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Ascorbyl palmitate vesicles (Aspasomes): formation, characterization and applications

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Abstract

Vesicles with biological activity or with a targeting function in addition to carrier properties will have an added advantage. Vesicles prepared with amphiphiles having antioxidant property may have potential applications towards disorders implicated with reactive oxygen species. Ascorbyl palmitate (ASP) was explored as bilayer vesicle forming material. It formed vesicles (Aspasomes) in combination with cholesterol and a negatively charged lipid (dicetyl phosphate). Aspasomes were prepared by film hydration method followed by sonication in which aqueous azidothymidine (AZT) solution was encapsulated in aqueous regions of bilayer. Aspasomes were obtained with all compositions containing 18–72 mol% cholesterol. Differential scanning calorimetric data of aspasome dispersion and anhydrous mixtures of ascorbyl palmitate, cholesterol and dicetyl phosphate confirm the formation of bilayered vesicles with ascorbyl palmitate.

Cholesterol content in aspasome did not exhibit any relation with vesicle size, zeta potential or percent entrapment. A substantial change in release rate of azidothymidine from aspasome was noticed on varying the proportion of cholesterol. Release rate and cholesterol content in Aspasomes did not exhibit any relation. A preparation with 45 mol% of cholesterol showed maximum retardation in release rate, than other compositions. The change in capture volume with time (latency) was studied for 8 h and with such a short duration study it was difficult to predict long term stability of these vesicles. But release experiments do indicate stability upto 18 h.

Percent reducing activity of aspasome was estimated by measuring the absorbance of α,α-diphenyl-β-picrylhydrazyl (DPPH) at 517 nm after addition of test antioxidant samples. These studies revealed that the antioxidant potency of ascorbyl moiety is retained even after converting ascorbyl palmitate into vesicles (Aspasomes). The antioxidant potency of Aspasomes was assessed by measuring the protection offered by this preparation against quinolinic acid induced lipoperoxidation of whole human blood in vitro, where in the lipoperoxidation was monitored by measuring thiobarbituric acid reactive substances (TBARS) levels. Aspasome rendered much better antioxidant activity than ascorbic acid.

Transdermal permeation of aspasomal AZT, ASP-AZT aqueous dispersion and AZT-solution across excised rat skin was investigated in vitro using Franz diffusion cell. Permeation of aspasomal AZT was much higher than the other two preparations.

Abbreviations: AZT, zidovudine which is chemically 3′-azido-3′-deoxythymidine or azidothymidine [CAS-30516-87-1]; ASP, ascorbyl palmitate; CHOL, cholesterol; DCP, dicetyl-phosphate; KI, potassium Iodide; HPLC, high performance liquid chromatography; ZP, zeta potential; DSC, differential scanning calorimetry; CV, capture volume; PBS, phosphate-buffered saline; MDA, malondialdehyde bis(dimethyl acetal) or 1,1,3,3-tetramethoxypropane; QA, quinolinic acid; TBARS, thiobarbituric acid reactive substances; DPPH, α,α-diphenyl-β-picrylhydrazyl; %RA, percent reducing activity; TBA, 2-thiobarbituric acid; ROS, reactive oxygen species

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However, ASP-AZT aqueous dispersion has also enhanced permeation of AZT significantly over the AZT-solution, indicating skin permeation enhancing property of ascorbyl palmitate.

Keywords: Niosomes; Ascorbyl palmitate; Azidothymidine (AZT); Ascorbyl palmitate vesicles; Aspasomes; Release; DSC; Lipoperoxidation of blood in vitro; Antioxidant potency; Transdermal permeation

1. Introduction

Niosomes (nonionic surfactant vesicles) are formed from the self assembly of hydrated surfactant monomers. Several niosome forming materials have been identified (Uchegbu and Florence, 1995a; Uchegbu and Vyas, 1998a). Niosomes have been applied as carriers for drug delivery. Extensive work is reported on controlled/sustained or site specific release properties of these vesicles (Uchegbu and Florence, 1995a; Uchegbu et al., 1995b; Uchegbu and Vyas, 1998a; Bouwstra and Hofland, 1994; Kiwada et al., 1985).

From the vast literature on vesicles, it appears that not much attention was focused on exploring the possibility of converting amphiphilic moieties that are biologically active, or can serve as targettable ligands, into bilayer vesicles. However, as such a very few materials with dual qualities such as amphiphilicity and biological activity may be available. Hence, it would be instructive to synthesize such amphiphilic materials which can be converted into bilayer vesicles. The recent work of Uchegbu (1998b) was on these lines. They have synthesized palmitoyl muramic acid and *N*-palmitoyl glucosamine and converted them into niosomes. The former is useful to improve the immuno-adjuvant effect and the latter improved targeting to malignant tissue.

1-*O*-Alkyl glycerols have exhibited several biological actions (Weber, 1988) and a prominent effect on blood brain barrier permeability. They markedly improved brain uptake of cancerostatic agents (Hallgren, 1983; Weber, 1988; Erdlenbruch et al., 2000). Since 1-*O*-alkyl glycerols are amphiphilic, we converted them into bilayer vesicles-Algosomes (Gopinath et al., 2002).

Ascorbic acid (vitamin-C) is a major antioxidant in human plasma as well as in and across cell membranes (May, 1999; Frei et al., 1990). It reduces α-tocopherol as well as peroxides and reactive oxygen species such as superoxide (Buettner, 1993).

Several fatty ester derivatives of ascorbic acid were synthesized in order to transfer the peculiar antioxidant properties of ascorbic acid in lipophilic media and to improve its stability (Spicilin et al., 2001; Capuzzi et al., 1996a). All of them retained the antioxidant property of the ascorbyl moiety. Ascorbic acid esters are amphiphilic and studies delineating their surface active properties and applications were reported (Lo Nostro, 1997; Lo Nostro et al., 2000a,b; Palma et al., 2002).

Ascorbic acid and its derivatives are widely used in a number of cosmetic and dermatological preparations, since it has many favorable effects on the skin (Silva and Maja Campos, 2000). Because of its capability to suppress pigmentation of the skin and decomposition of melanin, it can be used to whiten the skin. It also improves elasticity of the skin by promoting the formation of collagen (Darr et al., 1992; Djerassi, 1997; Fox, 1997).

Ascorbyl palmitate is more stable than ascorbic acid. Its lipophilic character is beneficial for its skin penetration. Microemulsions of ascorbyl palmitate have been prepared with a view to apply them for topical use (Spicilin et al., 2001).

We have investigated the possibility of converting ascorbyl palmitate into bilayered vesicles with a view to exploit them as carriers for drug delivery. We report here the formation, characterization and antioxidant potency of ascorbyl palmitate vesicles-Aspasomes. Also these vesicles were evaluated for in-vitro transdermal delivery of AZT.

2. Materials and methods

Ascorbic acid-6-palmitate (U.S.P.), cholesterol, dicetyl phosphate, quinolinic acid, 2-thiobarbituric acid, butylated hydroxytoulene, α,α-diphenyl-β-picrylhydrazyl (DPPH), malondialdehyde bis(dimethyl acetal) or 1,1,3,3-tetramethoxypropane and dialy-

sis tubing (cellulose membrane, 12,400 M_w cut-off) were obtained from Sigma Chemical Company, St. Louis, MO, USA. Zidovudine which is chemically 3′-azido-3′-deoxythymidine or azidothymidine (CAS-30516-87-1) (AZT) was donated by Burroughs Wellcome Research Laboratories, Research Triangle Park, NC, USA and paracetamol was a gift given by Parke Davis, Hyderabad, India. Ammonium acetate, acetic acid and trichloroacetic acid of analytical grade were purchased from s.d. fine chemicals, Boisar, India. Chloroform of analytical grade was purchased from Ranbaxy fine chemicals, S.A.S. Nagar, India. Methanol (HPLC grade) and acetonitrile (HPLC grade) were procured from Qualigens Fine Chemicals, Mumbai, India.

2.1. Aspasome preparation

Aspasomes were prepared by film hydration method under nitrogen atmosphere in which 200 μmol of lipid mixture (ascorbyl palmitate: cholesterol in varied molar ratio with dicetyl phosphate included at 10 mol% of the total lipid) was dissolved in 9 ml of chloroform and 1 ml of methanol in a round bottom flask and was kept under reduced pressure in rotary evaporator (Rotavapor R-114, Buchi Lobortechnik AG, Flawil, Switzerland) at 50 °C till it formed a thin dry film on the walls of the flask. The dried thin lipid film was hydrated with 10 ml of phosphate buffered saline (PBS, pH 7.4) containing Zidovudine (AZT) (2 mg/ml) maintained at the same temperature. The vesicles were then sonicated for 2 min using ultrasonicator (LABSONIC L, B. Braun Biotech International, USA) on which power was set at 50% of maximum output. The Aspasomes were stored in nitrogen-purged vials.

2.2. Characterization of Aspasomes

2.2.1. Photomicrography

Photomicrographs of unsonicated Aspasomes were taken using LABORLUX S microscope (Leitz Wetzlar, Germany) fitted with camera at various magnifications, so as to confirm the formation and to understand the nature of vesicles.

2.2.2. Size and size distribution

Size and size distribution of aspasome was determined by photon correlation spectroscopy (PCS) using Zetasizer 3000HSA (Malvern Instruments, Malvern, Worcs, UK). Each sample was diluted to a suitable concentration with filtered distilled water. Analysis was performed at 25 °C with an angle of detection of 90°. The mean size and standard deviation (±S.D.) was directly obtained from the instrument.

2.2.3. Zeta potential

Zeta potential (ZP) of suitably diluted aspasome dispersion was determined using Zetasizer 3000HSA (Malvern Instruments, Malvern, Worcs, UK). Charge on vesicles and their mean ZP values with standard deviation (±S.D.) were obtained directly from the instrument.

2.2.4. Percent entrapment

The percent entrapment of AZT in Aspasomes was assessed by Centrifugal ultra-filtration using Centrisart I (20,000 Da cut-off, Sartorius AG, Gottingen, Germany). The details of method are previously reported by us (Gopinath et al., 2001).

2.2.5. Latency

Change in capture volume of Aspasomes with time was studied by centrifugal ultrafiltration over a period of 8 h using Centrisart I (20,000 Da cut-off, Sartorius AG, Gottingen, Germany). Capture volume (CV) was expressed as μl/μmol of lipid.

2.2.6. Effect of osmotic gradient

Osmotic sensitivity and aggregation tendency of Aspasomes was investigated by monitoring changes in vesicle diameter using Zetasizer 3000HSA. One milliliter of aspasome suspension was challenged with 1 ml KI solution containing 5, 10, 15, 20, 40, 60, 80 or 100 mM of KI. The mixture was allowed to stand for 1 h and the vesicle size was measured.

2.2.7. Differential scanning calorimetry

Aspasome dispersions and anhydrous mixtures of ascorbyl palmitate/cholesterol/DCP were studied by Differential Scanning Calorimeter (Mettler DSC 821e, Mettler-Toledo, Switzerland) to understand the thermotropic properties and phase transition behavior. Analysis was carried out in conventional aluminium pan with a heating rate of 5 °C/min.

2.3. In vitro release

Aspasome preparation was subjected to release studies using dialysis apparatus fabricated in our laboratory. The detailed description of this apparatus and methodology adopted was reported in our earlier publication (Gopinath et al., 2001).

Prior to release study, maximum extent of unentrapped AZT was removed by dialyzing the original preparation. Five milliliters of dialyzed aspasome preparation was subjected to release studies. One hundred milliliters of PBS was placed in receptor cell. Dialyzed aspasome preparation or AZT solution (in PBS, pH 7.4) was transferred to donor cell. Five-milliliter samples were drawn from the receptor cell at preset intervals over a period of 18 h. At each time immediately after removal of sample, the medium was compensated with PBS. The samples were analyzed for AZT by HPLC. AZT solution (in PBS, pH 7.4) served as control. All experiments were performed in triplicate and values are reported as mean ± standard deviation.

2.4. Transdermal permeation study

The skin permeation of aspasomal AZT (ASP: CHOL:DCP-45:45:10 mol%) was determined by using Franz (vertical) diffusion cell. The excised rat skin was mounted between the receptor and donor compartments with the stratum corneum side facing upwards. The donor compartment was charged with 2 ml of aspasome dispersion. The receptor compartment was filled with 12 ml of Phosphate Buffered Saline (PBS, pH 7.4) which was maintained at 37 ± 0.5 °C and stirred by magnetic bar at 200 ± 5 rpm. The receptor solution contained 0.02% (w/v) of sodium azide to retard microbial growth. The available diffusion area of cell was 3.16 cm^2. At predetermined time intervals, a sample (0.5 ml) was taken from the receptor cell and immediately replaced by an equal volume of fresh receptor solution. The content of AZT in the samples was analyzed by HPLC. Ascorbyl palmitate aqueous dispersion containing AZT (ascorbyl palmitate dissolved in little quantity of methanol and volume made with sufficient quantity of PBS, pH 7.4) and AZT-solution (in PBS, pH 7.4) were used as controls.

2.5. High-performance liquid chromatographic analysis

A HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with SCL-6A system controller, LC-6A solvent delivery unit, SPD-6AV variable UV spectrophotometric detector, C-R4A chromatopac data processor and injector (Rheodyne) fitted with 20 μl capacity loop was used for the analysis. An octadecylsilane reverse phase stainless steel analytical column (25 cm × 4.6 mm) was employed for chromatographic separation.

2.5.1. HPLC analysis of AZT

AZT in samples was analyzed by high-performance liquid chromatography method previously reported by us (Gopinath et al., 2001).

Mobile phase: methanol–water (20:80) to which ammonium acetate was added to make a final concentration of 10 mM. The pH was adjusted to 4.0 with acetic acid. Flow rate: one milliter per minute, UV detection at 267 nm and separation was at ambient temperature. The retention times were 3.4 and 6.2 min for internal standard (Paracetamol) and AZT, respectively. The concentrations of AZT in samples were determined using a standard graph.

The total AZT in the aspasome was estimated by mixing 0.2 ml of the preparation with 1 ml of methanol by vortexing (Cyclomixer, Remi Motors, Mumbai, India). To the lysed preparation, 1 ml of paracetamol in methanol (containing 20 μg/ml of the substance) was added as internal standard and then diluted with sufficient quantity of distilled water. AZT content was estimated using HPLC.

2.5.2. HPLC analysis of ascorbyl palmitate

Ascorbyl palmitate in samples was analyzed by high-performance liquid chromatography method reported previously (Spicilin et al., 2001).

Mobile phase: methanol–acetonitrile–0.02 M phosphate buffer (pH 2.5) (75:10:15). Flow rate: 1.5 ml per minute, UV detection at 254 nm and separation was at ambient temperature. The retention time was 4.1 min for ascorbyl palmitate. The concentration of ascorbyl palmitate in the samples was determined using a standard graph. Aspasome preparation (100 μl) was diluted to 10 ml with methanol and 20 μl was injected into HPLC column.

2.6. Determination of antioxidant potency

For the following studies aspasome dispersion encapsulating PBS (pH 7.4) was prepared and ascorbyl palmitate in such preparation was estimated by HPLC.

2.6.1. Effect of aspasome on kinetics of lipoperoxidation

The kinetics of quinolinic acid (QA) induced lipoperoxidation of whole human blood was studied by estimating thiobarbituric acid reactive substances (TBARS) (Ohkawa et al., 1979; Scoccia et al., 2001) generated in vitro. Blood withdrawn from healthy human volunteers of 'O' (Rh+) blood group was mixed with citrated-PBS (pH 7.4). One milliliter of blood was mixed with 2 ml of quinolinic acid (1 mM), and mixture was incubated at 37 °C for 0.25, 0.5, 0.75 1.0, 1.5, 2.0, 2.5, 4.0, 4.5, 6.0, 6.5 h. At the end of incubation period the samples were treated with 1 ml of butylated hydroxytoulene (0.5 mg/ml in methanol) and allowed to stand for 15 min at room temperature. To this 2 ml of tricholoroacetic acid (15% w/v solution) was added, tubes were sealed and heated for 15 min in a boiling water bath to release protein bound TBARS. The samples were cooled in ice bath and centrifuged at 2000 g for 20 min. To 4 ml of supernatant, 400 μl of thiobarbituric acid (1% w/v solution) was added, tubes were sealed and heated for 1 h in boiling water bath. The samples were cooled and the colour intensity was measured at 532 nm against blank using a spectrophotometer (Elico Private Ltd., Hyderabad, India). A calibration curve was prepared with malondialdehyde (MDA) standard. Results were expressed as nmol MDA/ml of blood. All samples gave results which were within the linear portion of the MDA standard curve. Results were expressed as mean ± S.D. Statistical analysis was performed by Student's *t*-test; P value < 0.05 was considered statistically significant.

In order to evaluate the effect of antioxidants on the kinetics of lipoperoxidation of whole human blood in vitro, the blood samples were mixed with 2 ml of ascorbic acid (AA) (1 mM solution) 30 min prior or after the treatment with QA. These samples were further treated and analyzed for TBARS content following the procedure described above. In order to evaluate the antioxidant potency of Aspasomes, a similar study was conducted wherein the blood samples were treated with 2 ml of aspasome dispersion containing ascorbyl palmitate equivalent to AA used in the above study.

2.6.2. Reducing activity

The percent reducing activity (RA%) is a measure of antioxidant potency of a compound. Antioxidant potency of ascorbic acid solution, ascorbyl palmitate aqueous dispersion and aspasome dispersion were measured by the method described by Lo Nostro et al. (2000b). Ethanol-PBS solution of α,α-diphenyl-β-picrylhydrazyl (DPPH; 10^{-4} mol/l) was mixed with equal volume of antioxidant preparations (10^{-4} mol/l) and allowed to stand for 20 min. Absorbance of mixture before (A_0) and after 20 min (A_{20}) was measured at $\lambda = 517$ nm using spectrophotometer (Elico Pvt. Ltd., Hyderabad, India). In case of aspasome dispersion, the sample mixtures were ultra filtered using Centrisart I apparatus (20,000 Da cut-off, Sartorius AG, Gottingen, Germany) and the absorbance of the filtrate was measured. The percent reducing activity was calculated according to formula,

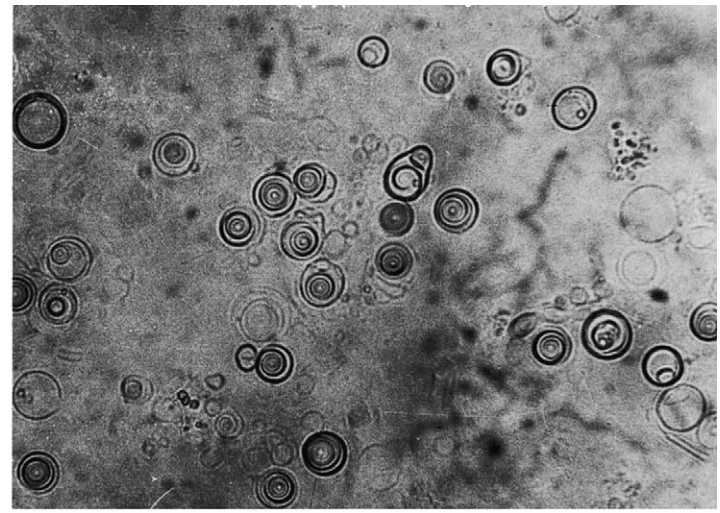
$$\mathrm{RA}(\%) = 100\left(\frac{A_0 - A_{20}}{A_0}\right)$$

3. Results and discussion

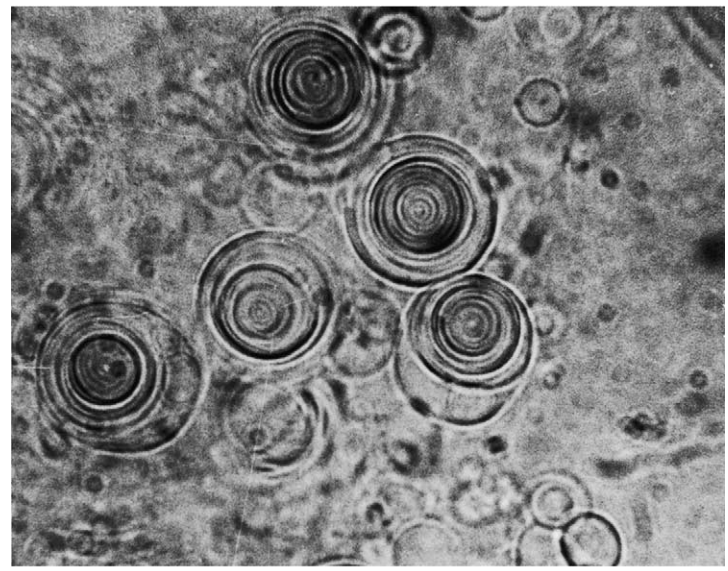
3.1. Preparation, characterization and release

According to some reports (Capuzzi et al., 1996a,b; Lo Nostro et al., 2000b) amphiphilic ascorbic acid esters can easily produce self assembled supramolecular aggregates such as monolayers, micelles or liposomes. However, these publications dealt with only micelles, monolayers and no attempt was made to convert any of the amphiphilic ascorbic acid esters/ethers into bilayered vesicles (liposome like structures).

We found that the thin film of ascorbyl palmitate on hydration did not form vesicles. In presence of cholesterol vesicles formed, but were very unstable. Ascorbyl palmitate and cholesterol in presence of negatively charged lipid dicetyl phosphate formed stable vesicles-Aspasomes.



(A)



(B)

Fig. 1. Photomicrographs of unsonicated aspasome dispersion formed with ASP:CHOL:DCP (45:45:10 mol%): (A) under 450× magnification; (B) under 1500× magnification.

Presence of vesicles in aspasome dispersion was confirmed by viewing unsonicated system using an optical microscope (Fig. 1). Vesicles were seen in all the preparations containing 18–72 mol% of CHOL. The vesicles were spherical and majority of them were multi-lamellar. Very few large unilamellar vesicles were also seen.

Effect of composition on vesicle size, zeta-potential and percent entrapment of AZT in Aspasomes is shown in Table 1. Vesicle size and zeta potential did not show any relation with the composition. With increase in CHOL concentration (from 18 to 54 mol%), entrapment (%) did not alter significantly. However, in aspasome dispersion containing

Table 1
Effect of aspasome composition on vesicle size, zetapotential and percent entrapment of AZT

Composition, ASP:CHOL:DCP (mol%)	Vesicle size (nm) (average ± S.D.)	Zeta potential (mV) (average ± S.D.)	Percent entrapment (average ± S.D.)
72:18:10	467.9 ± 17.5	−42.9 ± 0.5	23.90 ± 2.30
54:36:10	940.8 ± 18.2	−45.9 ± 1.3	26.32 ± 1.56
45:45:10	341.6 ± 5.1	−64.9 ± 2.9	30.58 ± 2.72
36:54:10	350.7 ± 6.3	−57.7 ± 0.9	29.27 ± 1.91
27:63:10	327.2 ± 14.3	−60.3 ± 2.3	19.10 ± 2.66
18:72:10	1363.6 ± 21.6	−76.3 ± 2.6	17.99 ± 3.05

63 and 72 mol% CHOL, the entrapment (%) decreased.

Percent AZT released versus time plots of Aspasomes are shown in Fig. 2. Significant changes in release were observed with change in CHOL content in the bilayer of aspasome. Aspasomal AZT showed much slower release rate than AZT solution. The release rate and CHOL content did not exhibit any relation. A rapid release rate due to 18 mol% CHOL decreased considerably when the CHOL concentration in the system was 45 mol%. Further increase in CHOL content gradually increased the release rate. Thus, systems with 18 or 72 mol% CHOL showed highest and system with 45 mol% CHOL showed the lowest release rates, respectively. The release profiles of Aspasomes reveal that presence of CHOL in aspasome stabilizes the bilayers and decreases their permeability. However, contrary to earlier reports (Bouwstra and Hofland, 1994) increase in CHOL did not gradually reduce the permeability. In fact at 45 mol% of CHOL, a stable aspasome is produced, with retarded AZT permeability. Above and below this concentration of CHOL, the bilayer permeability increased. Release rate did not exhibit any relation with vesicle size, zeta potential or entrapment.

Stability of the aspasome was assessed by studying changes in capture volume with time (Table 2). Capture volume did not change significantly during 8 h of storage. However, based on this short duration study long term stability of these vesicles could not be predicted. But our release experiments do indicate stability of vesicles up to 18 h.

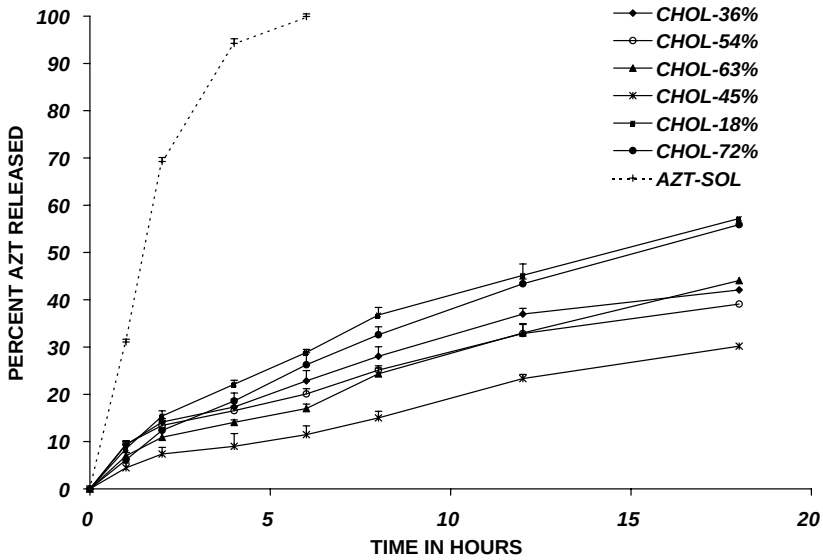


Fig. 2. In-vitro release profiles of AZT from Aspasomes containing varied molar concentrations of ascorbyl palmitate and cholesterol. Each point represents the mean ± S.D. ($n = 3$).

Table 2
Effect of cholesterol concentration on latency of Aspasomes

Composition, ASP:CHOL:DCP (mol%)	Change in capture volume (μl/μmol) with time					
	0	1 h	2 h	4 h	6 h	8 h
72:18:10	1.116 ± 0.087	1.517 ± 0.200	1.287 ± 0.050	1.413 ± 0.060	1.585 ± 0.005	1.272 ± 0.150
54:36:10	1.329 ± 0.159	1.365 ± 0.160	1.355 ± 0.230	1.681± 0.250	1.300 ± 0.050	1.290 ± 0.190
45:45:10	1.431 ± 0.051	1.346 ± 0.100	1.200 ± 0.007	1.365 ± 0.340	1.550 ± 0.006	1.258 ± 0.135
36:54:10	1.368 ± 0.150	1.257 ± 0.110	1.250 ± 0.240	1.215 ± 0.074	1.177 ± 0.120	1.053 ± 0.052
27:63:10	0.973 ± 0.120	0.993 ± 0.007	0.897 ± 0.050	0.928 ± 0.350	0.918 ± 0.060	0.850 ± 0.050
18:72:10	0.887 ± 0.070	0.881 ± 0.060	0.993 ± 0.050	1.100 ± 0.160	0.994 ± 0.190	0.828 ± 0.035

Each point represents the mean ± S.D. ($n = 3$).

Latency study revealed that during a short storage period of 8 h, the vesicle structure is intact and the system is stable. In contrast to 8 h latency data a significant permeation of AZT during in vitro release upto 8 h is noteworthy. In latency studies, AZT concentration inside and outside the vesicle is same and hence no concentration gradient exists. For release experiments dialyzed Aspasome dispersion devoid of unentraped AZT was used. Thus, in release experiments a concentration gradient is established due to continuous dilution of the solution outside the Aspasomes. A high AZT concentration inside and low concentration outside the aspasome is responsible for the significant AZT permeation observed in the release experiments.

The osmotic sensitivity of Aspasomes (ASP:CHOL: DCP: 45:45:10 mol%) was tested by challenging the system with KI solution (Fig. 3). Incorporation of an electrolyte (KI solution) will have two different effects on the aspasome dispersion. The osmotic gradient established by KI solution challenge will bring about osmotic shrinkage of vesicles. And the positive K^+ counter ions will undergo electrostatic interaction with negative ions in electrical double layer and will decrease double layer thickness there by reducing zeta potential value. Consequently, the vesicle aggregation will be invoked. Our studies on Aspasomes revealed that the osmotic effect is dominant when concentration of KI is in the range of 5–20 mM. Beyond 20 mM

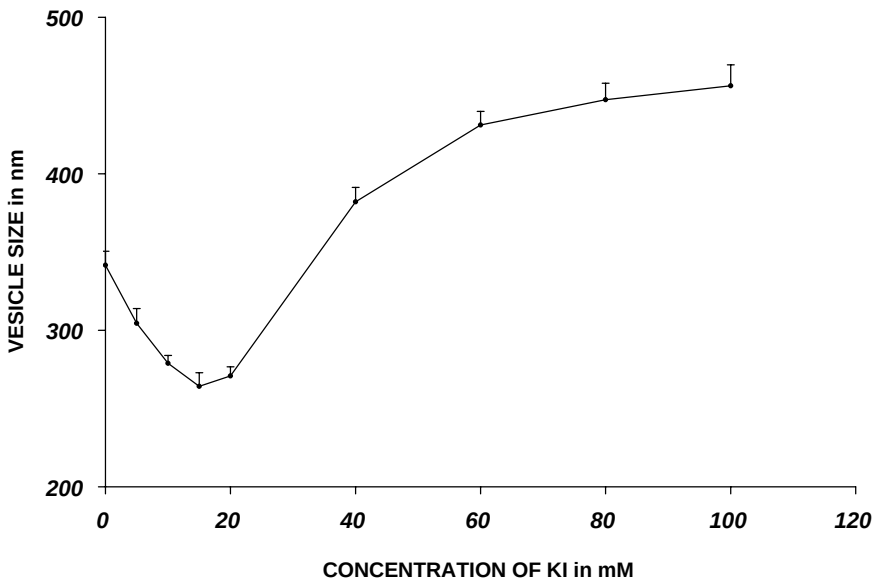


Fig. 3. Osmotic sensitivity of Aspasomes containing ASP:CHOL:DCP (45:45:10 mol%). Each point represents the mean ± S.D. ($n = 3$).

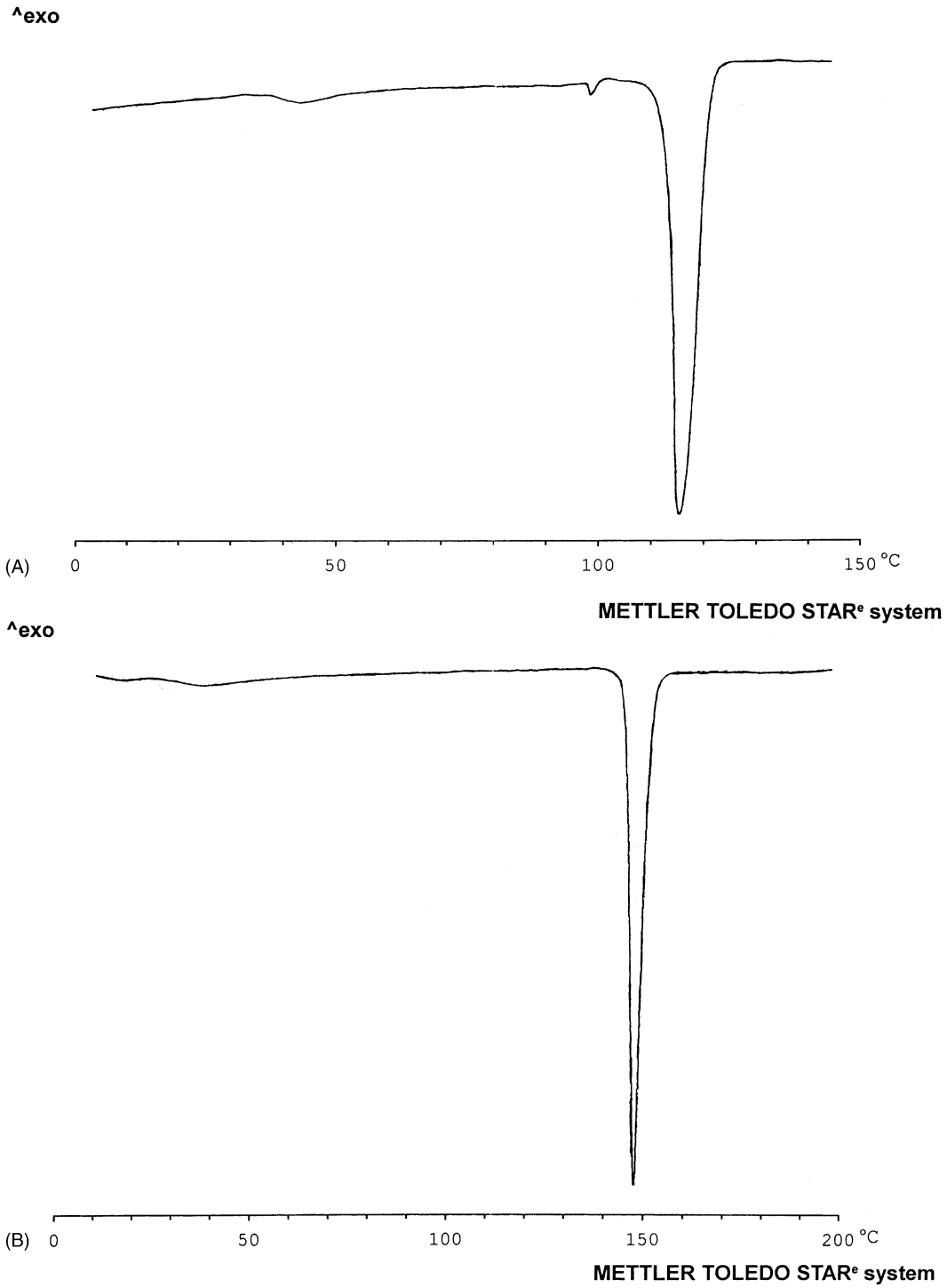


Fig. 4. DSC thermograms of (A) ascorbyl palmitate; (B) cholesterol.

concentration though the osmotic shrinkage of size is still prevalent, the electrostatic counter ion effect of K^+ seems to supercede and induce the aggregation of vesicles. As a result of this aggregation, an apparent increase in vesicle size was noticed (Fig. 3). We have observed similar kind of results with 1-*O*-alkyl glycerol vesicles (Algosomes) (Gopinath et al., 2002).

3.2. DSC studies

DSC scans of ascorbyl palmitate and cholesterol showed melting endotherms at 116.4 ± 2 °C and 149.7 ± 2 °C, respectively (Fig. 4A and B).

DSC scans of anhydrous mixtures of ascorbyl palmitate, cholesterol and dicetyl phosphate in various proportions are shown in Fig. 5. If one compares the

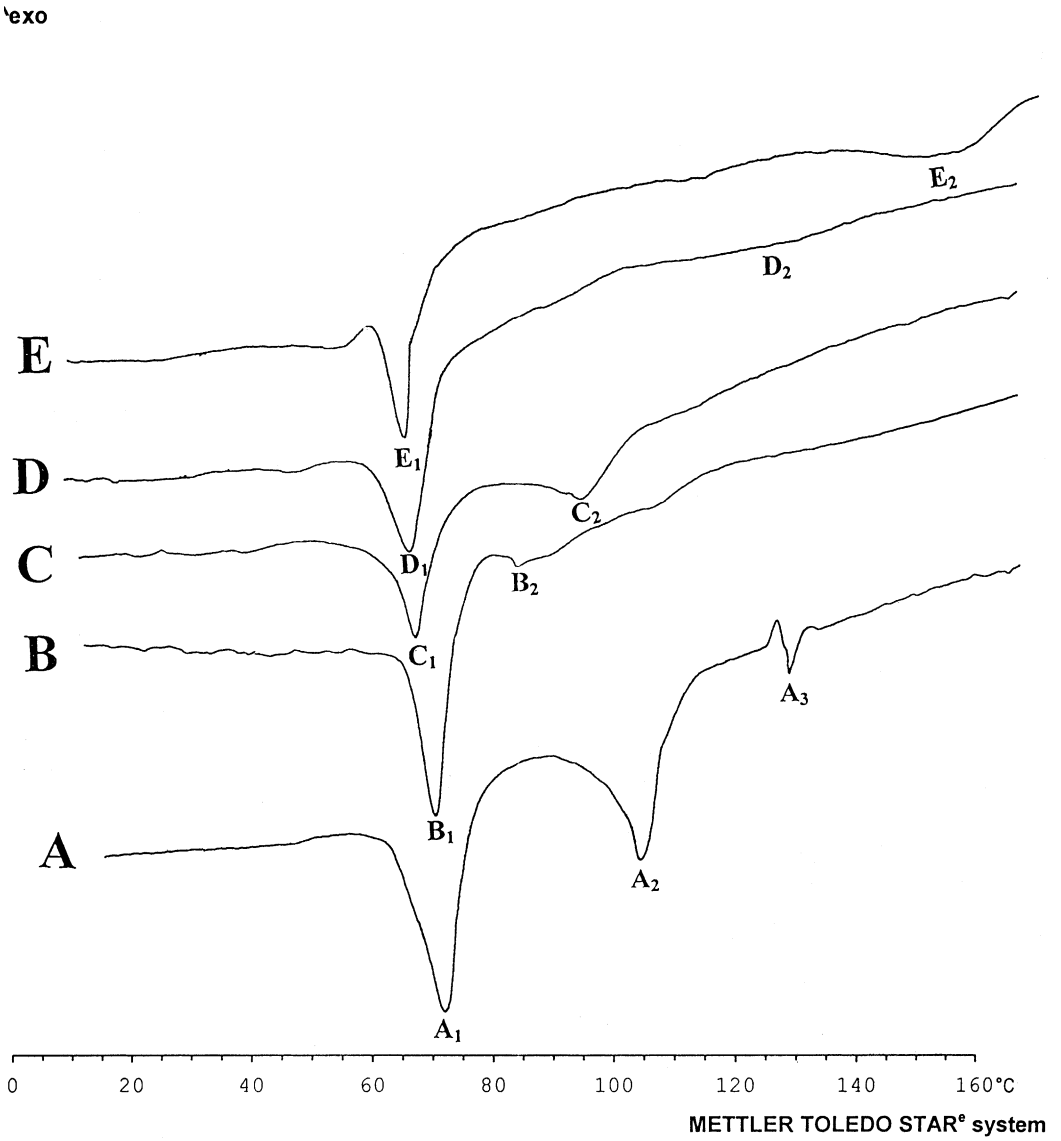


Fig. 5. DSC thermograms of anhydrous mixtures of ascorbyl palmitate, cholesterol and dicetyl phosphate in varied molar ratios: (A) 72:18:10; (B) 54:36:10; (C) 45:45:10; (D) 36:54:10; (E) 18:72:10.

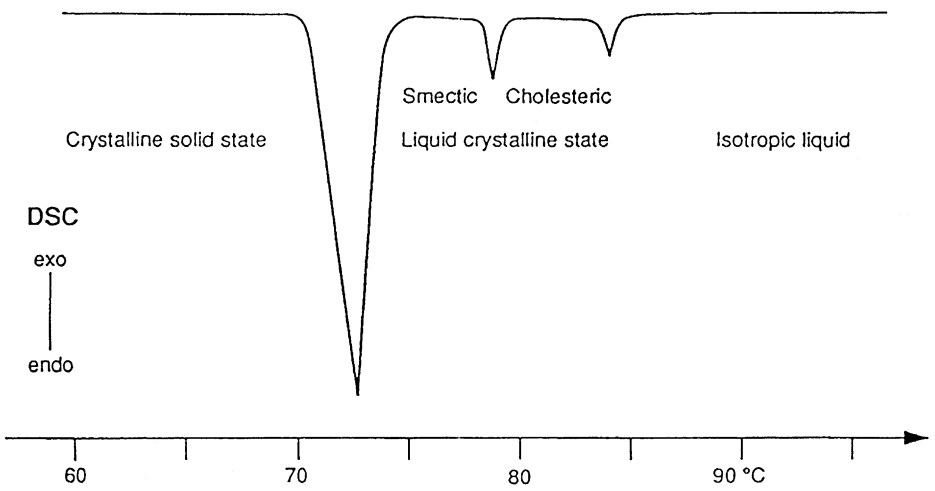


Fig. 6. DSC thermogram of pure cholesteryl myristate (from reference 32).

DSC trace of Cholesterylmyristate (Widmann, 1998) (Fig. 6) to our data, it is very similar to trace A (Fig. 5). The peak A1 is due to simple melting, e.g. eutectic mixture. But peaks A2 and A3 could be mesophasic transitions, may be smectic and cholesteric phases. Peak C2 and probably B2 (which is quite closer to B1) could also be mesophasic structures. But peak D2 (very broad and flat) and E2 could be of pure cholesterol, since these mixtures contain higher concentration of cholesterol compared to others.

The liquid crystalline state is a prerequisite for the formation of bilayered vesicles. There is some evidence for the formation of liquid crystals with ascorbyl palmitate, cholesterol and DCP anhydrous mixture based on DSC study.

The DSC scans of aspasome dispersions containing various concentrations of cholesterol encapsulating PBS are shown in Fig. 7. At 18 mol% of CHOL pre-transition (T_P) and main transition (T_M) temperatures were 47.7 and 55.41 °C, respectively. Several earlier workers have also (Mabrey, 1981; Posch et al., 1983; Biltonen and Lichtenberg, 1993; Taylor and Morris, 1995; Castile et al., 1999; Koyama et al., 2000) observed pre-transition and main transition temperatures for liposome dispersions. The large peak corresponds to gel to liquid crystal transition (Vance and Vance, 1991; Koyama et al., 2000) and the smaller peak is due to rearrangement of the individual lipid molecules within the bilayer (Hadley, 1985; Koyama et al., 2000). In Aspasomes at all concentrations of cholesterol above 18 mol%, pre-transition temperature peak (T_P) disappeared. At 36 mol% the main transition endotherm broadened and area under the curve, i.e., the heat of transition decreased. At 45 mol% a sharp transition was observed, indicating formation of a stable bilayer configuration. A sharp distinct transition was not detectable at 54 and 72 mol% of CHOL. At these concentrations formation of ASP-CHOL packing complexes with varying compositions are expected, which could have resulted in broadening of transitions. Such an effect reflects reduction of the cooperativity of the transition. The heat of transition of aspasome dispersion decreased with increase in cholesterol concentration (Table 3). Some earlier workers reported (Mabrey, 1981; Taylor and Morris, 1995; Koyama et al., 2000) decrease in heat of transition with increase in cholesterol concentration in liposome dispersions. Abolition of distinct transition at 50 mol% CHOL in liposomes was also reported (Mabrey, 1981; Biltonen and Lichtenberg, 1993; Taylor and Morris, 1995; Koyama et al., 2000). The thermotropic properties of Aspasomes are broadly similar to such earlier observations on liposomes.

The phase transition temperatures of aspasome dispersions and corresponding anhydrous lipid mixtures are shown in Table 3. With increase in cholesterol concentration in aspasome dispersions, the main transition temperature decreased slightly (Table 3). The phase transition temperatures of aspasome dispersions were significantly lower than their corresponding

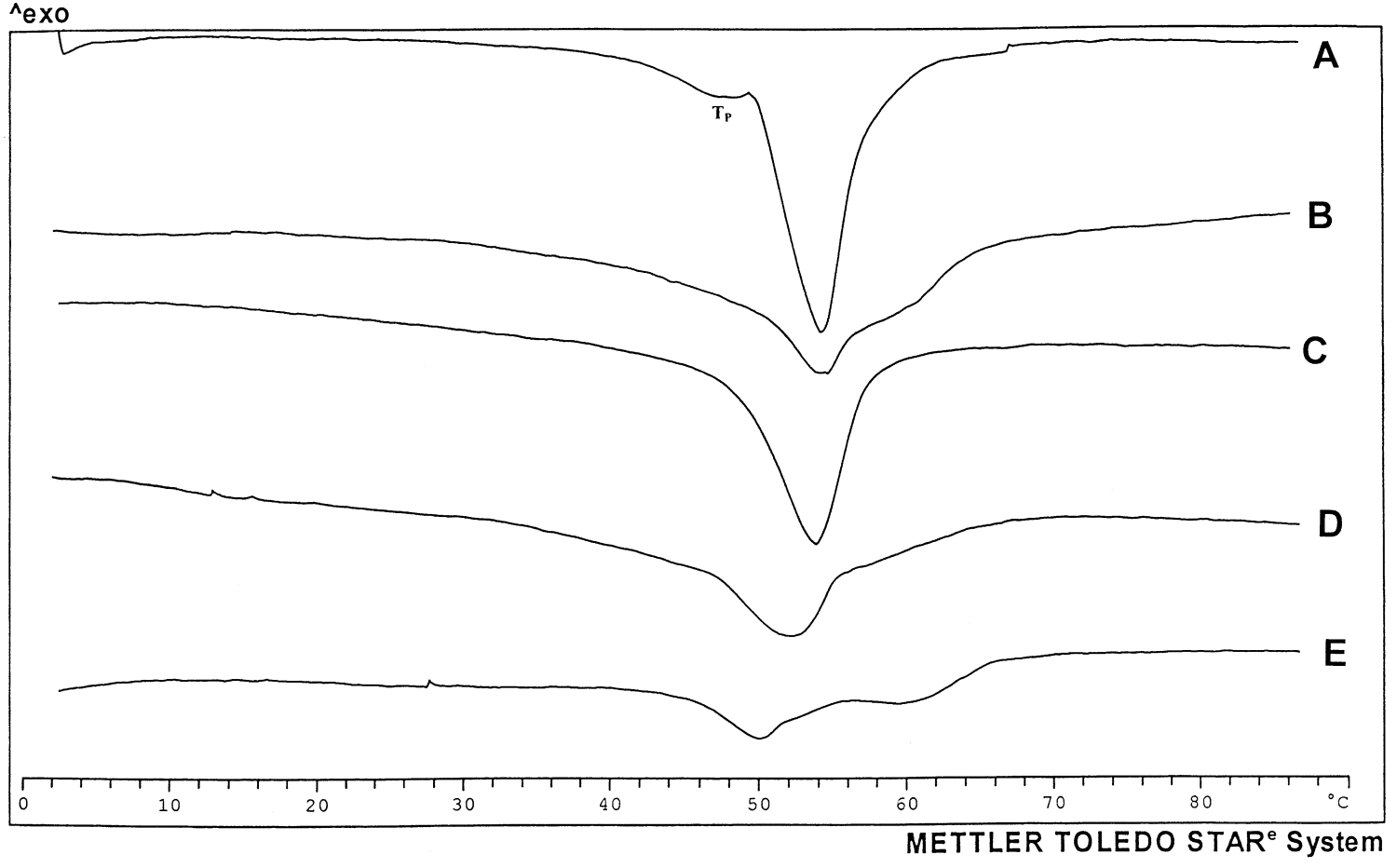


Fig. 7. DSC thermograms of aspasome dispersion (ascorbyl palmitate, cholesterol and dicetyl phosphate) in varied molar ratios: (A) 72:18:10; (B) 54:36:10; (C) 45:45:10; (D) 36:54:10; (E) 18:72:10.

Table 3
Transition temperatures and enthalpy of anhydrous mixtures of ascorbyl palmitate, cholesterol and dicetyl phosphate and aspasome dispersions

Composition, ASP:CHOL:DCP (mol%)	Anhydrous lipid mixtures		Aspasome dispersion	
	Maintransition peak (T_M, °C)	Heat (J/g)	Maintransition peak (T_M, °C)	Heat (J/g)
(A) 72:18:10	72.36 (A1)	−37.27	55.41	−4.42
(B) 54:36:10	73.37 (B1)	−25.60	55.01	−4.10
(C) 45:45:10	72.40 (C1)	−15.50	54.33	−3.12
(D) 36:54:10	73.02 (D1)	−12.55	52.66	−0.85
(E) 18:72:10	71.88 (E1)	−11.47	50.17	−0.44

anhydrous lipid mixtures. Ishigami and Machida (1989) compared phase transition temperature data of anhydrous sucrose fatty acid esters and their corresponding vesicle dispersions. The phase transition temperature of vesicle dispersions were always much lower than anhydrous lipid mixtures.

3.3. Antioxidant potency

Quinolinic acid is a neurotoxin and free radical inducer (Southgate et al., 1998; Southgate and Daya, 1999). We report here the QA induced lipoperoxidation of whole human blood in vitro. The purpose of this study was to evaluate the antioxidant potency of vesicular preparation obtained from ascorbyl palmitate (Aspasome).

In a control experiment, blood cells and QA mixture was incubated and formation of MDA was followed by measuring TBARS levels at different time intervals over a period of 6.5 h (Fig. 8). Up to 1.5 h TBARS levels increased steadily and reached maximum. Later upto 6.5 h negligible changes have occurred.

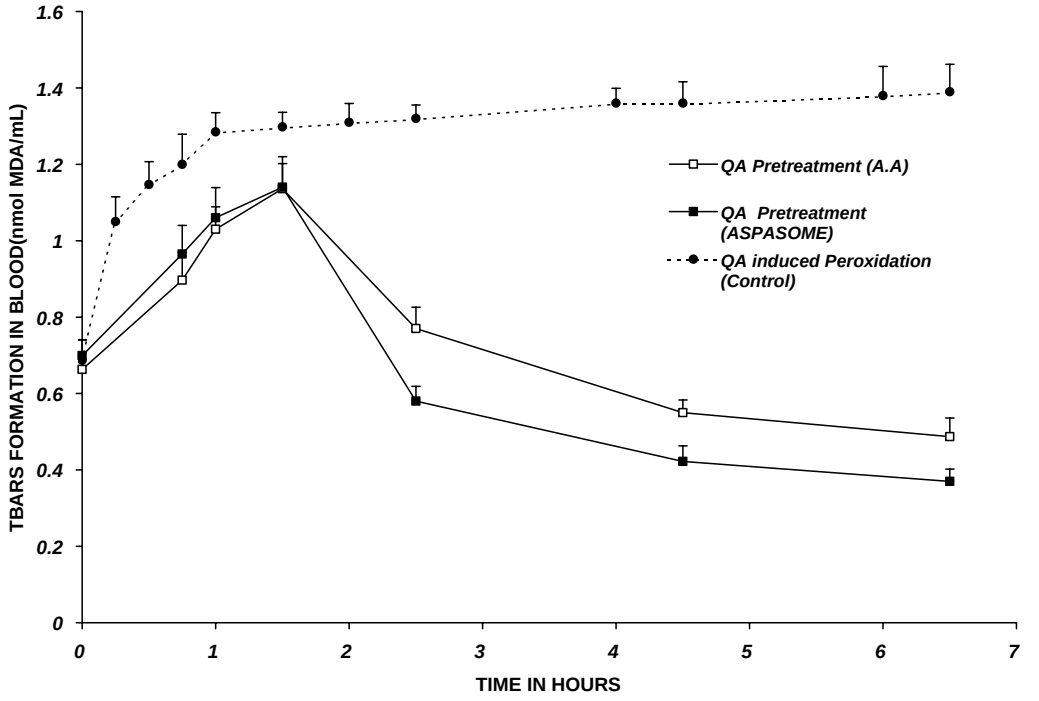


Fig. 8. Kinetics of in vitro lipoperoxidation assessed by TBARS formation upon treatment with quinolinic acid (QA) followed by treatment with antioxidants (ascorbic acid or aspasome). Each point represents the mean ± S.D. ($n = 3$). TBARS levels at all time points are significantly lower due to antioxidants (AA or aspasome) than control (t-test, $P < 0.01$). TBARS levels at 2.5, 4.5 and 6.5 h are significantly lower due to aspasome treatment than ascorbic acid (t-test, $P < 0.05$).

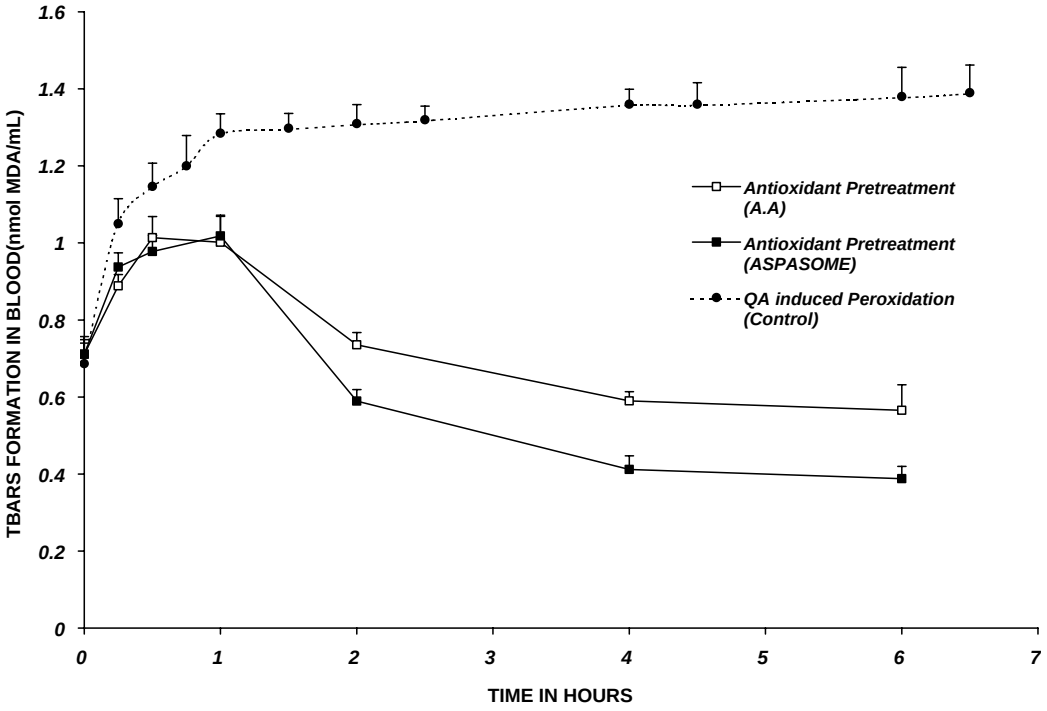


Fig. 9. Kinetics of in vitro lipoperoxidation assessed by TBARS formation upon treatment with antioxidants (ascorbic acid or aspasome) followed by treatment with quinolinic acid (QA). Each point represents the mean ± S.D. ($n = 3$). Due to antioxidant treatment (ascorbic acid or aspasome) TBARS levels at all time points are significantly lower than control (t-test, $P < 0.01$). Aspasome treatment has significantly lowered TBARS levels at 2, 4 and 6 h than ascorbic acid (t-test, $P < 0.05$).

Both antioxidants (AA or aspasome), upto 1.5 h in QA pretreatment and 1 h in antioxidant pretreatment showed lower TBARS levels than control (t-test, $P < 0.02$) (Figs. 8 and 9). During this period, since TBARS levels are continuously increasing, the rate of generation of lipoperoxides induced by QA is much faster than the protection offered by antioxidants. Both antioxidants seem to offer similar extent of protection. Later with increase in time in both treatments TBARS levels decreased exponentially. However, aspasome was more effective than ascorbic acid (t-test, $P < 0.05$) (Figs. 8 and 9).

Various blood constituents, viz. erythrocytes, leucocytes, platelets, circulating lipoproteins undergo lipoperoxidation (Yagi, 1982). In blood cells ROS can originate within the cell and cell membrane.

The cell membrane of the erythrocytes encounters oxidative stress coming from the cell interior and exterior. Intracellular oxidative stress originates from the metabolism of hemoglobin, which releases superoxide and H_2O_2 (VanDyke and Saltman, 1996) both of which readily permeate the membrane (Lynch and Fridovich, 1978). Such radicals potentiate lipid peroxidation of membrane phospholipids. Alternatively, iron-nucleotide complexes in erythrocytes might serve a similar function generating radical species with the capacity to initiate lipid peroxidation (Yagi, 1982).

Under oxidative stress, in blood cells, initially free radicals exist with in the cell and cell membrane, and once they accumulate to certain degree, leak out from cells into the surrounding environment (Yagi, 1982). A best antioxidant system should suppress free radicals of these three pools, viz. cell interior, cell membrane and cell surrounding medium.

Ascorbic acid being hydrophilic can effectively suppress leaked out free radicals into aqueous environment surrounding blood cells. Liu (1995) reported that AA is ineffective as antioxidant for lipoperoxidation initiated in hydrophobic phases. Whereas lipophilic

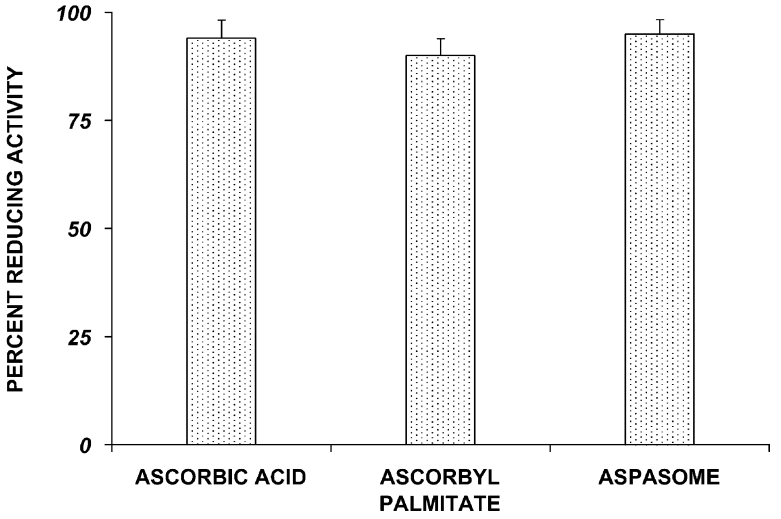


Fig. 10. Percent reducing activity by DPPH method. Each point represents the mean ± S.D. ($n = 5$).

6-*O*-alkanoyl ascorbic acids are much better than that of AA both in vivo and in vitro.

It was reported that amphipathic ascorbate derivative ascorbyl palmitate binds firmly to the exofacial surface of intact erythrocytes (Ross et al., 1999) with ascorbyl moiety projecting from the cell surface. Because of such an orientation its antioxidant potency is still retained even if is bound to cell surface via hydrophobic chain. In aspasome, polar ascorbyl head groups will be projected out of vesicle surface, where as palmitate chains assemble together due to hydrophobic interactions. The results of our in vitro study (Fig. 10) assessing reducing activity of aspasome dispersion reveal that antioxidant potency of aspasome is comparable to that of ascorbic acid or ascorbyl palmitate and thus, the antioxidant activity of ascorbyl moiety of ascorbyl palmitate is retained even after converting it into vesicles (Aspasomes).

The free radicals in aqueous environment of blood cell suspension can effectively interact with ascorbyl head group of aspasome vesicle and get neutralized. In addition, amphipathic nature of aspasome allows it to enter the hydrophobic domain of blood cell membrane, where it can suppress lipoperoxidation.

The nanosize of aspasome vesicle can bring about much higher adhesion to blood cell membranes and alternatively might allow them to localize intracellularly. Nanoparticles were shown to posses much higher adhesion to biomembranes than particles of micro/macro size (Muller et al., 2001). Other than erythrocytes, blood contains a large number of lymphocytes and neutrophiles, which are involved in phagocytosis. Internalization of liposomes and nanoparticles into these cells was reported extensively (Nishioka and Yoshino, 2001; O'Hagan et al., 1992; Oussoren et al., 1997; Yagi, 1986; Crommelin and Schreier, 1994; Kreuter, 1994; Finkelstein and Weissmann, 1981).

The free polar ascorbyl head groups, hydrophobicity/amphiphilicity and nanosize of aspasome might enable it to neutralize reactive oxygen species of blood in all the three pools mentioned above. Thus, its superior antioxidant activity than ascorbic acid is due to these mechanisms.

3.4. Transdermal permeation studies

Effect of liposomes and niosomes (Egbaria and Weiner, 1990; Knepp et al., 1990; Hofland et al., 1994; Schreir and Bouwstra, 1994; Touitou et al., 1994; VanHal and Bouwstra, 1996; Redziniak and Perrier, 1996; Seiller et al., 1996) on transdermal permeation of drugs and cosmetic materials has been studied extensively.

Although several studies were conducted for transdermal delivery of AZT using iontophoresis (Wearly and Chien, 1990; Oh et al., 1998), prodrugs, enhancers (Seki et al., 1990a,b; Kim and Chien, 1996) or vehicles (Jin et al., 2000; Thomas and Panchagnula, 2003), no

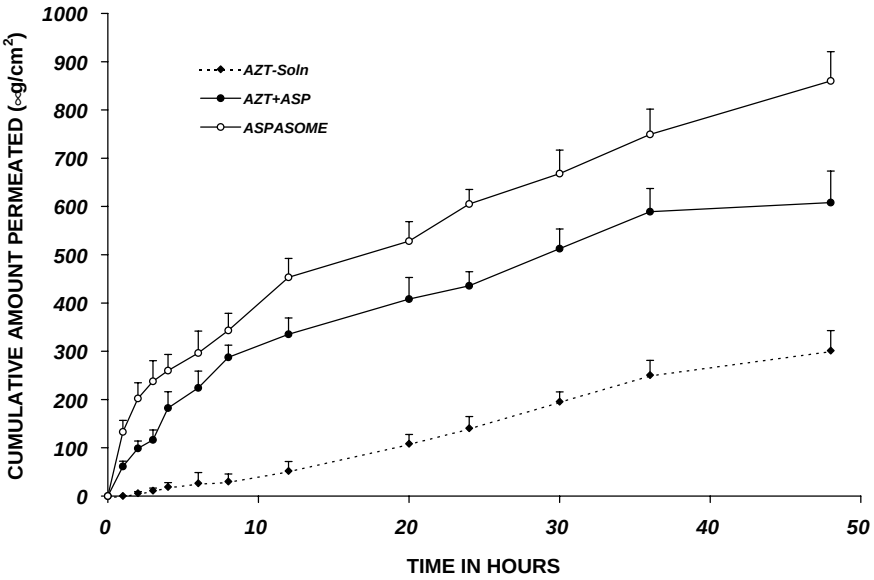


Fig. 11. In vitro permeation profiles of AZT across excised rat skin following treatment with various systems. All systems are significantly different from each other (*t*-test, $P < 0.05$). Each point represents the mean ± S.D. ($n = 3$).

data is available on transdermal delivery of AZT using colloidal vesicular carriers i.e., liposomes or niosomes.

We have studied transdermal permeation of AZT using aspasome preparation (ASP: CHOL: DCP-45:45:10 mol%). The in vitro transdermal penetration profiles of AZT-solution, AZT-ASP dispersion and aspasomal AZT across rat skin is shown in Fig. 11. The aspasomal AZT showed much higher permeation than AZT solution. However, even mere presence of ascorbyl palmitate has enhanced AZT permeation like many other fatty materials (Kim et al., 1993; Andega et al., 2001; Aungst et al., 1986; Loftsson et al., 1995; Oh et al., 2001) and surfactants (Sarpotdar and Zatz, 1986; Aungst et al., 1986; Smith and Maibach, 1995; Cappel and Kreuter 1991a,b). This advocates more studies to further elucidate its skin permeation enhancement property.

Although no detailed study was conducted to understand the mechanism by which aspasome is enhancing the permeation of AZT, it is speculated that due to its lipophilicity it partitions into lipids of the skin and by its amphiphilic character alters the intercellular space, thereby improving the AZT permeation (Delgrado-Charo et al., 1997; Schmalfuss et al., 1997).

4. Conclusions

Ascorbyl palmitate formed vesicles (Aspasomes) in presence of cholesterol and charge inducer dicetyl phosphate, encapsulating AZT solution. The antioxidant potency of aspasome was much better than that of ascorbic acid. Thus, they may find applications as drug delivery system in disorders implicated with reactive oxygen species. Aspasomes enhanced the transdermal permeation of AZT. The antioxidant property and skin permeation enhancing property indicate a promising future for aspasome as a transdermal drug delivery system.

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