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In vitro evaluation of drug release kinetics from liposomes by fractional dialysis

Ingrid Henriksen *, Sverre A. Sande, Gro Smistad, Tove Ågren, Jan Karlsen

Department of Pharmaceutics, University of Oslo, P.O.B. 1068 Blindern, 0316 Oslo, Norway

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Abstract

A new dialysis method was designed with the purpose of studying drug release rate from liposomes. The liposomes were diluted directly in the continuous phase and dialysed against a small volume of buffer. The dialysate was changed at 10 min intervals and the amount of carboxyfluorescein released was calculated from a standard curve. To evaluate the new method, parallel measurements were recorded with the traditional carboxyfluorescein assay, which is based on direct measurements on liposomes containing a self-quenching concentration. The new method is called fractional dialysis, and is shown to have sensitivity similar to that of the carboxyfluorescein assay. In certain cases the fractional dialysis method gives a more accurate release profile. It is suggested that fractional dialysis can be a useful alternative to other techniques for recording the in vitro release characteristics of specific drugs from liposomes or other colloidal carriers.

Keywords: Microparticle; Liposome; Drug release rate; Dialysis; Carboxyfluorescein

1. Introduction

An essential point in the evaluation of drug delivery systems is the rate at which the drug is released from the carrier. Dissolution/release tests are generally used not only in the quality control of the drug formulation, but also to predict in vivo behaviour and to study the structure of the dissolving matrix.

Colloidal drug carriers offer special challenges in this regard: In order to evaluate the amount of drug released, the carrier must with a few exceptions be separated from the bulk solution. This must occur rapidly and efficiently, but without any influence on the release profile of the system under study. Such separation has been found to be difficult in the case of small colloidal particles.

Washington (1990) has given a review of the experimental techniques used so far. He divides the methods for determination of release profiles from disperse systems into four broad groups: (1) membrane diffusion methods; (2) sample-and-separate methods; (3) in situ methods; and (4) continuous flow methods

All these methods have their advantages and disadvantages. In the present paper, we concentrate on the particular problems related to the

* Corresponding author.

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study of liposomes. Since they have non-solid, flexible structures, they will, depending on the lipid composition, suffer from stability problems different from other colloidal carriers.

In the sample-and-separate method, the carrier is diluted with buffer, and samples are taken at intervals. The carrier is then separated from the continuous phase by filtration or centrifugation (e.g., Tsukada et al., 1984; Taylor et al., 1990), and the drug released is assayed. Considering liposomal carriers, the problem is to achieve a rapid and clean separation: For relatively small liposomes (less than about 300 nm) high centrifugal force is required to sediment the vesicles. This will often lead to an increase in the drug release rate (Lelkes and Tandeter, 1982) and may even damage the liposomes. In their liquid crystalline state, liposomes can also squeeze through filters of a pore size smaller than their diameter and may be destroyed by the filtering pressure. However, a low pressure ultrafiltration technique has been reported (Magenheim et al., 1993) that may possibly be applied to liposomes. Gel permeation chromatography is also used for separation in release studies, but includes a dilution of the sample which can influence the release.

The most popular method to study the permeability of the liposomal membrane, has been the in situ method, using fluorescent water-soluble markers such as 5(6)-carboxyfluorescein (Weinstein et al., 1977) or calcein (Allen and Cleland, 1980). The markers are self-quenching at high concentrations, providing the advantage that the released marker in the bulk solution in low concentration can be assayed directly, without separating it from the liposomes, encapsulating a high concentration. This method is henceforth referred to in the present paper as the CF assay. The simplicity of the method makes it a good choice for studies of different liposomal compositions intended for the encapsulation of water-soluble drugs. However, the liposomal formulation chosen must still be tested with the drug in question, which probably will have other physical and chemical characteristics than the marker.

Membrane diffusion techniques permit the determination of the release of a variety of drugs from the carrier. The carrier is suspended in a small volume of the continuous phase, and separated from a large bulk of sink phase by a dialysis membrane permeable to the drug. The drug diffuses out of the sample and through the membrane to the sink, wherein it is periodically assayed. This approach has been criticized by Washington (1990, 1989), since the experiment is not performed under perfect sink conditions. The encapsulated drug will be in equilibrium with drug in the continuous phase, and the partition coefficient rather than the true release rate at large dilution is then measured. The method, therefore, has limited value in predicting the in vivo behaviour of colloidal carriers intended for intravenous or peroral administration. On the other hand, it has been used successfully to study the shelf-life of liposome/drug systems with respect to drug retention (Margalit et al., 1991).

Levy and Benita (1990) have compared the widely used dialysis bag technique with a proposed reverse dialysis technique. In the latter, the colloidal drug carrier is diluted directly in the sink solution. Then several dialysis bags, previously filled and equilibrated with the sink solution, are immersed in it. With the traditional dialysis bag technique, the dialysis membrane is rate limiting, and the method is therefore not appropriate. With reverse dialysis, the limitation of the dialysis membrane diffusion prevents the resolution of release profiles lasting less than 1 h. As a matter of fact, the dialysis membrane is also rate limiting in this case, since both the drug solution and the formulations of the drug give similar release profiles, with about 90% released within 1 h. In cases where the carriers release their drugs over a longer period of time, however, the data obtained with this method should be useful for computing the real drug release kinetics, assuming sink conditions.

On this background we propose a new dialysis method, where the liposomes are diluted directly with the continuous phase and dialysed against a small volume of buffer. The dialysis is not continuous and the model does not require an equilibrium between the two sides of the membrane. With proper planning of the experiment, a rapid release as well as a slow release profile can be studied.

2. Materials and methods

2.1. Materials

5(6)-Carboxyfluorescein (CF, approx. 99% by HPLC), Triton X-100 and egg L-α-phosphatidylcholine (PC, approx. 99%) were supplied by Sigma Chemical Co., USA. Tris of analytical grade were from Prolabo, Paris.

2.2. Preparation of liposomes

Liposomes were prepared by the film method as follows: PC chloroform solutions were rotary evaporated to dryness in a 250 ml flask. The resulting film was further dried under reduced pressure for 15 min. The aqueous CF solution (in 60 mM Tris buffer, adjusted to pH 8.0 with HCl) was then added to give a concentration of phospholipids of 50 mg/ml. This mixture was gently shaken for 10 min at 40°C. The liposomes were exposed to five freez-thaw cycles in liquid nitrogen and a 40°C water bath, then extruded 10 times through 200 nm polycarbonate membrane filters (Nuclepore) with a Lipex extruder.

Non-encapsulated material was separated from the liposomes with prepacked PD10 Pharmacia G-25 columns. 1 ml sample was applied to the pre-equilibrated column and eluted with 3.5 ml Tris/saline buffer. The concentration of NaCl in the Tris/saline buffer was adjusted to give approximately the same osmolarity as inside the liposomes (0.35 M NaCl with 100 mM CF and 0.05 M NaCl with 20 mM CF). The eluate was immediately diluted to 500 or 250 ml with the appropriate Tris/saline buffer, filled in a dark brown bottle and assayed for leakage at 37°C as described in section 2.6. The sample was stored at 37°C throughout the experiment.

2.3. Size measurements

The size of the liposomes was determined by photon correlation spectroscopy (PCS) after extrusion. A Coulter N4 MD was used at a 90° angle, as previously described (Henriksen et al., 1994). The diameter reported here is calculated with size distribution processor analysis (SDP), which does not require that the sample is unimodal or has a log Gaussian distribution.

2.4. Degree of encapsulation

The degree of encapsulation was calculated as the percentage of the amount of CF added to the separation column. For the 100 mM CF liposomes, the degree of encapsulation was also found as the exact CF/lipid ratio.

The lipid content was determined by gas-liquid chromatography after transesterification of the PC fatty acids to the methyl esters using sodium methoxide (1%) in methanol. Standard solutions of PC were derivatized and analysed by exactly the same method.

2.5. Fractional dialysis; theoretical background

The design of the MacroDialyzer provides important advantages to the more frequently used dialysis bags: it offers a constant membrane area available for diffusion, and a sample sink in a large volume can be analysed. This permits the calculation of a dialysis standard curve, based on the following mathematical considerations.

Consider the kinetic situation shown in Fig. 1. Compartment V (representing the sample in the sink) and M (representing the dialysate) are separated by a dialysis membrane. We make two assumptions; that the drug's coefficient of diffusion through the membrane, K_m, is the same in both directions ($K_m = K_{-m}$ in Fig. 1) and that the number of molecules diffusing in one direction is proportional to the concentration in the

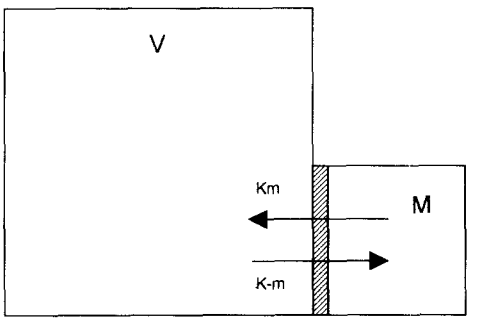

Fig. 1. Kinetic scheme for sink dialysis.

compartment from which they diffuse (first-order diffusion).

The change in number of molecules in the sample compartment may then be described as follows:

$$\frac{dX_m}{dt} = K_m \frac{X_v}{V_v} - K_m \frac{X_m}{V_m} \quad (1)$$

where X is the number of molecules in the respective compartments, and V represents their volume. Assuming $X_m = 0$ at t_0, Integration gives:

$$X_m = \frac{X_{0v} \cdot V_m}{V_m + V_v} \cdot [1 - e^A] \quad (2)$$

where

$$A = -K_m \frac{V_m + V_v}{V_v \cdot V_m} \cdot t$$

and t is the time. Since the release medium is continuously pumped through the dialysis cell, V_v represents the entire release volume and we may assume $V_v \gg V_m$:

$$\frac{X_m}{V_m} = C_m = C_{0v} \cdot [1 - e^B] \quad (3)$$

where

$$B = \frac{-K_m}{V_m} \cdot t \text{ and } C_{0v} = \frac{X_{0v}}{V_v}$$

Now, if t is a constant time period (e.g., 10 min as in our case), the exponent term B is also a constant, and the equation simplifies to:

$$C_m = K \cdot C_{0v} \quad (4)$$

This direct proportional relationship permits the construction of a standard curve relating the concentration measured in the dialysate (C_m) to the actual concentration in the dissolution medium, provided C_v is only subject to insignificant changes during the sampling period.

2.6. Fractional dialysis; experimental

Dialysis was performed in a Spectra/Por© MacroDialyzer, 7.5 ml half-cell volume and with Spectra/Por© CE membrane disks of 50 000 Mol. Wt cut-off.

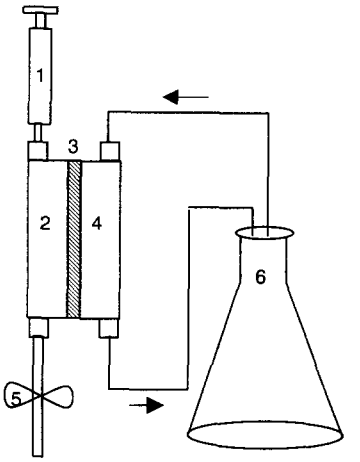

Fig. 2. Model of dialysis equipment. 1, syringe to add and remove dialysate; 2, dialysate half-cell; 3, dialysis membrane; 4, sample half-cell; 5, closure for air inlet/outlet; 6, sample compartment.

The dialysis cell was coupled as illustrated in Fig. 2. The sample was pumped into the system immediately after dilution and circulated at a speed of 3.5 ml/min. The other cell half was coupled to a syringe containing the buffer fraction (the dialysate). At suitable time intervals, a fixed buffer volume of 7.3 ml was introduced into the cell, the outlet closed and the entire dialysis cell was immersed in a water bath at 37°C. After 10.0 min the dialysate was removed, cooled to room temperature and assayed for CF. The concentration of CF at the sample side of the membrane was calculated from a dialysis standard curve previously determined (Fig. 3) with 10 min dialysate fractions from standard solutions of CF.

At the end of each day's dialysis, the cell was emptied and both halves flushed with distilled water. The cell and membrane was stored in a refrigerator overnight. A new membrane was inserted at least every 5 days. The liposome samples were stored in a 37°C water bath during the period of analysis.

To evaluate the linearity, dialysis was performed with three fractions from each of eight standard solutions ranging in concentration from 10^{-7} to 1.5×10^{-5} M (Fig. 3). All standards were diluted to a final volume of 250 ml.

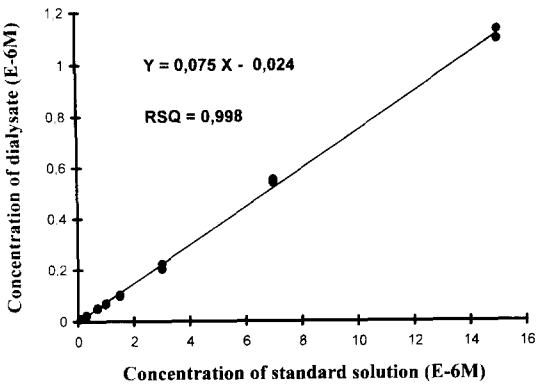

Fig. 3. Standard curve for 10 min fractional dialysis of carboxyfluorescein in Tris buffer (pH 8.0) at 37°C. Each data point represents one measurement. Three fractions were measured for each of the eight standards. The coefficient of variation calculated for six fractions from each of four standards was 2.0–5.4%.

To determine the variance of the dialysis method, a standard curve was prepared with six fractions from each of four standard CF solutions. The standards were diluted from the same stock solution to reduce other sources of error. The standards were dialysed in random order, and with dialysis membranes of varying 'age'.

2.7. Fluorescence measurements

CF fluorescence was measured at an emission wavelength of 520 nm and an excitation wavelength of 480 nm, and with 4 nm slits. Free concentrations measured were always below 3 μM (self-quenching is completely relaxed below about 30 μM (New, 1990)). The measurements were performed at room temperature with a Perkin Elmer MPF-3 Fluorimeter. Direct measurements of CF leakage (the CF assay method) was measured from the same sample and at the same time points as with the dialysis method. A sample of 1 ml was diluted to 10 ml and the fluorescence measured.

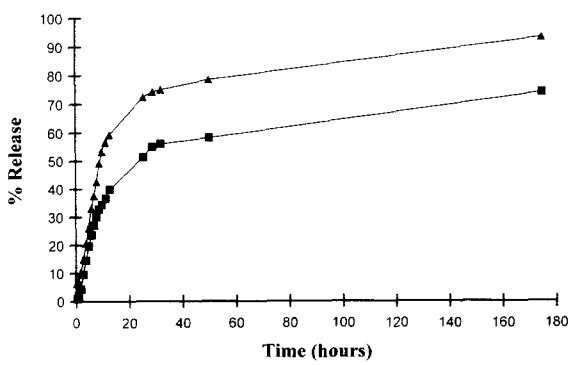

Fig. 4. Release rate of CF from liposomes prepared with 20 mM CF solution. (▲) CF assay; (■) fractional dialysis. One example of paired data from the same parallel is shown.

The presence of Triton X-100 (0.05%), liposomes, or NaCl at the concentrations used, did not influence the fluorescence.

The total amount of CF (F_t) was determined by adding 50 μl 10% Triton X-100 to 1 ml of the sample and subsequent dilution with buffer to 10 ml. The release was calculated as:

$$\% \text{ release} = F/F_t \times 100$$

where F is the amount of CF released at a given time point.

3. Results

3.1. Liposomal size and degree of encapsulation

Liposomes encapsulating 100 mM CF measured 202 nm, while those with 20 mM CF were 227 nm.

The liposomes produced with 100 mM CF showed a degree of encapsulation of 9.7% (minimum 9.6, maximum 9.8, $n = 3$), while 20 mM CF liposomes encapsulated 13.3% (minimum 12.6, maximum 14.2, $n = 3$). The molar CF/lipid ratio after gel filtration for the 100 mM CF liposomes was 0.14 μmol CF/μmol PC.

3.2. Standard curve of dialysis

Based upon the rate of diffusion across the dialysis membrane and the rate of release from liposomes, 10 min was found to be a suitable time interval.

The calculated standard curve showed a good fit to theory (Fig. 3) with a regression coefficient of 0.998. No significant change was seen when the slope and intercept were calculated for the lower and upper concentration range separately.

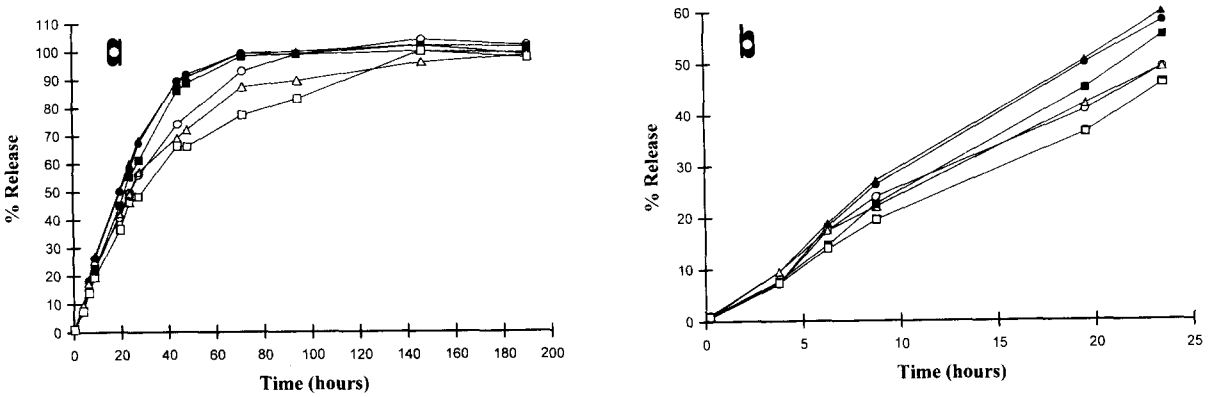

Fig. 5. Release rate of CF from liposomes prepared with 100 mM CF solution. (Filled symbols) CF assay; (open symbols) fractional dialysis. Three parallels are presented. Panel b gives a closer view on the first 25 h.

The standards showed no change in concentration after six dialysate fractions had been collected. The coefficient of variation ranged from 2 to 5.4%, which includes contributions from the fluorimeter (no temperature control).

3.3. Dialysis of CF liposomes

In Fig. 4 and 5 the direct measurement of CF release is compared with the release profile as calculated from the dialysis data. In Fig. 4, a 20 mM CF solution was encapsulated in the liposomes, while in Fig. 5 the concentration was 100 mM. In the first case, the measurements started at 7% released (CF assay) and 1% (dialysis), while the second commenced at 1 and 1%, respectively. In Fig. 4, only one parallel is shown, since this gives the best illustration of a paired set of data with the two methods.

In general, Fig. 4 and 5 show that the results obtained with both methods correlate when the liposomes still contain a high concentration of encapsulated CF.

4. Discussion

The salt concentration was not seen to influence the standard curve, as long as the same buffer was used on both sides of the dialysis membrane. Likewise, the presence of empty liposomes in a standard did not influence the dialysis rate. However, the nature of the drug itself had an effect. Experiments with quinine sulphate as the marker gave a standard curve with a slope of 0.088, while the slope was 0.075 for CF. The viscosity of the sample also affected the standard curve, as the presence of 0.67% of a high molecular weight polymer was seen to reduce the slope for quinine sulphate from 0.088 to 0.072. Dialysis at room temperature also reduced the speed of dialysis. These effects are all according to general dialysis theory as described by, for example, Van de Merbel et al. (1992).

The CF assay has been used here to evaluate the principle of a fractional dialysis method. In this evaluation it is essential to bear in mind the theory behind a direct CF measurement: The quenching factor is a function of the CF concentration. Weinstein et al. (1981) have provided a general expression for the leakage of CF from lipid vesicles. They demonstrated that the quenching is above 90% for concentrations of CF above 50 mM. Below this, the quenching decreases very rapidly, and is only about 25% in a 4 mM solution.

This means that a release profile can be recorded without any correction for the quenching as long as the concentration inside the vesicles is high, and the leakage is low. This simple approach is satisfactory for many purposes, and is accordingly used by most authors. The definition of what is a sufficiently high concentration seems, however, to vary. 100 or 200 mM CF is usually

recommended (e.g., New, 1990) if the total release profile is desired. Some authors report sufficient quenching even at 5 mM CF (97%; Pajean et al., 1991). 40 mM CF (e.g., Lelkes and Tandeter, 1982) or 50 mM CF (e.g., Bramhall et al., 1987; Domingo et al., 1993) are also commonly used. It is likely that the degree of quenching will be influenced by factors such as purity of the CF, ionic strength, pH, the presence of other components like phospholipids and whether the quenching is determined for CF in solution or encapsulated inside the liposomes. The best approach would be to prepare liposomes with different CF concentrations and construct a quenching curve from the results obtained. This is, however, time consuming. Our release data are therefore presented without corrections, as has been done in the majority of the publications.

The implications for the release rate profile in Fig. 5 are that the last half of the CF assay curve will appear with an excessively rapid release, the error increasing all the time up to 100%. Near this point the method loses its ability to distinguish encapsulated from released CF. This point is further illustrated in Fig. 4: The error in the release profile is already significant after a few hours. The starting point can be taken to indicate the percentage quenching, which would then be approx. 93% at 20 mM CF concentration.

Fig. 5 shows that the two methods give similar results up to about 25% release. Some interesting details should be pointed out from the dialysis curve:

Firstly, the data show at the most a 15% difference between the parallels. Several facts indicate that it is a real difference: This deviation is much larger than that seen in the standard curve. Furthermore, the difference between parallels is not random (i.e., the same parallel is always the lowest), and there are only small deviations at the beginning and at the end of the curve (ends at 98–102%).

Secondly, in the first part of the curves the two methods confirm each other, with two similar parallels and the third somewhat lower. The lack of scatter between parallels at the second half of the CF assay curve can be predicted from this method's lack of sensitivity at this point (the sensitivity is expected to decrease as the % quenching inside the liposomes decreases).

On this basis, we conclude that the fractional dialysis method provides accurate data, as confirmed by the first half of the curve in the traditional CF assay (Fig. 5). We further find indications that the new method is more sensitive because it gives a more correct presentation of the second half of the release profile. However, it is not easy to explain why three parallels from the same liposome production should show such diversity in their release behaviour.

The main advantages of the fractional dialysis method is that it is conducted under perfect sink conditions and can be applied to any drug substance for which a sensitive method of quantitation exists. Several data points can be collected within the first hour of release, depending on the length of the fraction interval. The drug release from a colloidal carrier is not influenced by the sampling process, and little substance is lost through the sampling. The equipment is cheap and simple, and also flexible, as it permits the study of a number of parameters on the release process. Such parameters could be, e.g., buffer, temperature, radiation, pH, and last but not least possible interactions between drug and carrier, such as adsorption phenomena.

The disadvantage of the method is that it requires a sensitive method of quantitation of the drug. Compared to sample and separate methods, only a fraction of the concentration of free drug is detected. However, this fraction can be increased by using a longer sampling interval. This means that fewer data points can be collected per hour.

The method also requires that a standard curve is determined for the system in question, since the rate of diffusion through the dialysis membrane may change with parameters such as pH, temperature, viscosity and drug characteristics.

The Spectra/Por© MacroDialyzer dialysis cell is most suitable for systems that release their contents over a period of a few hours, or a day. In such a case, the sample can be pumped continuously through the cell during the whole experiment. Slower release rates mean that the sample must be returned to the reservoir for storage

between measurements, to measure other samples or to allow cleaning of the system. This may induce a variation in the hydrodynamic conditions over the release period. Another dialysis cell may be more convenient in this case: Precut dialysis tubes in different sizes are now on the market and feature a screw top opening at one end and a seal at the other end. These could then be immersed in the sample sink, removed at the end of the interval and the contents assayed. The main prerequisite is that the surface area is constant, so that a standard curve can be calculated.

5. Conclusion

The suggested method of fractional dialysis for the analysis of drug leakage from liposomes shows a good fit to the theory. The method is confirmed by comparison to the traditional carboxyfluorescein assay, and is more sensitive than the latter as the CF concentration in the liposomes decreases. Fractional dialysis can therefore be a useful alternative to the already existing techniques for recording the in vitro release characteristics of specific drugs from colloidal carriers.

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