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Method for evaluating drug release from liposomes in sink conditions

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Abstract

An in vitro method was developed for the evaluation of the drug release from disperse systems, such as liposomes, under sink conditions. Determination of in vitro release of lipophilic drugs from liposomes requires a dissolution medium that maintains sink conditions without damaging the lipid membrane and a method to separate the released drug from the liposomal drug. We propose a new in vitro technique for the evaluation of drug release from liposomes using a hydrophilic β-cyclodextrin derivative in the dissolution medium to maintain sink conditions. A liposomal drug dispersion was placed in a magnetically stirred dialysis bag (Mw cut-off 300 000) containing cyclodextrins to provide sink conditions. The released drug was sampled from the outside of the bag. Release of hydrocortisone and budesonide from different multilamellar liposomes was measured. True release rate was evaluated from the release data of liposomal and free drug from the dialysis bags, respectively. Release of steroids from the liposomes was relatively fast, but they were retained longer in gel-phase, distearoyl-L-α-phosphatidylcholine (DSPC) liposomes than in liquid-phase, L-α-phosphatidylcholine (EPC) liposomes. Leakage of encapsulated calcein from the liposomes was not affected by cyclodextrins suggesting that they do not disturb the structure of the lipid bilayers. This method is capable of distinguishing different true release rates of drugs from colloidal carriers and it is easy to perform. © 1997 Elsevier Science B.V.

Keywords: Liposomes; Drug release; Dialysis; Steroids; Cyclodextrin; Hydrocortisone; Budesonide; Calcein

1. Introduction

Colloidal systems, such as liposomes and nanoparticles, have gained increasing attention as drug carriers. Evaluation of the drug release from colloidal dispersions is problematic. Owing to the very small size of the colloidal carriers (< 10 μm diameter), there are technical difficulties in the separation of the released drug from the bound rug. However, the release rates should be mea-

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sured in sink conditions, otherwise the release is dependent on the test conditions and does not describe the properties of the delivery system. In the case of lipophilic drugs the solubility in the aqueous phase is low and large volumes are needed for the receptor phase, but this may cause difficulties in detection.

Washington (1990) has reviewed the commonly used methods in release studies from disperse systems. In the traditional dialysis technique, the carrier is suspended in a small volume of continuous phase and separated from a sink solution by a permeable dialysis membrane (Margalit et al., 1991; Elorza et al., 1993; Yerushalmi and Margalit, 1994). This does not, however, provide sink conditions inside the dialysis bag resulting in a partition-controlled erroneous release profile. Levy and Benita (1990) developed a reverse dialysis technique, where the carrier is directly placed into a large volume of release medium. The dialysis bags, containing a small volume of the dissolution medium, were immersed in the solution and later withdrawn from the solution at fixed time intervals for analysis. Diffusion of the drug into the dialysis bag was rate-limiting. More permeable dialysis membranes and efficient mixing inside the dialysis bags should to be used, in particular for systems with rapid release profiles. Recently, Henriksen et al. (1995) introduced a fractional dialysis system with a constant area of dialysis membrane, but this technique did not provide sink conditions.

Surfactants or organic solvents have been used to maintain sink conditions in dissolution testing of water-insoluble drugs from tablets or controlled release systems (Shah et al., 1989; Takahashi et al., 1994; Veiga and Alvarez de Eulate, 1994; Maggi et al., 1996). They increase drug solubility, but their use with liposomes is not possible without disruption. It is also difficult to separate the solubilized drug in micelles from the liposomes or other colloidal systems. Albumin was used to increase the aqueous solubility of lipophilic drugs that are released from submicron emulsions (Magenheim et al., 1993), but albumin increases the solubility only moderately and its separation from the released drug by extraction is necessary before analyzing the drug concentration. Optimal solubilizers should have small molecular weights, to enable easy separation from the colloid and they should increase drug solubility efficiently without solubilizing the dosage form.

In this study, we introduce a simple release test method that maintains sink conditions with cyclodextrins. The method is appropriate for compounds which have poor aqueous solubility and which complex strongly with cyclodextrins. We studied the release of steroids from multilamellar (MLV) liposomes with different lipid compositions using hydrophilic cyclodextrins to ensure the sink conditions in the receiving solution. Dialysis bags were used to separate the released drugs from the liposomes.

2. Materials and methods

2.1. Materials

Budesonide was obtained from Orion Pharmaceuticals (Kuopio, Finland), hydrocortisone was purchased from Oriola Oy (Kuopio, Finland). L-α-Phosphatidylcholine (EPC) from egg yolk was purchased from Avanti Polar Lipids (AL, USA), dipalmitoyl-L-α- phosphatidylglycerol (DPPG) and cholesterol (Chol) were from Sigma (St. Louis, MO), dipalmitoyl-L-α-phosphatidylcholine (DPPC) and distearoyl-L-α-phosphatidylcholine (DSPC) were gifts from Orion-Farmos Pharmaceuticals (Turku, Finland). 2-Hydroxypropyl-β-cyclodextrin (HP-β-CD, Encapsin®; DS = 0.6; Mw = 1297.4) was purchased from Janssen Biotech (Olen, Belgium). Hepes and calcein were from Sigma (St. Louis, MO) and Sephadex® G-50 from Pharmacia LKB (Uppsala, Sweden). Dialysis membranes (Mw cut-offs 12 000–14 000, 25 000, 100 000, 300 000) were from Membrane Filtration Products (San Antonio, USA) and Spectrum Medical Industries (Houston, TX). All other chemicals were of reagent grade and used as received.

2.2. Solubility studies

Solubility enhancement of steroids in the presence of HP-β-CD (4–72 mM) was measured using the phase solubility method (Higuchi and Connors, 1965) at room temperature. The apparent $K_{1:1}$ complexation constants for steroid–CD complexes were calculated from the phase-solubility diagrams (Saarinen-Savolainen et al., 1997).

2.3. Preparation of liposomes

Multilamellar liposomes (MLV) were prepared by the hand-shaking method (New, 1990b). Liposomes were prepared using either a single phospholipid (EPC, DSPC) or lipid mixtures of following molar compositions: DPPC:Chol (2:1), DSPC:DPPG (10:1). Phospholipids (100 mg) and steroids (5 mg) were dissolved in 5 ml of chloroform–methanol mixture (2:1) and the solvent was removed under reduced pressure using a rotary evaporator, at the temperature above the transition temperature (T_c) of the lipids used (EPC at 30°C, DPPC at 46°C, DSPC at 60°C), to deposit a thin film of dry lipid on the walls of the flask. Evaporation was continued for 15 min after the dry residue appeared, then 5 ml of isotonic Hepes buffer (20 mM, pH 7.4) and glass beads were added. The flask was attached to a rotary evaporator and shaken at a temperature above T_c at room pressure for 30 min. Excess, unencapsulated drug was separated from the liposomes by centrifugation at $16\,000 \times g$ for 15 min (Eppendorf Centrifuge, Model 5415 C, Eppendorf–Netheler–Hinz GmbH 2000, Hamburg, Germany). The resulting supernatant was replaced by the same volume of fresh dissolution medium (72 mM CD in 20 mM Hepes, pH 7.4) and the release test was started immediately after adding the CD–buffer solution. Negatively charged liposomes (DSPC:DPPG) were used without centrifugation because their sedimentation was incomplete. Budesonide is, however, well encapsulated into the DSPC or EPC liposomes (85–95% of the total drug) and therefore the amount of free drug can be estimated to be also rather low in DSPC:DPPG liposomes. Large unilamellar calcein liposomes (LUV) were prepared as described earlier (Saarinen-Savolainen et al., 1997). The size distribution of the liposomes was determined by quasi-elastic light-scattering (Nicomp Submicron Particle Sizer, Model 370, Santa Barbara, CA). The fraction of the drug in liposomes was determined by centrifuging the liposome dispersion ($16\,000 \times g$ for 15 min) and dissolving the liposome pellet in ethanol or in a mixture of acetonitrile–water (55:45). The encapsulated amount of steroids were determined by HPLC.

2.4. In vitro release of drugs from liposomes

The dialysis membranes were kept overnight in the dissolution medium before dialysis to ensure the thorough wetting of the membrane. Two milliliters of the drug-loaded liposomes in CD–Hepes (72 mM CD, 20 mM Hepes, pH 7.4) solution or free drug in CD–Hepes solution were placed into the dialysis bag which was then transferred into 45 ml of CD–Hepes solution. The solution in the dialysis bag was stirred with a magnetic stirrer and the solution outside the bag with an electric stirrer. Samples of 200 μl were withdrawn at fixed time intervals from outside of the bag and replaced with equal volumes of CD–Hepes solution. Samples were analyzed for budesonide and hydrocortisone by HPLC.

The steroids were analyzed by HPLC consisting of a Beckman solvent module, a Beckman ultraviolet detector set at 215 nm, a System Gold data module (Beckman Instruments, San Ramon, USA), a Marathon autosampler (Spark Holland, Emmen, The Netherlands) equipped with column thermostat and a Rheodyne 7080–080 injection valve with a 20 μl loop. Hydrocortisone and budesonide were analyzed with a LC-8 column (Ultratechsphere; 5 μm, 150×4.6 mm, Macclesfield, Cheshire, UK). The mobile phase was a mixture of acetonitrile–water (55:45) and the flow rate was 1.0 ml/min. The quantitation limit (i.e. response $> 5 \times$ noise level) was 0.1 μg/ml. The dose-response curve was linear ($r > 0.999$) in the concentration range of 0.1 μg/ml–1.05 mg/ml. The reproducibility of detector response (RSD %) was 6.2% for 4–6 repeated assays of a budesonide standard solution (0.1 μg/ml).

The release of calcein from EPC liposomes into 72 mM CD–Hepes solution was measured fluorometrically (Perkin-Elmer Luminescence Spectrometer Model LS 50 B, UK, excitation and emission wavelengths were 494 and 515 nm, respectively, and widths of excitation and emission slits were 5 nm). Samples of 2.0 ml were withdrawn from the receptor phase and replaced with equal volumes of CD–Hepes solution. The fluorescence intensity of the sample was measured. One milliliter of 2.5% Triton X-100 solution was added to the fluorometer cuvette and the measurement of the fluorescence intensity was repeated. If the intact liposomes are able to pass through the pores of the dialysis membrane into the receiver phase, addition of Triton should disrupt the liposomes and increase fluorescence. Intact calcein liposomes do not fluoresce due to the self-quenching concentration (61 mM) of calcein in the liposomes.

2.5. Simulations

Kinetic simulations on hydrocortisone release were carried out using STELLA 2.2.2. (High Performance Systems) program. We assumed first-order release from liposomes and first-order rate of hydrocortisone diffusion from the dialysis bag. The rate constant for the latter (0.65 h^{-1}) was determined experimentally. The effect of the release rate from liposomes on drug appearance outside of the dialysis bag was simulated.

3. Results and discussion

3.1. Solubility of steroids

The intrinsic solubilities (S_o) of budesonide and hydrocortisone in Hepes buffer (pH 7.4) at room temperature were 0.03 and 0.83 mM, respectively. The solubility of budesonide was increased $\approx$ 100-fold (3.25 mM) and that of hydrocortisone 45-fold (37.21 mM) in the presence of 72 mM HP-β-CD. A linear relationship was observed between the drug solubility and the concentration of CD indicating the formation of 1:1 drug–CD complexes. Determined apparent complexation constants ($K_{1:1}$) were 1551 M^{-1} for budesonide and 1104 M^{-1} for hydrocortisone in the presence of HP-β-CD. On the basis of the complexation constants about 99% of budesonide and 98% of hydrocortisone are as CD complexes inside the dialysis bag.

3.2. Size distribution and entrapment of steroids into liposomes

The light scattering size analysis showed that the mean size by volume of the liposomes was 0.8–8.7 μm. Sizes of all the liposomes were much greater than the pore size of the membrane (Mw cut-off $> 10\,000$), which allowed diffusion of drug–CD complexes (Mw < 3000).

Lipophilic budesonide was encapsulated almost completely ($> 95\%$) into MLV liposomes consisting of EPC or DPPC while encapsulation was 85% in the more ordered, longer lipid chain DSPC liposomes. The lower encapsulation of hydrocortisone into EPC (71%) and DSPC (29%) MLVs may be due to its more hydrophilic character compared with budesonide.

3.3. Release studies

Release of the free and CD-complexed drugs through the dialysis membrane must be rapid to enable measurement of the true drug release rate from the liposomes. Release from the dialysis bag is affected by the stirring inside the bag as well as the membrane permeability. Diffusion of budesonide (1 mg/ml in 72 mM HP-β-CD) was increased with magnetic stirring inside the CelluSep 25 000 bag. With magnetic stirring both inside and outside the bag, 50% of the drug diffused from the bag in 5 h, while it took 13 h with stirring only outside the bag.

The increasing molecular cut-off limit allowed significantly faster drug diffusion from the bag (Fig. 1) and, therefore, Spectrapor 300 000 membrane was chosen for further experiments. Previously, mostly dialysis membranes with Mw cut-off of 12 000–14 000 have been used (Levy and Benita, 1990; Margalit et al., 1991; Boman et al., 1993), but these membranes severely limit drug diffusion.

Release of budesonide (1 mg/ml) in 144 mM (20%) HP-β-CD from dialysis bags with Mw cut-off of 300 000 was also studied. The increased viscosity of the 144 mM HP-β-CD solution, compared with 72 mM (10%) HP-β-CD solution, decreased the diffusion rate of budesonide from the bag. Using the Spectrapor 100 000 membrane, 50% of budenoside diffused in 5 and 7 h when 72 mM and 144 mM CD solutions were used, respectively.

REV liposomes entrapped with calcein were used to study the diffusion of intact liposomes from the dialysis bag (Spectrapor 300 000) and the effects of CDs on liposomal bilayers. Pure calcein solution (1 μM, 72 mM HP-β-CD, 20 mM Hepes) diffused from the dialysis bag into 72 mM CD–Hepes solution in 3 h. With the liposomes, only the remaining external calcein was diffused in 5 h reflecting that CD, at the concentration used, does not alter the integrity of liposomal membrane. Addition of Triton X-100 did not increase the fluorescence intensity of the samples. Thus, intact liposomes do not pass through the pores of the dialysis membrane into the dissolution medium. No quenching of the calcein fluorescence was observed in the presence of 72 mM CD.

Release of budesonide and hydrocortisone from MLV liposomes into 72 mM HP-β-CD was measured. The release of steroids from EPC liposomes was faster than from DSPC liposomes (Fig. 2a,b). Leakage of budesonide from DPPC–Chol liposomes was similar to the leakage from EPC liposomes. The results show that the release of drugs from liposomes is dependent on the lipid bilayer composition, membrane order and lipophilicity of the drug. Incorporation of steroids in short-chain aliphatic lipids (EPC, DPPC) is increased compared with that of long-chain lipids (DSPC) due to increased chain–chain interactions and a decreased number of voids for the drug molecules to be incorporated in the structure of DSPC bilayer. Short-chain lipids produce more fluid membranes than membranes of long-chain lipids (Lenaz, 1979). EPC (phase-transition temperature, $T_c = -15$ to -7°C) is at room temperature in the

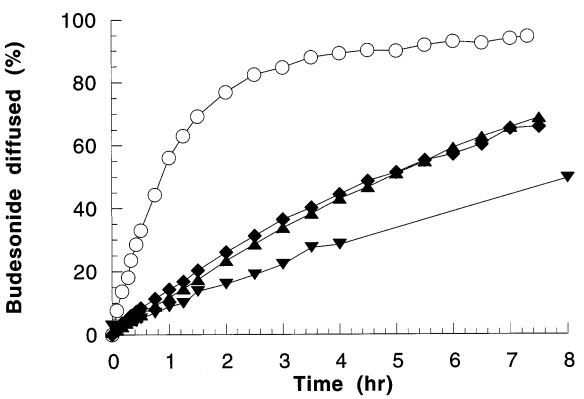

Fig. 1. Diffusion of budesonide into 72 mM HP-β-CD solution through dialysis membranes of different molecular weight cut-off at 25°C. (○) Spectrapor 300 000; (◆) Spectrapor 100 000; (▲) CelluSep 25 000; (▼) CelluSep 12 000–14 000. Each point represents the mean value of two different determinations.

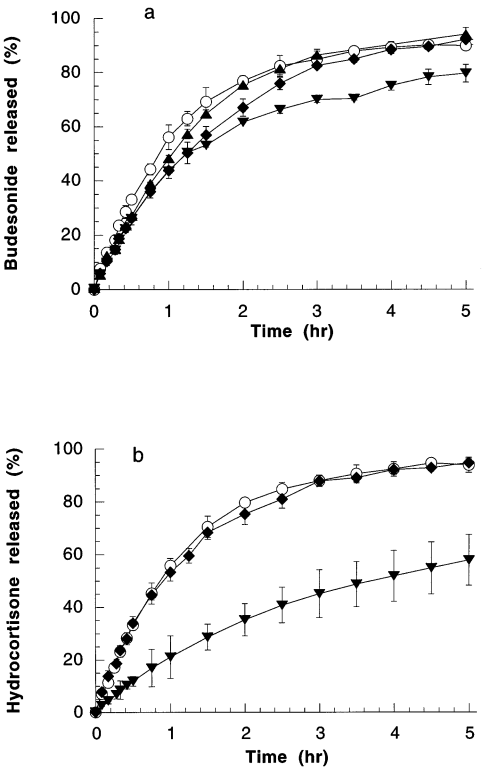

Fig. 2. Release of budesonide (a) and hydrocortisone (b) from MLV liposomes of different lipid compositions at pH 7.4 at 25°C. (a) (○) Budesonide solution; (◆) EPC; (▲) DPPC–Chol (2:1); (▼) DSPC; (b) (○) Hydrocortisone solution; (◆) EPC; (▼) DSPC. Mean ± S.D., $n = 3$–6.

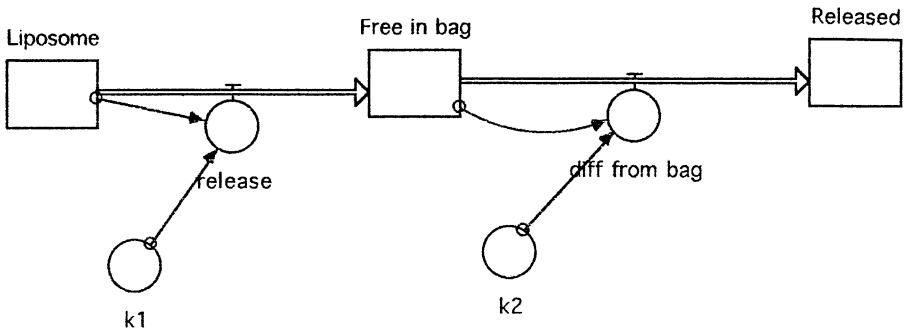

Fig. 3. Diagram of STELLA simulation model for drug release from liposomes and further escape from the dialysis bag.

liquid crystalline form whereas DPPC ($T_c = 41°C$) and DSPC ($T_c = 55°C$) are in the ordered gel-phase. The molecular geometry of the drug may also modulate drug incorporation (Sarmento et al., 1993). Location of budesonide and hydrocortisone in the membrane may be close to that of cholesterol due to their structural similarities. The cholesterol molecule is located in the membrane in the outer hydrophobic cooperativity region of the bilayer, with its rigid steroid ring extending to the depth of 7–10 carbon atoms of lipid alkyl chains (New, 1990a). Below the phase transition temperature, the packing of the headgroups of the phospholipids is weakened due to cholesterol, leading to the increased fluidity of the gel-phase and increased permeability of the membrane (New, 1990a; Taylor et al., 1990). Strongly lipophilic budesonide may be located within the lipid bilayers, while the more hydrophilic hydrocortisone may locate partly within the aqueous domains of the MLV liposomes. That may partly explain the slower release of hydrocortisone compared with budesonide from DSPC liposomes.

The release of calcein from REV liposomes was much slower than the release of steroids from MLVs. In 5 h, only 25% of the calcein was released from the liposomes.

The STELLA model that we used is shown in Fig. 3. Kinetic simulations show that at true release rates, more than 2 h^{-1}, only small changes are seen in hydrocortisone release from the dialysis bag (Fig. 4). Therefore, we can only estimate that the true rate of hydrocortisone release from EPC liposomes is more than 2 h^{-1}. The release rate from DSPC liposomes appears to be in the region of 0.2 h^{-1}, while calcein is released at a rate lower than 0.1 h^{-1}.

In conclusion, the conventional simple dialysis bag technique can be used to determine drug release from colloidal carriers when using permeable dialysis membranes, magnetic stirring inside the bag and cyclodextrins. These factors provide separation of free drug, and sink conditions during release. The technique also allows the evaluations of the true drug release rates from liposomes.

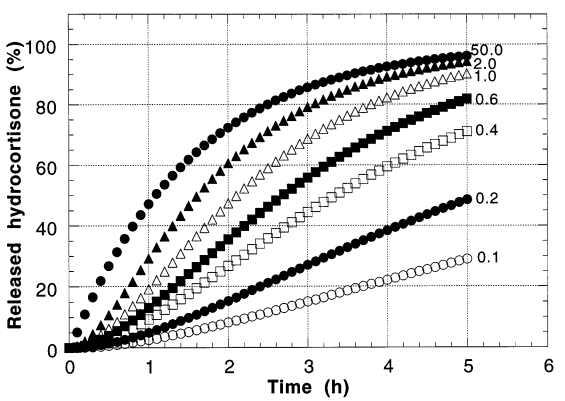

Fig. 4. Simulated cumulative release of hydrocortisone from the dialysis bags. The numbers denote the release rate constants (h^{-1}) for hydrocortisone from liposomes. Escape of free hydrocortisone from the bag took place at the rate of 0.65 h^{-1}.

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