J. Pharm. Pharmacol. 1994, 46: 704–707
Received August 25, 1993
Accepted March 10, 1994

A Study of Modified Betaines as Cryoprotective Additives

ANDREW W. LLOYD, CEDRIC J. OLLIFF AND KEN J. RUTT

Pharmaceutical Sciences Research Group, Department of Pharmacy, University of Brighton, Moulsecoomb, Brighton BN2 4GJ, UK

Abstract—Glycinebetaine and N-modified betaines have been previously shown to be effective at reducing leakage from liposomes on freeze-thaw procedures. This study involved the preparation of a series of other modified betaines and the comparison of their abilities to reduce leakage from frozen multilamellar liposomes. All the compounds investigated, with the exception of the octyl ester of betaine, reduced the degree of leakage on freezing and thawing with additive concentrations up to 0·6 M. The betaine esters were less effective than betaine as cryoprotective additives and caused an increase in the leakage from unfrozen liposomes. Taurinebetaine, a sulphobetaine, was also less effective at reducing leakage on freezing than betaine and again increased leakage from unfrozen liposomes. Increasing the number of methylene groups between the carboxylate group and the nitrogen improved the ability to reduce leakage, particularly at lower additive concentrations.

Glycinebetaine (betaine) has been studied as a possible cryoprotectant for the prevention of freeze-thaw damage to liposomes (Higgins et al 1984), erythrocytes (Brearley et al 1987) and erythrocyte ghosts (Brearley et al 1988, 1990). Higgins et al (1986) showed glycinebetaine to be as effective as glycerol in protecting egg lecithin multilamellar vesicles (MLV) from freeze-thaw damage at various cooling rates. Glycinebetaine was also shown to be superior to dimethylsulphoxide at concentrations of 0·35 and 0·7 M in the external solution at cooling rates greater than 50°C min^{-1} (Higgins et al 1985). Increasing the internal concentration of glycinebetaine was found to increase the degree of leakage of streptomycin from MLV at cooling rates greater than 10°C min^{-1} (Higgins et al 1987).

Previous studies have investigated the effects of *N*-alkylation of betaine on the ability of betaine to reduce freeze-thaw damage to MLV (Lloyd et al 1992a). Those studies showed that the fully methylated nitrogen atom is essential for optimal activity. The purpose of this study was to investigate the effects of further structural modifications on the ability of betaine to reduce freeze-thaw damage to liposomes.

Materials and Methods

Preparation of (carboxymethyl)trimethylammonium chloride esters (glycinebetaine esters)

The glycinebetaine esters were prepared by modifications of the methods of Balle & Eisfield (1937) and Linfield et al (1963) by treating the appropriate alkylchloroacetate with trimethylamine. Methyl chloroacetate and ethyl chloroacetate were purchased from Aldrich Chemical Co. (Dorset, UK) and purified by base extraction and distillation. Propyl chloroacetate and octyl chloroacetate were prepared by the following general method adapted from Vogel (1978).

Chloroacetic acid (0·5 mol) was dissolved in a mixture of the appropriate alcohol (1 mol), toluene (100 mL) and concentrated sulphuric acid (1 mL). A Dean and Stark apparatus was fitted and the mixture refluxed until no further water was collected in the Dean and Stark trap. An additional 10 mL of the appropriate alcohol was then added and the reflux continued for 30 min. The reaction was transferred to a separating funnel and washed successively with water (100 mL), 1% sodium bicarbonate solution (100 mL) and water (100 mL). The organic phase was dried over anhydrous magnesium sulphate before distillation using a short well-lagged fractionating column.

The (carboxymethyl)trimethylammonium chloride esters ($(CH_3)_3N^+CH_2COO(CH_2)_nCH_3Cl^-$) were prepared from the alkyl chloroacetates using the following generalized method.

Ethanolic trimethylamine (4·2 M, 25 mL) was added to the appropriate alkyl chloroacetate (0·1 mol) and the reaction mixture was stirred until the exothermic reaction had ceased. The ethanol was removed by reduced pressure rotary evaporation to give the product. This was recrystallized from acetonitrile and dried in-vacuo.

(Carboxymethyl)trimethylammonium chloride methyl ester. Yield: 10·3 g (62%), mp 164–165°C, analysis (expected values): carbon 42·5% (42·9%), hydrogen 9·0% (8·4%), nitrogen 7·7% (8·3%), chloride 21·2% (21·2%).

(Carboxymethyl)trimethylammonium chloride ethyl ester. Yield: 9·2 g (51%), mp 161–161·5°C, analysis (expected values): carbon 46·3% (46·3%), hydrogen 8·8% (8·8%), nitrogen 7·5% (7·7%), chloride 20·2% (19·6%).

(Carboxymethyl)trimethylammonium chloride propyl ester. Yield: 17·9 g (92%), mp 130–131°C, analysis (expected values): carbon 49·0% (49·1%), hydrogen 9·2% (9·2%), nitrogen 7·2% (7·2%), chloride 18·7% (18·2%).

(Carboxymethyl)trimethylammonium chloride octyl ester. Yield: 12 g (48%), mp 77–78°C, analysis (expected values): carbon 59·2% (58·8%), hydrogen 10·2% (10·5%), nitrogen 5·5% (5·3%), chloride 13·3% (13·4%).

Preparation of taurine betaine

Taurinebetaine ($(CH_3)_3N^+CH_2CH_2SO_3^-$) was prepared by

Correspondence: A. W. Lloyd, Pharmaceutical Sciences Research Group, Department of Pharmacy, University of Brighton, Moulsecoomb, Brighton BN2 4GJ, UK.

adaption of the method of Barnhurst (1961). Trimethylamine was quaternized with ethylene bromide to give 2-bromoethylammonium bromide: dibromoethane (1 mol) was stirred with ethanolic trimethylamine (4·2 M, 25 mL) for 90 h during which a white precipitate formed. The solid was filtered off, washed with diethylether, recrystallized from ethanol and dried at 65°C. Yield: 19·8 g (80%), mp 200°C (decomposed), analysis (expected values): carbon 24·5% (24·3%), hydrogen 5·4% (5·3%), nitrogen 6·0% (5·7%).

Treatment of 2-bromoethylammonium bromide with sodium sulphite gave the sulphobetaine: 2-bromoethylammonium bromide (0·1 mol) was refluxed with 5% molar excess of sodium sulphite (13·4 g) in water (40 mL) for 3 h. The majority of the water was removed by evaporation. The residue was acidified with concentrated HCl until no further precipitation occurred. The inorganic solid was removed by filtration through a glass-sintered funnel (No. 3) and the filtrate collected. The filtrate was further concentrated in an evaporating dish to a thick syrup. Addition of propan-1-ol caused precipitation of the product as a white solid. The product was filtered off, washed with propan-1-ol and recrystallized from ethanol/water. Yield: 15·4 g (90%), mp > 300°C, analysis (expected values): carbon 35·9% (35·9%), hydrogen 7·8% (7·8%), nitrogen 8·0% (8·4%).

Preparation of (trialkylammonio)carboxylates

The (trialkylammonio)carboxylates ($(CH_3)_3N^+(CH_2)_nCOO^-$) were prepared from their corresponding (carboxyalkyl)trimethylammonium halides ($(CH_3)_3N^+(CH_2)_n COOH, X^-$.) by ion-exchange chromatography as described previously (Lloyd et al 1992a). The other (carboxyalkyl)-trimethylammonium halides were prepared by modification of the methods of Balle & Eisfield (1937), Linfield et al (1963) and Beckett & Woodward (1963).

An appropriate ethyl haloacetate (0·1 mol) was reacted with ethanolic trimethylamine (4·2 M, 25 mL). The mixture was stirred for 3 h during which precipitation of a white solid occurred. The ethanol was removed from the reaction mixture by rotary evaporation and the residue dissolved in 3·6% w/v HCl (100 mL). This mixture was refluxed for 3 h. Evaporation of the solvent under reduced pressure gave a white product which was recrystallized from ethanol.

(2-Carboxyethyl)trimethylammonium chloride. Yield: 11·1 g (66%), mp 189–191°C, analysis (expected values): carbon 43·2% (43·0%), hydrogen 8·7% (8·4%), nitrogen 7·5% (8·4%).

(4-Carboxybutyl)trimethylammonium bromide. Yield: 7·2 g (25%), mp 182–184°C, analysis (expected values): carbon 39·7% (40·0%), hydrogen 7·6% (7·5%), nitrogen 5·4% (5·8%).

(5-Carboxypentyl)trimethylammonium bromide. Yield: 6·2 g (25%), mp 185·5–186·5°C, analysis (expected values): carbon 42·5% (42·5%), hydrogen 8·0% (7·9%), nitrogen 5·5% (5·5%).

The structures of all compounds were confirmed by infrared spectroscopy and ^{1}H NMR spectroscopy. The melting points are uncorrected. The microanalyses were performed on a Perkin-Elmer 240 elemental analyser by Butterworth Laboratories (Middlesex, UK).

Liposome preparation

This method has been described in detail previously (Lloyd et al 1992a). In brief, a film of lipid which had been previously dried onto the inside of a round-bottomed flask was hydrated, using a rotary evaporator, with a 1% w/v solution of a water soluble dye, amaranth in 0·02 M pH 7·0 phosphate buffer (5·0 mL). The liposome suspension was stored at −20°C until required. After thawing, the liposome suspension was freeze-thawed three times by direct immersion of the tube in liquid nitrogen followed by immersion in a waterbath at 50°C. The resultant liposome suspension was maintained at room temperature (21°C) for 1 h to anneal the liposome structure. The liposomes were then washed to remove the unentrapped amaranth, before resuspension in 0·02 M phosphate buffer to give a stock solution containing 10 mg mL^{-1} lipid for use in freeze-thaw experiments.

Liposomes prepared in this way had a slight positive zeta potential (+7 mV, Coulter Delsa) when suspended in the buffer solution; they were multilamellar (electronmicroscopy) and had a particle size of 1–10 μm (Malvern Mastersizer). Of the amaranth solution added, 2·6% was entrapped giving an entrapment of 2·5 mg amaranth/100 mg lipid.

Freeze-thaw protocol and determination of entrapped amaranth

The liposomes were diluted with cryoprotectant solutions and portions of the liposome suspensions (200 μL) were placed in 1·5-mL polypropylene Eppendorf centrifuge tubes. Samples were frozen in a circular rack by immersion into liquid nitrogen (2 min) and then thawed in a waterbath at 50°C (2 min). The rate of cooling (between −20 and −50°C) on immersion into liquid nitrogen was 720°C min^{-1} and the rate of warming in a water bath at 50°C over the same temperature span was 840°C min^{-1} (Lloyd et al 1992a). Phosphate buffer (1·0 mL) was added to each tube and the liposomes pelleted by centrifugation at 15 000 rev min^{-1} (15 600 *g*) in an Eppendorf centrifuge (model 5414S). The supernatant was discarded and the liposome pellet washed by resuspension in buffer and further centrifugation. The liposome pellets were finally solubilized in 50% propan-1-ol (1·2 mL). The amaranth remaining entrapped was determined by measuring the absorbance of each solution at 522 nm using a Perkin-Elmer Lambda 2 spectrophotometer.

The percentage of amaranth remaining entrapped after the sample had been freeze-thawed was used as a measure of cryoprotective activity.

Results and Discussion

Triplicates of amaranth-containing liposomes suspended in buffer alone or in buffer with different concentrations of the various betaines were freeze-thawed as outlined above. In control experiments, in which the liposomes were not frozen, there were no differences in leakage over the duration of the experiment in the absence of any additives. Fig. 1 shows the effect of freezing liposome samples in the presence of various concentrations of the methyl, ethyl and propyl

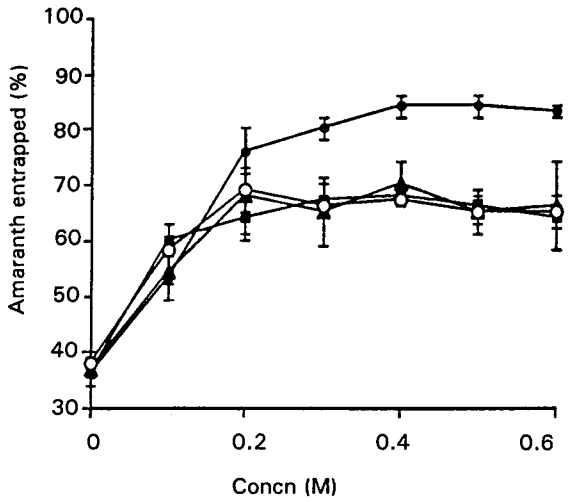

FIG. 1. The effect of glycinebetaine (●), glycinebetaine methyl ester (■), glycinebetaine ethyl ester (▲) and glycinebetaine propyl ester (○) on amaranth loss from liposomes on freeze-thaw. Bars indicate range of values (n = 3).

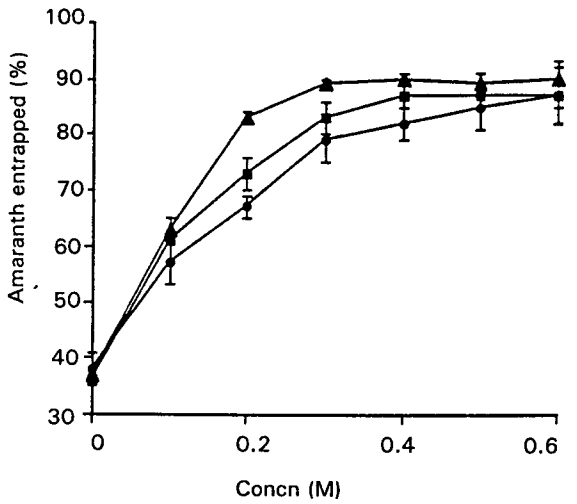

FIG. 3. The effect of glycinebetaine (●), 3-(trimethylammonio)-propionate (■) and 6-(trimethylammonio)hexanoate (▲) on amaranth loss from liposomes on freeze-thaw. Bars indicate range of values (n = 3).

betaine esters. All three compounds were found to be less effective than betaine at concentrations above 0·1 M. They were all found to cause more leakage in the absence of freezing than betaine (not shown). The octyl ester was found to cause solubilization of the liposomes at concentrations as low as 0·05 M. Fig. 2 compares the abilities of glycinebetaine and taurinebetaine to reduce leakage of amaranth from freeze-thawed MLV. Taurinebetaine appears to be much less effective than glycinebetaine at reducing leakage from MLV on freezing and thawing, particularly at higher additive concentrations. Fig. 3 shows the abilities of the (trimethylammonio)carboxylates to reduce leakage from MLV on freezing and thawing. In control experiments, in which the liposomes were not frozen, there were no differences in leakage over the duration of the experiment for any of these compounds. The results suggest that in general the ability of the compound to reduce leakage on freezing and thawing increases with the number of methylene bridging groups, particularly at the lower additive concentrations. In a further study the 6-(trimethylammonio)hexanoate, the inner salt of (5-carboxymethyl)trimethyl ammonium bromide was found to be significantly more active than glycinebetaine at 0·1 M ($P < 0{\cdot}001$, n = 12).

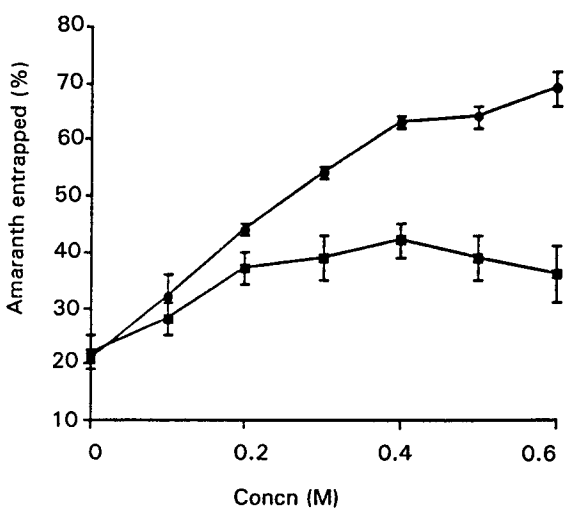

FIG. 2. The effect of glycinebetaine (●) and taurinebetaine (■) on amaranth loss from liposomes on freeze-thaw. Bars indicate range of values (n = 3).

The processes resulting in drug leakage on the freezing of liposomes and the mechanisms by which glycinebetaine and *N*-modified betaines reduce these effects have been extensively discussed (Higgins et al 1986; Lloyd et al 1992a). The variation in the cryoprotective activity of various betaines has been previously attributed to a combination of factors including differences in both water activities of the solutions and the ability of the additives to promote glass formation (Lloyd et al 1992b). The latter effects may explain the differences observed for the betaine derivatives studied in this work. Thus the addition of solutes in high concentrations alters the activity of extracellular water (Bryant & Wolfe 1992). This changes the pressure and consequently the intracellular water activity, at any given chemical potential, which modifies the stress on the membrane. Increasing the hydrogen-bonding capacity of the carboxylate group increases the structuring of localized water and thus decreases the extracellular water activity. This reduces the extracellular pressure and the stress on the membrane at any given temperature during the freezing and thawing process. Furthermore, increasing the structuring of localized water around the betaine molecule will suppress the anomalous structuring of bulk water which occurs in supercooled water, decreasing molecular mobility at low temperatures so that ice nucleation is less likely, thereby promoting vitrification of the solution (Macfarlane & Forsyth 1990).

Both these effects are therefore related to the ability of additives to structure local water molecules through hydrogen bonding. The effect of esterification may, therefore, simply reflect the importance of the carboxylate group for optimal reduction of leakage from MLV on freezing; by

esterifying the carboxylate group the molecule becomes much less negatively charged, has less hydrogen bonding capacity and the cryoprotective activity of the compound is reduced. Alternatively, these effects may be attributed to the differences in the number of ionic species in the ester solutions compared with the betaine solutions. This would increase the osmotic imbalance across the membrane and may increase the initial shrinkage of the liposomes at any given additive concentration. However, studies on the cryopreservation of erythrocytes, which are much more susceptible to osmotic damage, suggest that this is probably not the only factor involved in this cryoprotective process (Lloyd et al 1990).

The effects of taurinebetaine and glycinebetaine may be attributed to the differences in hydrogen bonding capacity as the carboxylate group is a stronger hydrogen bond acceptor than the sulphonate group (Friedman & Kreshnan 1973), although solubility could play a role in this case.

The difference in activity between the different (trimethylammonio)carboxylates may also be attributed to an increase in hydrogen-bonding capacity of the carboxylate group. As the distance between the positively-charged nitrogen atom and the negatively-charged carboxylate group increases, more charge resides on the oxygen atoms increasing their hydrogen bonding capacity.

The results of these studies show that the ability of glycinebetaine to reduce leakage from frozen liposomes can be improved by chemical modification. However, further studies are required to understand the mechanisms by which such compounds work. Given that glycinebetaine is administered at a dose of 6 g per day for the treatment of homocystinuria with no apparent toxic effects (Wilchen et al 1983), compounds of this type may offer great potential as cryopreservatives for the long-term frozen storage of liposome formulations.

References

Balle, G., Eisfield, K. (1937) Quaternary ammonium compounds and a process of preparing them. US Patent 2,087,565

Barnhurst, J. D. (1961) Dipolar ions related to taurine. J. Org. Chem. 26: 4520–4522

Beckett, A. H., Woodward, R. J. (1963) Surface-active betaines: *N*-alkyl-*N*, *N*-dimethylglycines and their critical micelle concentrations. J. Pharm. Pharmacol. 15: 422–431

Brearley, C. A., Hodges, N. A., Olliff, C. J. (1987) Optimal concentrations for non-penetrating cryopreservatives of human blood. J. Pharm. Pharmacol. 39 (Suppl.): 30P

Brearley, C. A., Lloyd, A. W., Olliff, C. J., Hodges, N. A. (1988) A comparative study of the cryopreservation of human erythrocytes, ghosts and liposomes. Biochem. Soc. Trans. 16: 354

Brearley, C. A., Hodges, N. A., Olliff, C. J. (1990) The retention of entrapped molecules within erythrocyte ghosts during cryopreservation. J. Pharm. Pharmacol. 42: 297–301

Bryant, G., Wolfe, J. (1992) Interfacial forces in cryobiology and anhydrobiology. Cryo. Lett. 13: 23–36

Friedman, H. L., Kreshnan, C. U. (1973) Thermodynamics of ionic hydration. In: Franks, F. (ed.) Water: A Comprehensive Treatise Vol. 3. Plenum Press, New York, pp 96–97

Higgins, J., Hodges, N., Olliff, C., Phillips, A. (1984) The use of cryoprotectives to minimise drug leakage from liposomes during freezing and thawing. J. Pharm. Pharmacol. 36 (Suppl.): 24P

Higgins, J., Hodges, N., Olliff, C., Phillips, A. (1985) The evaluation of betaine as a liposome cryoprotective. J. Pharm. Pharmacol. 37 (Suppl.): 55P

Higgins, J., Hodges, N. A., Olliff, C. J., Phillips, A. J. (1986) Factors influencing cryoprotective activity and drug leakage from liposomes after freezing. J. Pharm. Pharmacol. 38: 259–263

Higgins, J., Hodges, N. A., Olliff, C. J., Phillips, A. J. (1987) A comparative investigation of glycinebetaine and dimethylsulphoxide as liposome cryoprotectants. J. Pharm. Pharmacol. 39: 577–582

Linfield, W. M., Abend, P. G., Davis, G. A. (1963) The synthesis and surface active properties of certain amphoteric compounds. J. Am. Oil Chem. Soc. 40: 114–117

Lloyd, A. W., Rutt, K. J., Olliff, C. J. (1990) The synthesis and comparison of modified betaines as cryoprotective agents. J. Pharm. Pharmacol. 42 (Suppl.): 75P

Lloyd, A. W., Baker, J. A., Smith, G., Olliff, C. J., Rutt, K. J. (1992a) A comparison of glycine, sarcosine, *N*, *N*-dimethylglycine, glycinebetaine and *N*-modified betaines as liposome cryoprotectants. J. Pharm. Pharmacol. 44: 507–511

Lloyd, A. W., Baker, J. A., Olliff, C. J., Rutt, K. J. (1992b) The evaluation of *N*-modified betaines as erythrocyte cryoprotectants. Cryo. Lett. 13: 337–348

Macfarlane, D. R., Forsyth, M. (1990) Recent insights on the role of cryoprotective agents in vitrification. Cryobiology 27: 345–358

Vogel, A. (1978) Practical Organic Synthesis. Longman Inc., New York

Wilchen, D. E. L., Wilchen, B., Dudman, N. P. B., Tyrrell, P. A. (1983) Homocystinuria—the effect of betaine in the treatment of patients not responsive to pyridoxine. N. Engl. J. Med. 309: 408–453