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The measurement of beclomethasone dipropionate entrapment in liposomes: a comparison of a microscope and an HPLC method

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Abstract

The purpose of this study was to examine the methodologies that may be used to estimate the maximum incorporation (< 5 mole% drug) of beclomethasone dipropionate (BDP) in dipalmitoylphosphatidylcholine (DPPC) multilamellar liposomes. Two approaches are described. First, differential interference contrast (DIC) microscopy and cross-polarisation microscopy have been used to measure the concentration at which BDP crystals become apparent in BDP-containing liposome preparations, thereby allowing a semi-quantitative but simple estimation of entrapment. An alternative method is described whereby the unentrapped solid drug is separated from the liposomes via suspension in D_2O, followed by centrifugation and HPLC analysis. The method resulted in an estimate of 1.5–2 mole% BDP, while the HPLC method yielded a value of 2.52 mole% BDP. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Beclomethasone dipropionate; Crystal; HPLC; Liposome; Microscopy

1. Introduction

Liposomal formulations of the glucocorticoid beclomethasone dipropionate (BDP) for delivery from nebulisers have been investigated as a means of improving pulmonary delivery of the steroid and minimising adverse effects, such as hoarseness and *Candida* infections in the upper respiratory tract (Waldrep et al., 1994b; Vidgren et al., 1995; Waldrep et al., 1997). In order to optimise the manufacture and therapeutic effectiveness of such formulations, it is clearly necessary to understand and control the entrapment of the steroid within the liposomal system. BDP is a highly hydrophobic steroidal drug which is formulated as an aqueous suspension for nebuliser therapy (Webb et al., 1986). When incorporated into phospho-

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lipid films or bilayers, BDP, as a result of its hydrophobic nature, would be expected to be associated with the hydrocarbon chain region of the lipid molecules.

However, steroids and steroid esters have limited solubilities in phospholipid systems (Fildes and Oliver, 1978; Smith et al., 1980), hence maximum entrapment levels are known to be low, with excess drug being present in a crystalline form. Studies with hydrocortisone have shown that whilst it may interact with phospholipid head-groups (Cleary and Zatz, 1973) the palmitate ester has limited affinity for the phospholipid, with the excess forming a discrete phase or acting as insoluble particles stabilised by adsorbed phospholipid (Shaw et al., 1976). Crystalline drug may be external or internal to the liposome, hence release rates may be partially attributable to the dissolution of this excess material rather than efflux from liposomes. The initial rapid release of synthetic steroids from liposomes has also been attributed to an incompatible fit of the steroid in the liposome bilayer (Shaw et al., 1976; Radhakrishnan, 1991). Radhakrishnan (1991) has suggested that BDP tends to crystallise in liposomal formulations due to an incompatible steric fit between the steroid and the bilayer, resulting in large amounts of crystalline steroid being detected after extrusion and on storage. Waldrep et al. (1994a,b) reported acceptable levels of drug entrapment in dilaurylphosphatidylcholine (DLPC) MLV formulations using a BDP:lipid ratio of 1:21.

In general, the knowledge base regarding the factors influencing the liposomal incorporation of drugs which exhibit low-level entrapment is limited, even to the extent that the means of quantifying entrapment levels have not been widely studied.

In order to calculate the maximum incorporation of drug in a liposome formulation, it is almost invariably necessary to separate free drug from liposomally-associated material. A number of techniques, including gel-filtration, dialysis and centrifugation are available for the study of drugs having medium or high aqueous solubility, whereby the non-associated drug is present as an aqueous solution in the surrounding medium (Tyrell et al., 1976). However, when examining drugs which exhibit poor aqueous solubility, the separation of unentrapped material becomes considerably more problematic, since excess drug may be present as suspended particles in the aqueous phase. If there is a marked difference between the size of the crystals and the liposomes, ultracentrifugation alone may be used to separate the two components (Sharma et al., 1994; Al-Muhammed et al., 1996) or combined with filtration (Moribe et al., 1999). Optical microscopy (Khokhar et al., 1991; Sampedro et al., 1993) and freeze-fracture electron microscopy (Perez-Soler et al., 1987) have been used to visually confirm that liposomes sedimented by ultracentrifugation are free from unincorporated drug.

The difficulties associated with quantifying the incorporation of hydrophobic materials in liposomes are compounded when the material in question exhibits low levels of incorporation and when the sizes and densities of liposomes and drug crystals are similar (as is the case with the systems under study here), then serious difficulties occur for separation and subsequent quantitation of entrapment. In such instances, density-gradient ultracentrifugation may be necessary to achieve separation of liposome-associated and non-associated material (Fraley et al., 1980; Versluis et al., 1998).

In this study, two approaches have been used to estimate the maximum entrapment of BDP in dipalmitoylphosphatidylcholine (DPPC) liposomes. First, differential interference contrast (DIC) microscopy and cross-polarisation (CP) microscopy have been used to estimate the concentration at which BDP crystals are seen in liposomal preparations. This is a simple approach, in which the concentration at which crystals appear is noted, yielding a semi-quantitative assessment of entrapment. Second, a method has been developed for the removal of unentrapped drug crystals which then allows assay of drug loading using HPLC. By comparing both the practicalities and the results obtained using both approaches, it is intended that reliable measurement of maximum entrapment values may be facilitated.

2. Materials and methods

2.1. Preparation of liposomes

BDP-containing multilamellar vesicles (MLVs) were manufactured by weighing appropriate amounts of DPPC; approximately 99%, Sigma Chemicals, UK) and BDP monohydrate (GlaxoWellcome, UK) into a round-bottomed flask, and adding chloroform (HiPerSolv, BDH Chemicals, UK) to dissolve both components. Chloroform was removed by rotary evaporation under vacuum in a water bath at 55°C for 15 min. The flask was then flushed with nitrogen for 1–2 min to remove any traces of residual solvent. An appropriate volume of deionised water (Model WP 700, Whatman, UK), further purified by passing through an Elgastat Ultra High Quality Purification System (Elga Ltd., UK) was added to the dry film in the flask to give a final phospholipid concentration of 10–50 mg/ml. Glass beads were added to aid mixing, the flask flushed with nitrogen and gently rotated for 30 min in the water bath, and shaken to produce MLVs. The suspension was annealed for a further 2 h in the water bath at 55°C before storage under nitrogen in a refrigerator at 2–8°C.

2.2. Microscopy investigations of BDP-containing liposome preparations

A small drop of the liposomal sample (DPPC concentration 10 mg/ml) containing BDP monohydrate (1, 1.5, 2, 3, 4, 5 and 10 mole% of phospholipid content) was deposited onto a clean glass slide, a glass cover-slip was gently placed on top and the sample viewed with the microscope. The microscope (Olympus BX50, Olympus, Japan) used in these studies could be used in either the DIC microscopy or CP microscopy mode. DIC introduces contrast into non-absorbent specimens. Plane polarised light is split into two beams which pass through adjacent points on the sample. A prism placed above the objective recombines the beams and the phase change between the two is converted into an amplitude of colour difference. The samples were examined using both techniques with a view to detecting the presence of crystalline drug in the surrounding aqueous medium.

2.3. Separation of unentrapped BDP from liposome suspensions

In order to allow HPLC analysis of the entrapment of BDP in liposomal formulations, it was necessary to remove the excess solid BDP, hence a separation method was developed which was based on the density difference between the liposomes and the BDP crystals. While centrifugation of liposomes results in the sedimentation of suspended particles, suspension in a medium of greater density may result in flotation of the liposomes (Fraley et al., 1980). The methodology employed in this study has investigated whether differences in the densities of H_2O (0.9982 g/ml at 20°C; Weast, 1988b) and deuterated water, D_2O (1.053 g/ml at 20°C; Weast, 1988a) may be used as a means of separating liposomes from unincorporated BDP crystals. Consequently, the effects of preparing and suspending the liposomes in H_2O and D_2O have been studied, as well as preparing the liposomes in H_2O and suspending in D_2O and vice versa.

A DPPC liposomal suspension containing 10 or 20 mg/ml phospholipid and 10 mole% BDP was prepared in 5 ml deionised water, this concentration of drug being in clear excess of the maximum entrapment value. 1-ml samples were added to either 5 ml of deionised water or deuterated water (Sigma Chemicals, UK), in centrifuge tubes, which were then centrifuged for 1 h at 2280 rpm in a bench centrifuge (Minor S, M.S.E. Ltd., UK). The liposome fraction following centrifugation in D_2O was present as floating material, and was removed using a Pasteur pipette and observed using DIC and CP microscopy.

After liposomes prepared in D_2O were centrifuged, the floating layer was removed and resuspended in 5 ml D_2O, and centrifuged at 2280 rpm for a further 1 h. In addition, liposomes containing 10 mg/ml DPPC and 10 mole% BDP were prepared in 5 ml of D_2O, and 1 ml of these liposomes was suspended in 5 ml of D_2O and centrifuged at 3040 rpm for 1 h. This was repeated three times and each fraction was removed

in the same way as before and viewed for the presence of crystals.

2.4. HPLC assay for the determination of BDP in the presence of DPPC

Samples for the HPLC assay were dissolved in methanol (HiPerSolv, BDH Chemicals, Poole, UK). The mobile phase was methanol:deionised water in a ratio of 7:3 (v/v). The HPLC apparatus (Gilson, Anachem, Beckenham, UK) was used with a mobile phase flow rate of 2 ml/min, with a sample injection volume of 50 μl and UV detection at 238 nm. All solvents were deaerated by sonication in an ultrasonic bath (Decon Ultrasonics Ltd., Sussex, UK), for 10 min. The chart recorder (Servogor 120, John Minster Instruments, Folkestone, UK) parameters were set at 10 mV with a chart speed of 3 cm/min. All samples were assayed a minimum of four times, and the column was rinsed with injections of mobile phase between each assay. From the resultant chromatograms, peak areas were calculated manually from peak height × half base width, and a calibration curve for BDP monohydrate in methanol at concentrations of 0, 4, 6, 8, 10, 16 and 20 μg/ml prepared. The assay was validated using calibration solutions of BDP in the absence and presence of DPPC.

2.5. Determination of the maximum amount of BDP entrapped in DPPC liposomes

Liposomes containing 50 mg/ml DPPC and 5 mole% BDP were checked microscopically for the presence of crystals, which were sedimented by centrifugation in D_2O (Section 2.3). The top layer of concentrated liposomes was then removed and deposited onto a tared glass slide. This was checked microscopically for the absence of crystals and placed in an oven at 50°C. The weight of the sample was monitored until no further weight loss was observed, indicating that all the water in the sample had been removed. The dried sample was weighed, dissolved in and made up to 10 ml with methanol and assayed by HPLC to determine the concentration of BDP that had been incorporated in the DPPC liposomes (calculated as the hydrate). BDP present in the aqueous phase of these preparations was considered negligible, given the insolubility of the drug and the high concentration of liposomes/lipid in the collected fraction.

3. Results and discussion

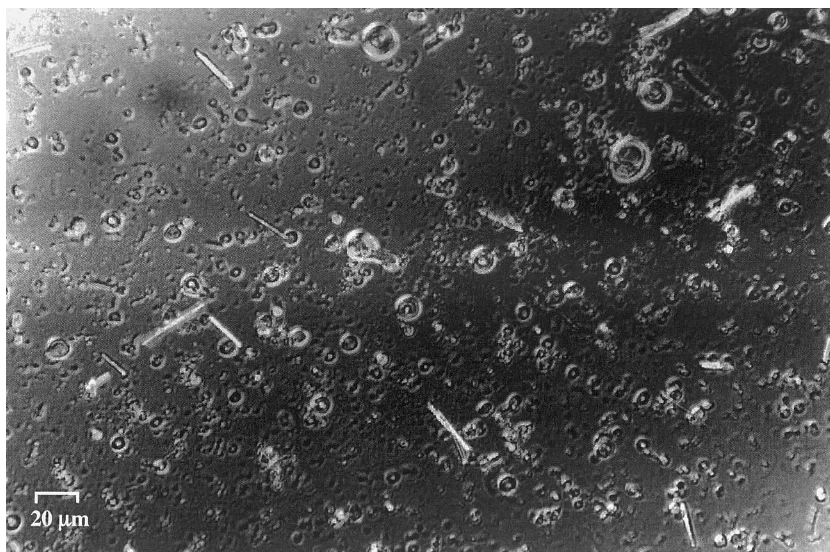
3.1. DIC and CP microscopy

DIC and CP microscopy showed the presence of drug crystals in liposomal preparations containing 2, 3, 4, 5 and 10 mole% BDP, with representative images for the 2% systems shown in Fig. 1a and b. The BDP crystals are particularly well visualised using DIC microscopy (Fig. 1a) whilst liposomes are easily identified by the distinctive Maltese crosses produced when viewed using CP microscopy (Fig. 1b). However, BDP crystals were not detected in samples containing 1.5 mole% BDP using DIC (Fig. 2a) or CP (Fig. 2b) microscopy, nor those with 1 mole% BDP, suggesting that the limiting concentration for crystal formation in DPPC liposomes is between 1.5 and 2 mole% BDP. Interestingly, samples containing 1.5 mole% BDP showed the presence of crystals after 3 days of storage in the refrigerator at 4°C, under an inert atmosphere of nitrogen, suggesting instability of BDP in DPPC liposomes and/or the temperature dependency of BDP solubility in DPPC bilayers.

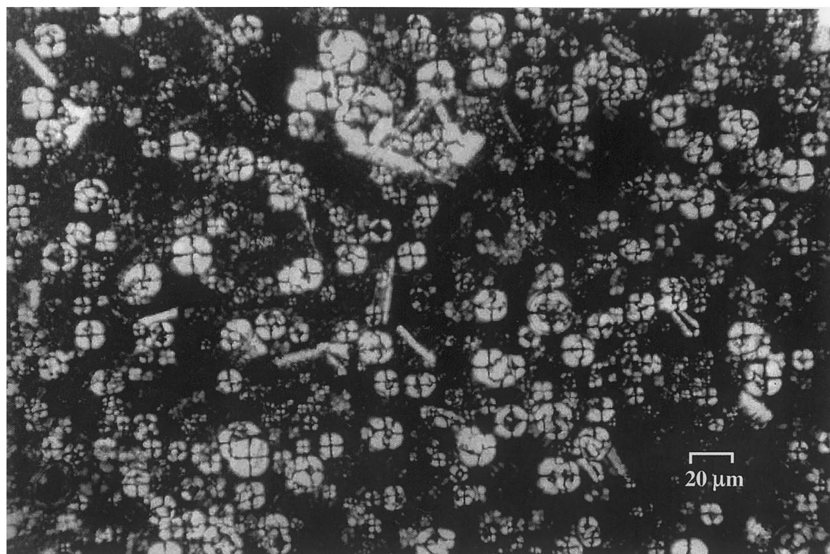
3.2. Separation of unentrapped BDP

The basis behind separation of free material by centrifugation is generally that liposomes containing entrapped material will be more dense than the suspending medium. Thus, when such liposomes are subjected to a high gravitational field, they would be expected to sediment, whilst any free material should remain in the supernatant. This has been the method of choice for many authors (Meisner et al., 1989; Taylor et al., 1990; Ma et al., 1991), though the method is unable to efficiently sediment small vesicles (Tyrell et al., 1976). As an alternative, liposomes may be suspended in a medium of higher density, resulting in

the liposomes and liposomally-entrapped material floating on top (Fraley et al., 1980). This may be useful if the entrapped material has a high molecular weight, or is entrapped at high concentrations, making sedimentation difficult. However, centrifugation may be problematic when non-liposome-associated drug is in a crystalline form and sediments or floats with the liposomes. For the purposes of the present study, it was clearly of interest to investigate whether manipulation of the relative densities of the liposomes and suspending medium may be used as a means of separating the crystalline phase. If this can be achieved, the separated liposomes may then be assayed for drug content and this figure used to establish maximum entrapment.



(a)



(b)

Fig. 1. (a) DIC micrograph of DPPC MLVs containing 2 mole% BDP; (b) CP micrograph of DPPC MLVs containing 2 mole% BDP.

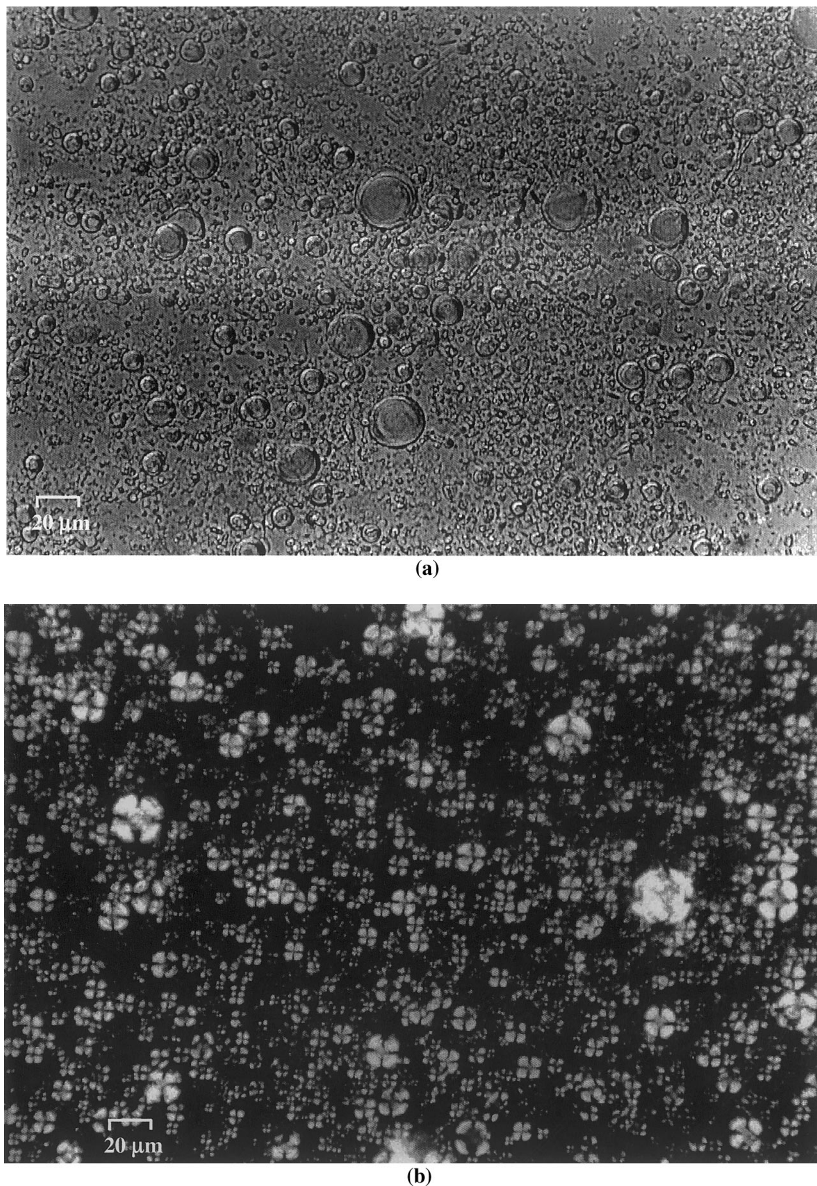


Fig. 2. (a) DIC micrograph of DPPC MLVs containing 1.5 mole% BDP; (b) CP micrograph of DPPC MLVs containing 1.5 mole% BDP.

In the first instance, liposomes prepared and suspended in H_2O were studied in terms of their behaviour on centrifugation. Using DIC and CP microscopy, both the supernatant and the pellet were found to contain liposomes and crystals, while no surface layer was apparent. Clearly, then, this method of preparation and suspension does not facilitate component separation by centrifugation. Liposomes prepared in H_2O and suspended in D_2O were then examined. In this case, a surface layer was formed reflecting the higher density of the suspending medium, which on mi-

croscopic examination was found to contain liposomes with a few small drug crystals. The supernatant contained a few liposomes and crystals, whilst the pellet contained a large number of crystals with no liposomes being apparent.

The converse system was then examined (liposomes prepared in D_2O and suspended in H_2O). As expected, the pellet was found to contain both liposomes and crystals, while no surface layer was formed. Finally, liposomes prepared and suspended in D_2O were examined. In this case, a floating layer comprising liposomes and a few smaller BDP crystals was observed. The supernatant was mostly clear, but some crystals and liposomes were present, whilst the pellet consisted of BDP crystals and a few liposomes. These studies indicated that the method achieving greatest fractionation of the preparations was the preparation and suspension of the liposomes in D_2O and this approach was, therefore, selected for further optimisation. It should be noted that this was, in many ways, an unexpected result, as one would have expected greater separation with the internal H_2O/ external D_2O systems. Furthermore, preparation of liposomes in H_2O would be a more realistic manufacturing method. However, for the purposes of this study, whereby proof of concept of the approach was being explored, it was considered preferable to examine further the systems with optimal separation.

In order to remove the small BDP crystals that still remained in the liposome fraction of liposomes prepared in D_2O after centrifuging in the same medium, the top layer from the sample was removed, re-suspended in 5 ml D_2O and centrifuged for a further 1 h at 3040 rpm. DIC and CP microscopy of the floating liposome fraction was found to contain no crystalline material (Fig. 3a and b, respectively) whilst the pellet consisted of densely-packed BDP crystals with some liposomes (Fig. 4a and Fig. 4b, respectively). Centrifugation in D_2O thus proved an effective method for separating liposome-associated and free BDP and has the advantages that it is relatively quick, simple, and particularly useful when small quantities of entrapped material are required for analytical purposes.

3.3. HPLC assay

All peaks for BDP were symmetrical with a retention time of approximately 8.5 min (Fig. 5a). A linear seven point calibration plot ($R = 0.98$) was constructed between 0 and 20 μg/ml BDP monohydrate. Validation of the assay in the presence of phospholipid was performed by assaying a solution DPPC:BDP (mole ratio 99:1) in methanol. At a concentration of 99 mole%, DPPC did not interfere with the HPLC assay for BDP (Fig. 5b). The assay of this solution produced peak areas which were used to calculate the corresponding BDP concentrations. The peak areas and retention times determined in the absence and presence of DPPC were not significantly different ($P < 0.05$). BDP alone (11.009 μg/ml) had a mean retention time of 8.663 ± 0.111 min with a mean peak area of 1.144 ± 0.088 cm^2. BDP (11.009 μg/ml) plus 99 mole% DPPC had a mean retention time of 8.625 ± 0.050 min with a mean peak area of 1.141 ± 0.035 cm^2.

3.4. Maximum incorporation of BDP in DPPC liposomes determined by HPLC

DPPC liposome formulations prepared with 5 mole% BDP contained drug crystals which were removed by centrifugation in D_2O. The concentrated liposomal sample was then dried in an oven at 50°C overnight to yield a powder (17.463 mg) which was dissolved in methanol and assayed by HPLC to determine the concentration of BDP. The average peak area was 2.841 ± 0.429 cm^2, corresponding to a BDP monohydrate concentration of 31.853 (± 4.810) μg/ml). This corresponds to a mean molar ratio of DPPC:BDP of 39.6:1 in the collected liposome fraction, and indicates that the maximum amount of BDP incorporated into the DPPC liposomes was 2.52 mole% BDP.

Thus, this HPLC assay proved to be a useful technique for the determination of BDP incorporation into liposomes. The assay was found to be unaffected by the presence of DPPC at high concentrations, and dehydrated samples tested for entrapment values of BDP in DPPC liposomes yielded values similar to those determined by microscopy techniques.

4. Conclusion

The concentration at which excess BDP formed crystals in liposomal suspensions, as detected using microscopy, was found to be between 1.5 and 2 mole% BDP.

D_2O was found to be a better suspending medium than H_2O for efficiently separating BDP crystals from liposomes by centrifugation. Employing two or more centrifugation stages at 3040 rpm for 1 h permitted the separation of the smallest BDP crystals from the liposomes. Validation of the HPLC assay in the presence of 99 mole% DPPC showed that this did not affect the

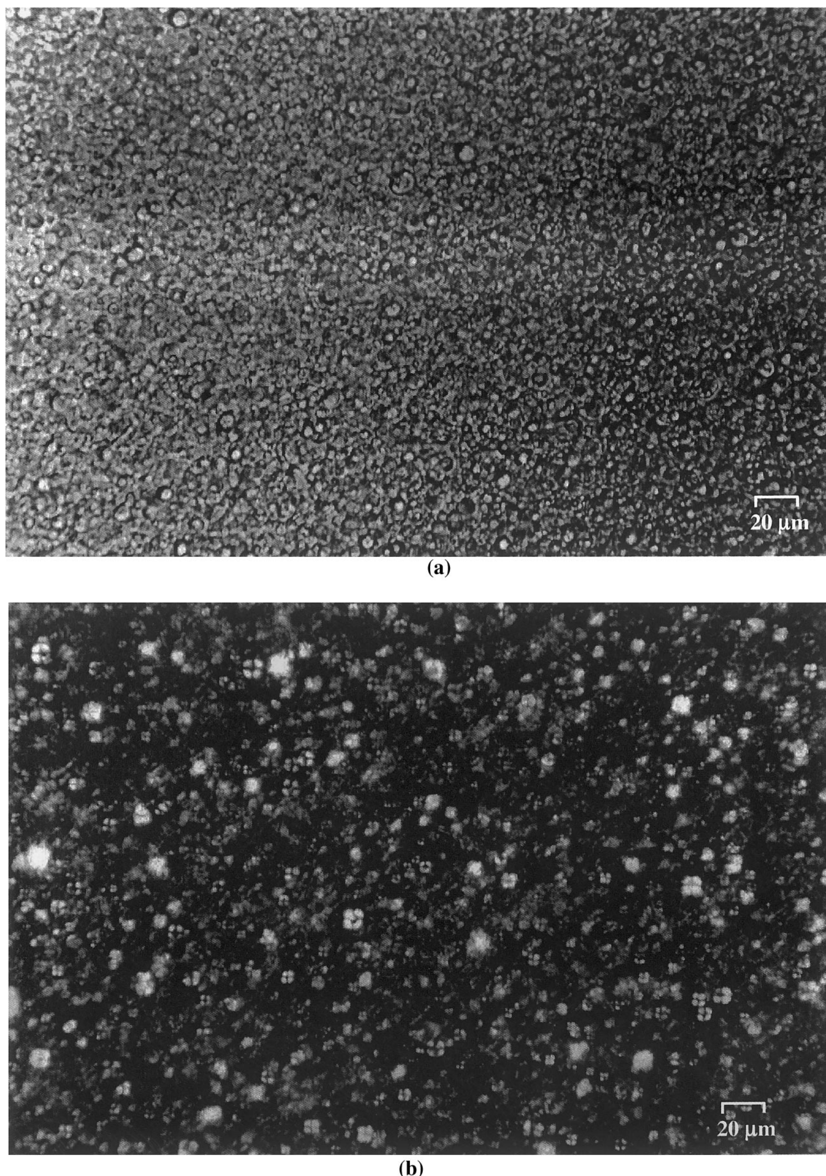
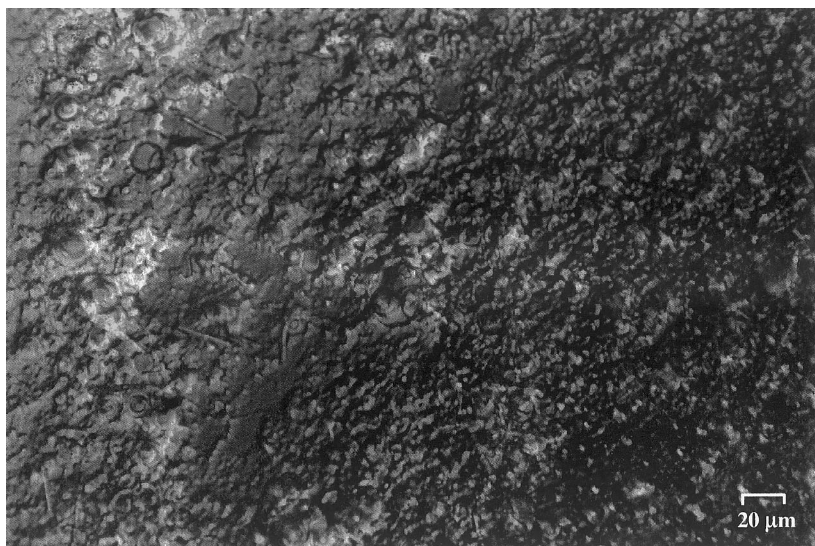
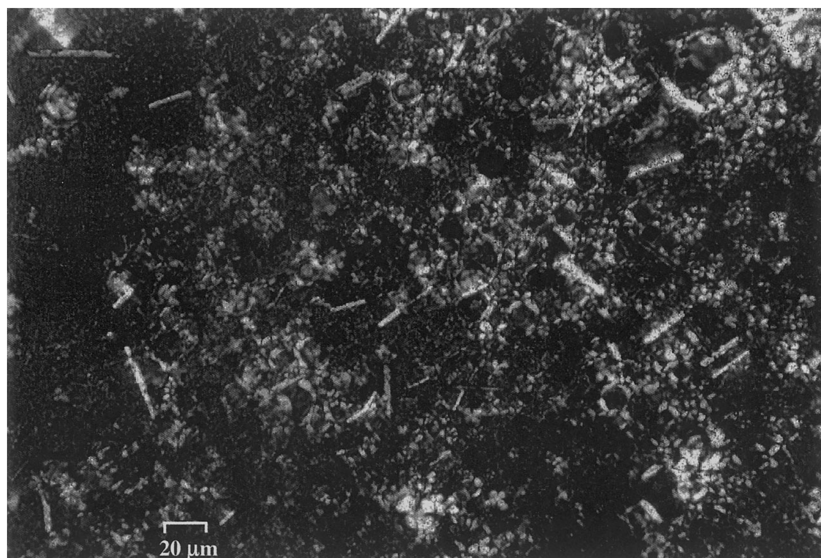


Fig. 3. (a) DIC micrograph of top layer after centrifugation of DPPC MLVs containing 5 mole% BDP, prepared and centrifuged in D_2O; (b) CP micrograph of top layer after centrifugation of DPPC MLVs containing 5 mole% BDP, prepared and centrifuged in D_2O.



(a)



(b)

Fig. 4. (a) DIC micrograph of pellet after centrifugation of DPPC MLVs containing 5 mole% BDP, prepared and centrifuged in D_2O; (b) CP micrograph of pellet after centrifugation of DPPC MLVs containing 5 mole% BDP, prepared and centrifuged in D_2O.

determination of BDP within the sample, or the retention of BDP on the column. The maximum amount of BDP that was completely associated with the DPPC liposomes was found using this method to be 2.52 mole%.

This study has explored two methods of assessing poorly incorporated drug solubility in liposomes. The microscopy method has the advantage of ease and simplicity and is probably satisfactory for many routine examinations. It does not, however, actually measure the amount within the liposomes; indeed it works on the assumption that the liposomes act as a simple solvent for the drug which, on saturation, causes the drug to be present as a separate solid phase. This may not be entirely accurate. Indeed, what the method may at

least be partially measuring is the solid solubility of the drug in the phospholipid film prior to hydration. The HPLC-assay method has the advantage of directly measuring the concentration in the liposomes and also presents a means of crystal separation which is in itself of interest. However, the approach suffers from the disadvantage of involving several stages and the use of D_2O which, to some extent, may alter the partitioning behaviour of the drug. However, the observation that both methods yielded very similar results is encouraging.

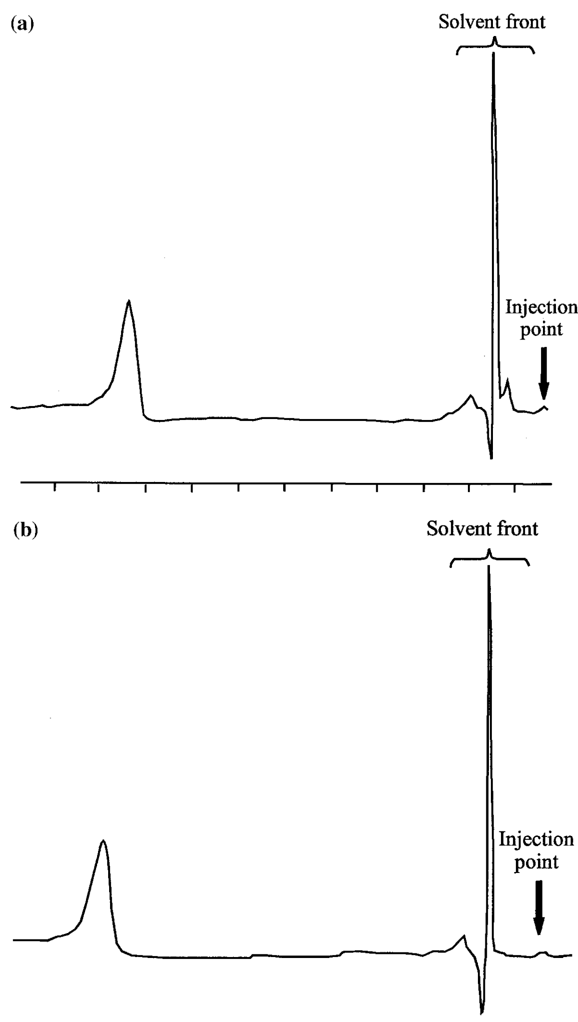


Fig. 5. (a) Chromatogram of BDP (11.009 μg/ml) in methanol; (b) Chromatogram of BDP (11.009 μg/ml) in methanol in the presence of 99 mole% DPPC.

Two further considerations that require further work are first that the measured 'solubility' in the liposomes may not be an equilibrium value, as was noted in this case by the appearance of crystals on storing the liposomes for 3 days. This leaking effect has not yet been adequately explored. Second, while all entrapment calculations were performed on the basis of the monohydrate, it is not yet known whether the BDP has associated water within the liposome bilayer. This is again an area which merits further examination. Overall, however, the study has presented two methods of estimating the maximal concentration of poorly entrapped drugs and a discussion of the concomitant advantages and disadvantages of each approach.

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