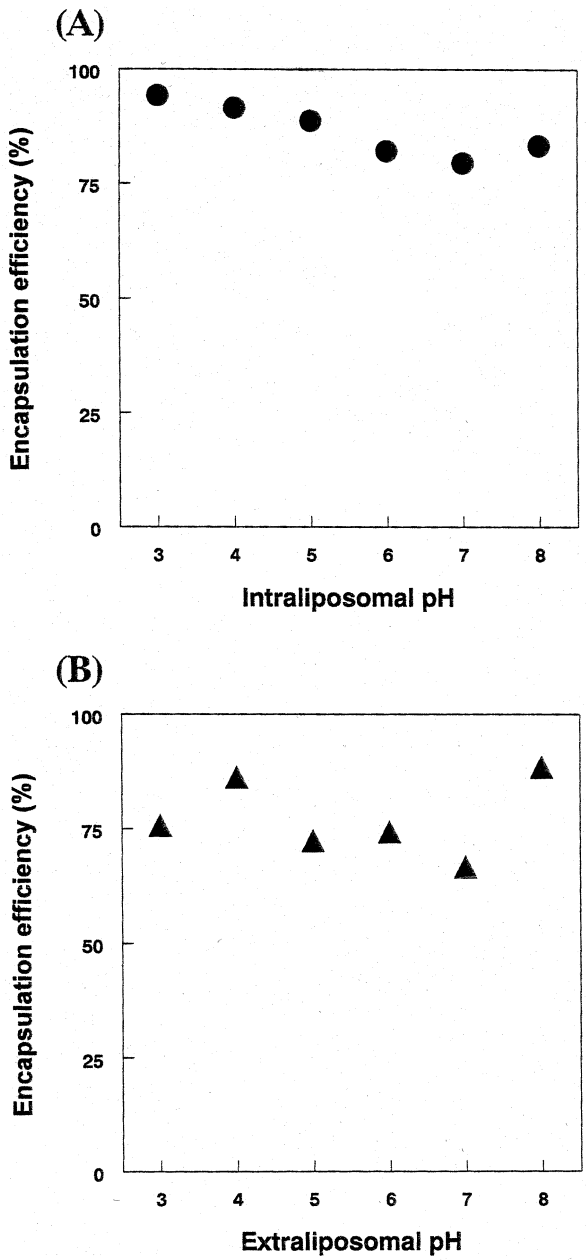

Fig. 3. (A) Effect of the pH of the intraliposomal aqueous compartment on fasudil uptake into preformed HSPC/CHOL liposomes (●). Liposomes were prepared in the presence of ammonium sulfate buffers (250 mM) at the indicated pHs. Liposomes were incubated in the presence of fasudil at 65 °C for 30 min after the vesicle external medium was exchanged with 10% (w/v) sucrose (pH 8.0). Encapsulation efficiency was determined as described in the Section 2. (B) Effect of the pH of the extraliposomal aqueous compartment on fasudil uptake into preformed HSPC/CHOL liposomes (▲). Liposomes were prepared in the presence of 250 mM ammonium sulfate buffer (pH 3.0). The vesicle external medium was then exchanged with 10% (w/v) sucrose containing 25 mM Trizma base with at indicated pHs. Liposomes were incubated in the presence of fasudil at 65 °C for 30 min. Encapsulation efficiency was determined as described in the Section 2.

3.2. Stability of the fasudil-loaded liposomes during storage at 4 °C

In order to be pharmaceutically acceptable, a prepared formulation should retain encapsulated fasudil for long periods, so that it can be stored in a form ready for injection. We followed the stability of liposomes loaded with fasudil at 4 °C with respect to retention of the encapsulated drug and changes in the size distribution. The latency of the drug in prepared formulations was calculated as drug:PL ratios after separation of the free drug divided by the initial drug:PL ratio prior to incubation. Nearly 100% of the encapsulated drug ($>95\%$) was retained inside the liposomes after 180 days in HEPES buffer (pH 7.4) at low temperature (4 °C) (Fig. 5A). No change in size distribution of the liposomes was observed for periods of up to 180 days (result not shown). These results indicate that the encapsulated drug, which is associated with the liposomes during storage, and long term storage did not cause disruption of the prepared liposomes. In addition, the chemical stability of fasudil loaded into the liposomes was investigated by HPLC analysis immediately after their preparation and after storage at 4 °C for 180 days. At right after the preparation, the extent of degradation of fasudil was below the detection limits, and after storage at 4 °C for 180 days, the degradation of fasudil was still below the detection limit (data not shown). Haran et al. (Haran et al., 1993) reported that no peroxidation of the HSPC acyl chains of liposomes could be detected after 2 months of storage at 4 °C and that the degradation of CHOL in the liposomes were below the limit of detection. Thus, the HSPC/CHOL liposomes used in a current study would be free of lipid chemical degradation products, as recommended for human trials (Barenholz and Amselem, 1993). It appears that the absence of any chemical degradation of the drug or of neurotoxic lysophosphatides (Blakemore et al., 1977; Smith and McDonals, 1980) in the solutions following storage for long periods at 4 °C constitutes further proof of their safety for intrathecal administration.

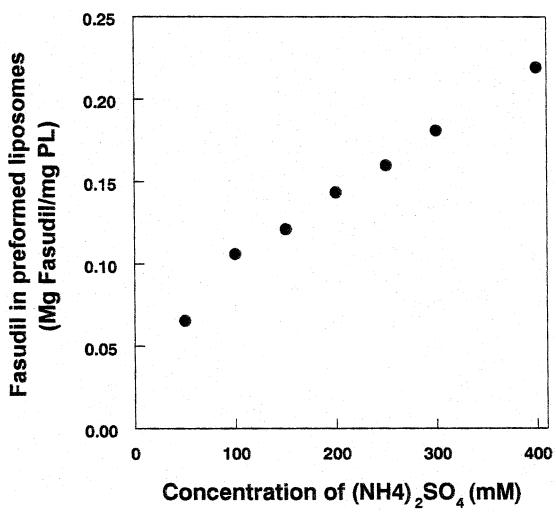

Fig. 4. Effect of intravesicular ammonium sulfate-concentration on fasudil uptake into preformed HSPC/CHOL liposomes. Liposomes were prepared in the presence of ammonium sulfate buffer (pH 3.0) having various concentarion (50–400 mM). The vesicle external medium was then exchanged with 10% (w/v) sucrose containing 25 mM Trizma base (pH 8.0). Liposomes were incubated in the presence of fasudil at fasudil hydrochloride:lipid ratio of 0.2:1 (w/w) at 65 °C for 30 min. Encapsulation efficiency was determined as described in the Section 2.

3.3. Drug release kinetics in human CSF at 37 °C

The advantages of using liposomes as drug carriers in the central nervous system arises from their ability to achieve a sustained release of therapeutically relevant concentrations of drugs directly into the CSF over period of days. The relatively slow release of a drug from liposomes mimicks some aspects of drug infusion, including a significant decrease in the toxicity of the free drug. As shown in Fig. 5B, the prepared formulations released the loaded fasudil in human CSF at 37 °C with a half-life of 7.27 days ($n=3$). This finding indicates that the encapsulation of fasudil in liposomes provides for a sustained-release of the drug in the subarachnoid space, thereby maintaining its concentration in the CSF above therapeutic range. In addition, in order to follow the kinetics of the release of passively encapsulated liposomal contents, CF, an aqueous phase marker and membrane impermeable dye (Haugland, 1992, 1993, 1994), was passively encapsulated into

liposomes, and the CF-loaded liposomes were then incubated in human CSF at 37 °C. As shown in Fig. 5B, more than 85% of the encapsulated CF still remained inside the vesicles after 14 days of incubation. This finding indicates that the release of fasudil from liposomes was caused by the diffusion of fasudil molecules through the lipid membranes, and not by the disruption of liposomes, during their incubation in the CSF.

We recently reported that a liposomal formulation of fasudil was beneficial in reducing side effects and increasing the therapeutic efficacy of the drug in a rat acute ischemia model (Takanashi et al., 2001). The in vitro slow release of fasudil over a period of days observed in this study (Fig. 5B) supports the conclusion, that the improved therapeutic efficacy of intrathecal administered fasudil was due to a sustained-release of the drug from the liposomal formulation of fasudil in the CSF in vivo. A sustained-release preparation of the drug, which is small enough to be applied in the subarachnoid space at the time of lumbar puncture, could continuously deliver drug into the subarachnoid space without the risk of infection associated with externalized catheters or intravenous administration. It is well known that many factors, including liposome composition and size, the physicochemical properties of the drug, and the method of drug loading into liposomes affect the drug release rate from liposomes (Allen et al., 1995). Hence, it will be possible to increase therapeutic efficacies of the drugs by producing liposomal formulations which have more sustained-release properties for intrathecal applications.

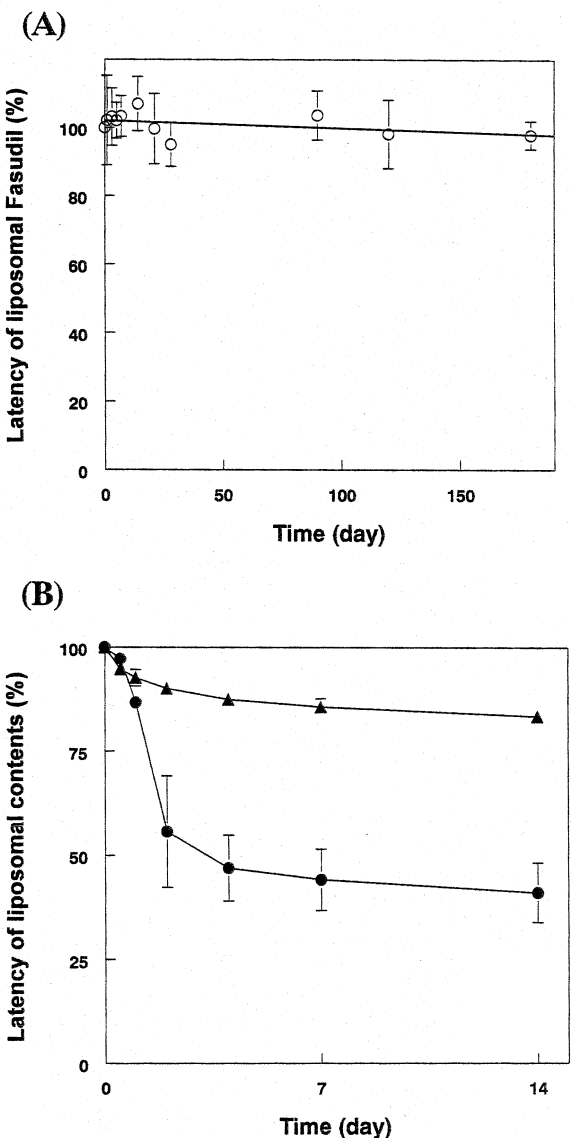

Fig. 5. (A) Release of the contents from fasudil-loaded liposomes in HEPES buffer (pH 7.4) at 4 °C (○). (B) Release of the contents from either fasudil (●) or CF (▲)-loaded liposomes in human CSF at 37 °C. Fasudil was encapsulated into preformed liposomes by means of remote-loading with an ammonium sulfate gradient. CF was passively encapsulated into the liposomes. Liposomes containing fasudil was incubated in HEPES buffer at 4 °C (A). Liposomes containing either fasudil or CF were incubated in the CSF at 37 °C (B). For the fasudil-loaded liposomes containing fasudil, aliquots were removed at specified time points and applied to a Sepharose CL-4B column which had been equilibrated with HEPES buffer (pH 7.4) to separate fasudil associated with the liposomes from the leaked drug. The latency of the drug was determined as described in Section 2. For the liposomes containing CF, aliquots were removed at specified time points, to determine the increase in fluorescence due to released CF. Data represents the mean ± S.D.

4. Conclusions

A major problem in the pharmaceutical application of liposomes is their efficient and stable

loading with drugs, and their long term storage in a form ready for injection. Fasudil was successfully encapsulated into preformed liposomes in response to an ammonium sulfate gradient (250 mM). The liposomal formulation of fasudil was stable for 24 weeks at 4 °C with respect to retention of the encapsulated drug and change in size distribution. The chemical stability of encapsulated fasudil is also a proven fact. Fasudil was continuously released for more than 3 days in human CSF in vitro. The fasudil-loaded liposomes, prepared using an active-loading method described here, would meet pharmaceutical demands for clinical use. Although, we have confirmed that liposome-entrapped fasudil can be safely applied in cisterna magna in an earlier report (Takanashi et al., 2001) and a current study, involving the circulation and kinetics of the drug in the subarachnoid space remain to be understood.

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