

In vitro release of hydrophilic and hydrophobic drugs from liposomal dispersions and gels

MOHAMED MAHMOUD NOUNOU
LABIBA K. EL-KHORDAGUI
NAWAL A. KHALAFALLAH
SAID A. KHALIL

*Department of Pharmaceutics
Faculty of Pharmacy
University of Alexandria, Egypt*

A method for determining the rate of hydrophilic and hydrophobic drugs release from different types of liposomal dispersions and gels using a dialysis method is described. Dibucaine base and 5-fluorouracil were used as model drugs for a hydrophobic and a hydrophilic drug, respectively. A dialysis technique was employed. Release rates were affected by the rate of rotation of the paddles of the tablet dissolution tester, temperature, and the volume of release medium. The method was used to evaluate the *in vitro* drug release from hydrophilic and hydrophobic drugs from liposomal dispersions and gels. The *in vitro* release study of dibucaine base showed no burst effect, while the *in vitro* release study of 5-fluorouracil showed a clear burst effect with an initial fast release phase followed by a sustained release phase.

Keywords: dibucaine, 5-fluorouracil, *in vitro* release, release stability, liposomal gel, Higuchi diffusion model

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An essential point in evaluation of liposomal drug delivery systems is the rate at which the drug is released from the liposomal carrier system. *In vitro* release tests are generally used not only in quality control of drug formulations, but also to predict their *in vivo* behaviour. But, for liposomal topical drug delivery systems, the *in vitro* release profiles could hardly describe the *in vivo* performance (1, 2).

Although there are well established physicochemical methods to characterize the various drug colloidal carriers under investigation, investigators are still facing technical difficulties to properly evaluate the *in vitro* drug release profile from these colloidal carriers.

Liposomal 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 (1) has reviewed the experimental techniques used so far. He divided the methods for determination of release profiles

* Correspondence, e-mail: nounousomes@gmail.com

from disperse systems into four groups: membrane diffusion, sample and separate technique, *in situ* methods and continuous flow methods. All these methods have their advantages and disadvantages.

The main aim of this study is to provide a convenient and reproducible technique for *in vitro* drug release of hydrophilic drugs such as dibucaine base and hydrophobic drugs such as 5-fluorouracil from liposomal dispersions and gels. The *in vitro* release study of hydrophobic drugs such as lidocaine, tetracaine and dibucaine base from liposomal systems presents a problem since the hydrophobicity of the drug is an obstacle to the true release pattern of the drug; besides, in this case, it is impossible to correlate *in vitro* and *in vivo* performance. This fact has been ensured by the work of Foldvari *et al.* (2). Since no *in vitro* release study was done during her assessment of topical liposomal local anesthetics.

EXPERIMENTAL

Materials

The following chemicals were used in the study: dibucaine base (Ward Blenkinsop Co., UK), 5-fluorouracil USP 25 (Nantong General Pharmaceutical Factory, China), cholesterol 99% extra pure (S.d. Fine-Chem Ltd., India), Lipoid E 80 (phosphatidylcholine 80% fat free egg lecithin) (LIPOID, Germany), dicetyl phosphate (Sigma Chemical, USA), hydroxypropyl methylcellulose of high viscosity grade (4000 c.p.) (Alexandria Pharmaceuticals, Egypt) and Spectra/Por[®] dialysis membrane 12,000–14,000 molecular mass cut off (Spectrum Laboratories Inc., USA).

All other materials and solvents were of analytical grade and doubly-distilled water was used.

Liposome preparation

Multilamellar liposomal vesicles (MLVs). – MLVs containing either 5-FU or dibucaine base were prepared according to the classical method of Bangham *et al.* (3).

The lipid components [egg phosphatidylcholine (EPC), either alone or in different molar ratios with cholesterol (CHOL), and either stearylamine or dicetyl phosphate (DP)] were weighed into a long-necked pear-shaped quick-fit round bottom flask and dissolved in chloroform/methanol (7/3, V/V). Dibucaine base was dissolved in the same solvent mixture and added to the lipid solution. The organic solvent was removed under reduced pressure at 55 °C using a rotary evaporator. In the case of dibucaine base, the resulting thin lipid film was slowly hydrated using bicarbonate buffer pH 9.0. In the case of 5-fluorouracil, the resulting thin lipid film was slowly hydrated using isotonic phosphate buffer saline (IPBS) pH 7.4 containing 1 mg mL⁻¹ 5-fluorouracil. The process of hydration was carried out by rotation at a low speed at 55 °C in a rotary evaporator under atmospheric pressure followed by hand shaking for 15 minutes at 55 °C in a thermostatically controlled water bath. The resulting liposomal dispersion was left to mature overnight at 4 °C.

Table I. Composition of dibucaine and 5-fluorouracil liposomes

| Code | Drug | Type | Hydration medium | Phosphatidylcholine /cholesterol/charge imparting agent molar ratio | Charge imparting agent |
|------|----------------|-------|-------------------------|---|------------------------|
| D1 | Dibucaine | MLVs | Bicarbonate buffer pH 9 | 7/3/0 | – |
| D2 | | | | 7/6/0 | – |
| D3 | | | | 7/6/1 | Dicetyl phosphate |
| D4 | | | | 7/6/1 | Stearylamine |
| FU1 | 5-Fluorouracil | SPLVs | IPBS pH 7.4 | 10/0/0 | – |
| FU2 | | | | 10/0/0 | – |
| FU3 | | | | 10/4/0 | – |
| FU4 | | | | 10/10/0 | – |
| FU5 | | | | 10/4/1 | Dicetyl phosphate |

5-Fluorouracil stable plurilamellar liposomal vesicles (SPLVs). – Stable plurilamellar vesicles (SPLVs) were prepared using the technique of »hydration from organic solvent« first introduced by Gruner *et al.* (4). The resulting thin lipid film formed as described before was redissolved in 8 mL of diethyl ether. The aqueous phase containing 5-fluorouracil was added in successive portions to the lipid solution and the mixture was sonicated. This resulted in the formation of a homogeneous opalescent dispersion. Sonication was continued for about 15 minutes until the organic solvent was evaporated and could no longer be smelled (4, 5). The resulting viscous gel was redispersed in IPBS pH 7.4. The liposomal dispersion was kept for 24 hours in the refrigerator to mature (6).

A total of four dibucaine base and five 5-fluorouracil liposomal dispersions were prepared. Their lipid composition and formulation codes are shown in Table I. Dispersions were prepared in triplicate.

Separation of liposomes

This was achieved by centrifugation at 16500 rpm (27800 × *g*) for 90 min at –5 °C. The liposomal concentrate was washed twice either with IPBS pH 7.4 in the case of 5-fluorouracil or with bicarbonate buffer pH 9.0 in the case of dibucaine base and recentrifuged for further 90 minutes (5). The resulting liposomal concentrates were refrigerated.

Entrapped 5-FU was determined by lysis of liposomes with chloroform/methanol (7/3, *V/V*). Concentrations of 5-FU and dibucaine were determined spectrophotometrically at 266.5 nm and 241 nm, respectively.

Preparation of dibucaine liposomal gel

Dibucaine base liposomal concentrate (D-3) was added to plain hydroxypropyl methylcellulose gel by trituration to yield finally a 1% (*m/m*) drug and a 2% (*m/V*) gelling agent concentration [dibucaine liposomal gel (DLG)].

Dibucaine liposomal gel loaded with both free and liposomal dibucaine was prepared by mixing dibucaine liposomal gel with dibucaine gel (HPMC gel incorporating 4% free dibucaine base) in the proportions shown in Table II.

Table II. Dibucaine and 5-fluorouracil HPMC gel formulations

| Code | Description | Properties | Concentration of HPMC gel (%) | State of the drug incorporated inside the gel |
|----------|---|---|---|---|
| DG | Dibucaine plain gel | Transparent, smooth, viscous, non tacky gel | 4 | 100% free dibucaine |
| DLG | Dibucaine liposomal gel | White, opaque, smooth, viscous, non tacky gel | 4 | 100% dibucaine encapsulated inside liposomes |
| DCG-25 | Dibucaine combination gel (free and liposomal drug) | | 4 | 25% of the drug is liposome-encapsulated and 75% is free drug |
| DCG-50 | | | 4 | 50% of the drug is liposome-encapsulated and 50% is free drug |
| DCG-75 | | | 4 | 75% of the drug is liposome-encapsulated and 25% is free drug |
| DCG-87.5 | | | 4 | 87.5% of the drug is liposome-encapsulated and 12.5% is free drug |
| FUG | | 5-fluorouracil plain gel | Transparent, smooth, viscous, non tacky gel | 2 |
| FULG | 5-fluorouracil liposomal gel | White, opaque, smooth, viscous, non tacky gel | 2 | 100% 5-fluorouracil encapsulated inside liposomes |

Preparation of 5-FU liposomal gel

A 5-FU topical gel was prepared using negatively charged 5-FU stable plurilamellar vesicles (FU-5) and 2% hydroxypropylmethyl cellulose (HPMC) as gelling agent. After gel formation, the amount of 5-fluorouracil liposomes (FU-5) required to obtain 0.1% (*m/m*) drug-loaded gel was incorporated in the gel by trituration.

Their formulation codes are shown in Table II. 5-FU plain gel (0.1% 5-FU in 2% HPMC gel) was used as control.

In vitro drug release from liposomal dispersions

Drug release from liposomes was studied using a dialysis method. Dialysis bags (spectra/Por® 2) were soaked before use in distilled water at room temperature for 12 hours to remove the preservative, followed by rinsing thoroughly in distilled water. The dialysis bags were fitted on the tablet dissolution tester paddle by attaching a stainless steel part to allow fixing the dialysis bags to it.

Liposomal concentrate (equivalent to 2 mg dibucaine base or 5-fluorouracil) dispersed in one mL of bicarbonate buffer pH 9 in the case of dibucaine liposomal dispersions or IPBS pH 7.4 in the case of 5-FU liposomal dispersions was placed in a dialysis bag of 10 cm initial length and 6.4 mm diameter. The bag was closed at both ends with cotton thread and tested for leakage. The final length of the bag after tying was 8 ± 0.2 cm. The dialysis bag was attached horizontally, fully stretched, to the paddle, which was then immersed in the tablet dissolution tester beaker containing either 250 mL of phosphate buffer pH 5.6 containing 7% (V/V) propylene glycol and 25% (V/V) methanol in the case of dibucaine base or 150 mL of IPBS pH 7.4 in the case of 5-fluorouracil as release medium. The bag was fully immersed under the surface. The temperature was set at 32 ± 0.2 °C and the rotation speed of the paddles was set at 100 rpm.

Control bags were prepared and tested along with the liposomal dispersions. Each control bag contained 2 mg dibucaine base or 5-fluorouracil dissolved in 1 mL of the release medium.

Aliquots of the release medium were withdrawn for analysis at different time intervals and replaced with fresh medium. Release runs were continued for 12 hours. The absorbance of the collected samples, diluted as necessary with release medium, was measured at λ_{\max} 244 nm and 266.5 nm in the case of dibucaine base and 5-fluorouracil, respectively. The results recorded are the mean value of three runs carried out for each liposome concentrate.

A representative dibucaine liposomal dispersion (D2) was examined by electron microscopy before and after the release run.

In vitro release from hydroxypropylmethyl cellulose liposomal gels

A dialysis method was applied using a molecular porous membrane (Spectra/Por® 2) allowing the free dibucaine base and free 5-fluorouracil released from the liposomes to pass freely through its pores.

The USP tablet dissolution tester (7) was used in the release studies. A known mass of each gel formulation shown in Table II was added into stainless steel cups (radius 1.5 cm, height 0.5 cm). The gel surface was made flat; the cups were fitted with the dialysis sheet with a rubber band and fixed at the bottom of the beakers of the tablet dissolution tester. Release profile was determined as previously detailed in the previous section.

Transmission electron microscopy

Dibucaine liposomal dispersion (D2) was examined by transmission electron microscopy before and after the release run.

Negative staining of liposomal samples, described elsewhere (8), was employed. The samples were examined by transmission electron microscopy at 40,000x magnification power at 80 kV using 2% (*m/V*) uranyl acetate as staining agent.

RESULTS AND DISCUSSION

It has been recommended as a rule of thumb that the drug concentration in the sink phase in release experiments should be kept below 10% of saturation. If the drug is poorly soluble in water like dibucaine base, nonaqueous solvents or solubilizing agents may be added to the sink.

In the case of dibucaine base liposomes, a release medium containing 7% (*V/V*) propylene glycol and 25% (*V/V*) methanol was employed to provide sink conditions. Other cosolvency release media were investigated, containing different proportions of methanol, ethanol and propylene glycol, throughout the study. The release medium used was the only system that has proven its reliability and efficacy in providing good sink conditions. The stability of the carrier system in this release medium was investigated. One of the dibucaine liposomal concentrates (D2) included in the release study was examined

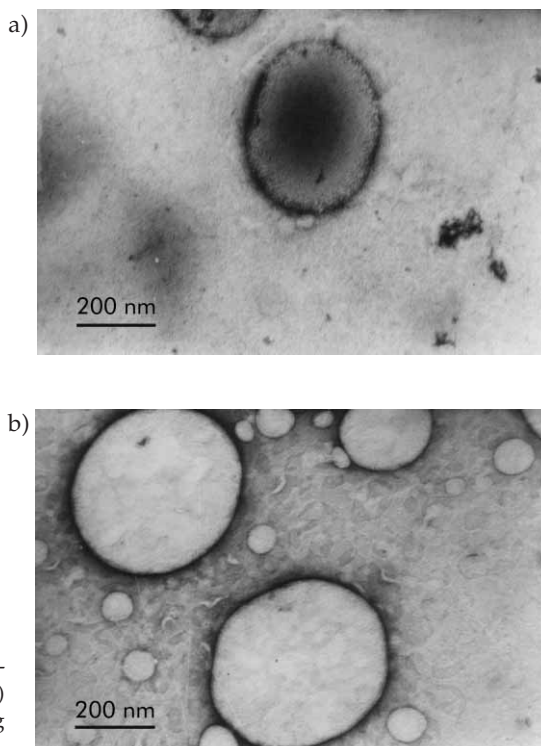


Fig. 1. Electron photomicrograph of dibucaine liposomal dispersion (D2) ($\times 40,000$): a) before the release run, b) after terminating the release run.

using the electron microscope before and after the release run to detect effects, if any, of release study conditions on the integrity of the liposomes (Fig. 1). No evidence of large scale lysis or coalescence of the vesicles could be found. Retention of sealed vesicular structures after the release run is evident. In the case of 5-fluorouracil IPBS pH 7.4 was sufficient to provide good sink conditions. Also other researchers (2) have employed a cosolvency system as a hydration medium for highly hydrophobic drugs.

The reproducibility and efficacy of the release study were ensured through a control sample containing only the drug in the free form. This could ensure that the dialysis membrane was not a barrier throughout the release study. The control sample in the case of 5-fluorouracil and dibucaine base was released in about two hours.

A constant surface area of dialysis membrane, constant amount of the drug inside the dialysis bag and constant volume or mass of final formulation should be employed to eliminate variation within identical patches. Any defects could lead to huge variations within the release profile of identical patches and would present untrue comparative data for different formulations.

If no sufficient agitation is employed, this could lead to stagnant layer formation, which will represent an opposing force to drug release from the formulation through the dialysis membrane, leading to a faulty and unsatisfactory release profile.

Cumulative percent dibucaine release values over 12 hours, determined for four of the liposomal dispersions, are shown in Fig. 2. The figure also includes, for comparison, the diffusion profile obtained for free untrapped dibucaine under the same conditions as the liposomal dispersions. Compared to the control data, dibucaine release from liposomes is prolonged.

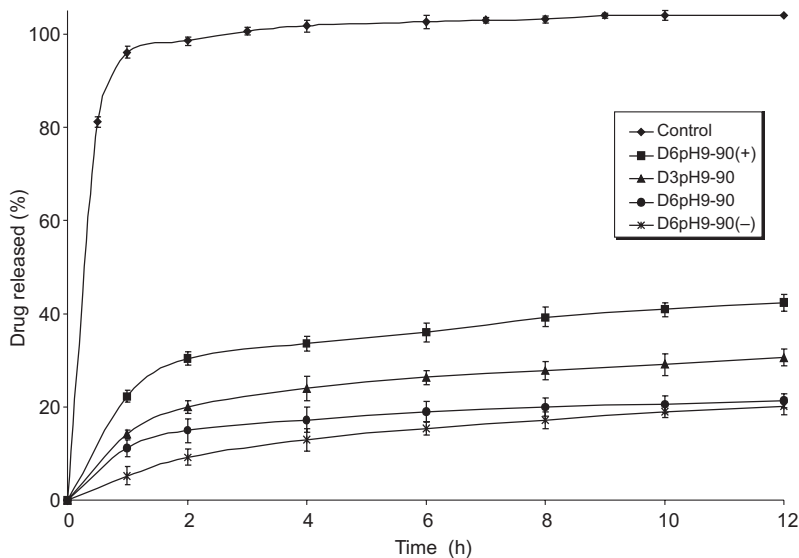


Fig. 2. Release profiles of dibucaine liposomal dispersions prepared in pH 9 hydration medium at 32 °C (mean \pm SD, $n = 3$).

Doubling the cholesterol ratio (D2 compared to D1) decreased the release rate. Positively charged liposomes (D4) exhibited the fastest release of all liposomal dispersions.

Judging by the cumulative percent released profiles (Fig. 2) approximately 10–30% of the drug is released at a relatively rapid rate during the first 2 hours, followed by slower release rates over the next ten hours. The initial rapid phase of drug release is less evident in the case of negatively charged liposomes.

Drug release profiles from multilamellar liposomes described appearing in the literature characteristically show an initial fast drug loss followed by slower rates of drug loss (9, 12). The initial fast rate of release is commonly ascribed to drug detachment from liposomal surface while the later slow release results from sustained drug release from the inner lamellae.

Zero-, first-order and Higuchi's equations were applied to *in vitro* release results. Correlation coefficient values were high in all cases but Higuchi's model was found to be the best fitting model, suggesting that drug transport out of the liposomes was driven mainly by a diffusion-controlled mechanism. Negatively charged liposomes exhibited low release rates. The negative charge imparting agent conjugated with the phospholipids bilayer structure presumably attracts the ionized part of dibucaine, decreasing its release (13).

To further validate the domination of the diffusion controlled mechanism rather than a zero- or first- order one, additional evidence bearing upon the relative validity of these two models was obtained utilizing the differential forms of their relative rate equations (14). When the release rates from the dibucaine liposomal dispersion (D2) were plotted as a function of Q' and $1/Q'$ (Q' trial drug released at a given time), linearity was obtained only in the latter case, indicating that the process was diffusional (Fig. 3).

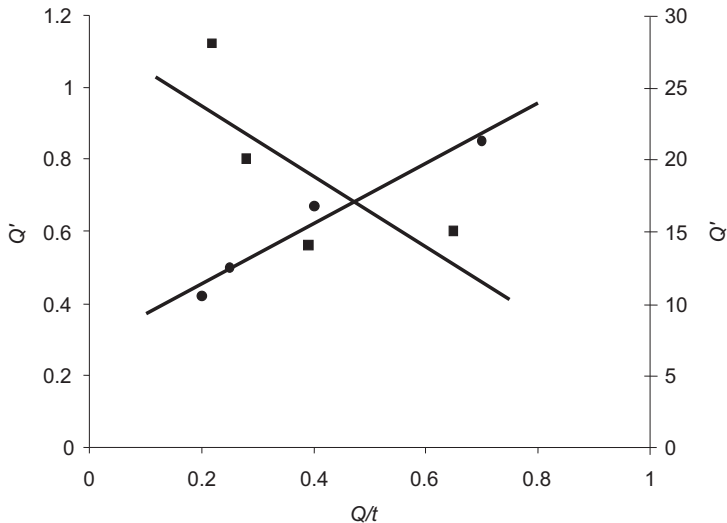


Fig. 3. Differential forms of the drug release rate Q'/t vs. dibucaine released (Q') or $1/Q'$ for dibucaine liposomal dispersion (D1).

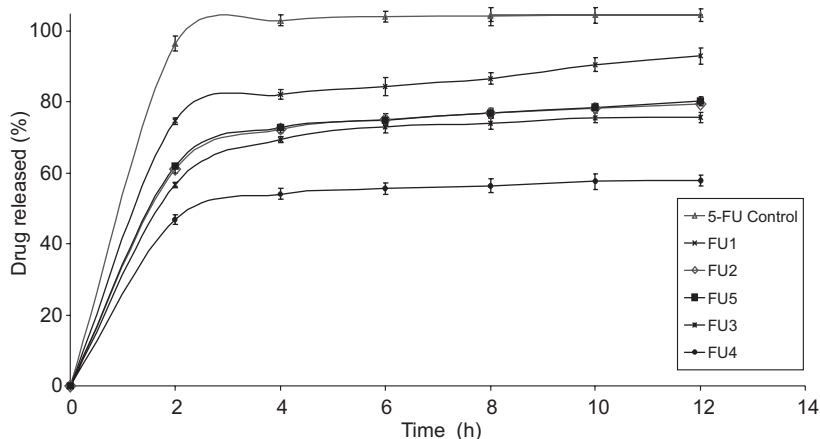


Fig. 4. Release profiles of 5-FU from the liposomal dispersions and drug control into IPBS pH 7.4 at 32 °C (mean \pm SD, $n = 3$).

Release data for 5-FU from five test liposomal dispersions into IPBS pH 7.4 at 32 °C using a dialysis method are shown as cumulative percent release over a 12 hour study period (Fig. 4). Diffusion of free 5-FU in solution through the dialysis membrane was used as control. The release profile of free 5-FU shows 100% diffusion in 2 h. Entrapment of 5-FU in liposomes expectedly reduced both the drug release rate and the cumulative amount released. It was observed that SPLVs exhibited slower drug release relative to MLVs of the same lipid composition. In general, the release profiles of liposomal dispersions were biphasic, showing a relatively large burst effect over the first two hours, followed by a slower release phase. The burst effect varies with the liposome type and lipid composition. Inclusion of cholesterol reduced the initial release rate, the effect being dependent on the EPC/CHOL molar ratio. Moreover, imparting a negative charge using dicetyl phosphate slightly enhanced drug release relative to neutral liposomes of the same composition.

The release process was characterized by treating release data mathematically using zero-order, first-order and Higuchi equations. Higher correlation coefficients were obtained for the Higuchi equation, indicating a diffusion controlled release model.

Release of 5-FU from liposomes into IPBS pH 7.4 at 32 °C was also considerably affected by the preparation method and lipid composition of the vesicles. The release profiles indicate a fast release initial phase (burst effect) (15), the magnitude of which could be related to the intercepts of the straight lines corresponding to the slower release phase while slopes of these lines are related to the drug release rate in this phase.

Both the intercept and slope values for the release of 5-FU from MLVs were higher than those for SPLVs, indicating higher stability of the SPLV liposomal structure. SPLVs proved to be more stable compared to MLVs (16). Inclusion of cholesterol in SPLVs further reduced the burst effect and release rate of 5-FU, the effect being more pronounced at higher phosphatidylcholine/cholesterol molar ratio. This can be accounted for by the effect of cholesterol on the bilayers microviscosity and permeability to solutes (17). Ne-

gatively charged SPLVs exhibited a higher 5-FU release rate compared to neutral vesicles of the same lipid composition, probably as a result of the repulsive forces between the 5-fluorouracil and the imparted charge on the liposomal surface due to dicetyl phosphate.

Release data expressed as percent dibucaine released over 12 hours determined for six dibucaine gels are shown in Fig. 5. The release profile of the free untrapped dibucaine base from its gel (DG) showed that 95% of the drug was released within four hours. In comparison, the release profile of dibucaine from the liposomal gel (DLG) showed that $27.8 \pm 1.3\%$ of the drug was released in 12 hours, reflecting the sustained release of dibucaine from the liposomal gel. The release profiles of the combination gels showed that $86.2 \pm 4.3\%$, $74.03 \pm 2.2\%$, $63.6 \pm 3.0\%$ and $56.0\% \pm 2.5\%$ were released in 12 hours from the combination gels containing 25, 50, 75 and 87.5%, respectively, of liposome-entrapped drug, indicating the effect on release of the changes in proportion of free to liposome-entrapped drug in the prepared gels.

Looking at the initial phase of drug release and judging by the percent drug released within one hour for the different gels, $53.0 \pm 4.1\%$, $29.2 \pm 1.2\%$, $22.5 \pm 3.4\%$, $17.1 \pm 2.3\%$, $14.0 \pm 3.0\%$ and $6.5 \pm 3.6\%$ of dibucaine was released within one hour, starting with the gel containing untrapped drug (DG) and ending with the gel containing fully entrapped drug (DLG).

Looking into the kinetics of dibucaine release from the gels, the release data from time zero to 12 hours were found to best fit Higuchi's diffusion model based on the magnitude of the correlation coefficient obtained for zero-order, first-order and Higuchi's diffusion model.

The release profiles obtained for the various freshly prepared gels (Fig. 5) indicated the potential to modulate dibucaine release from the gels by varying the proportion of free and liposome-entrapped drug in the gels. The Higuchi's release plot intercept value

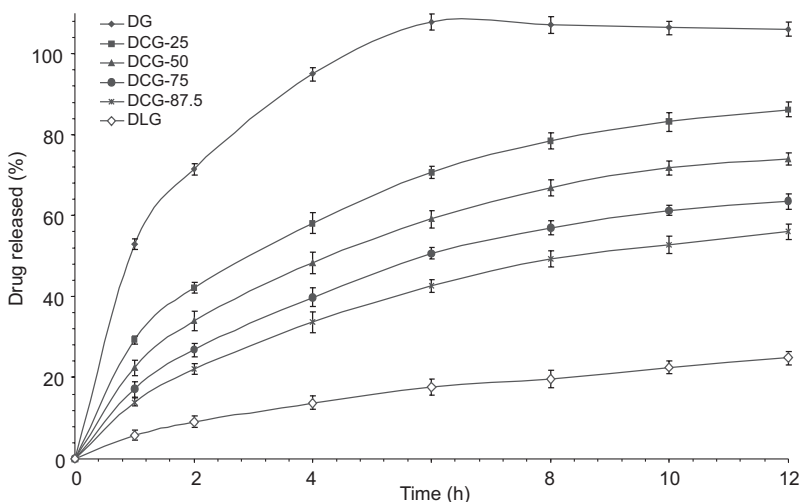


Fig. 5. Release profiles of dibucaine topical gel formulations at 32 °C (mean \pm SD, $n = 3$).

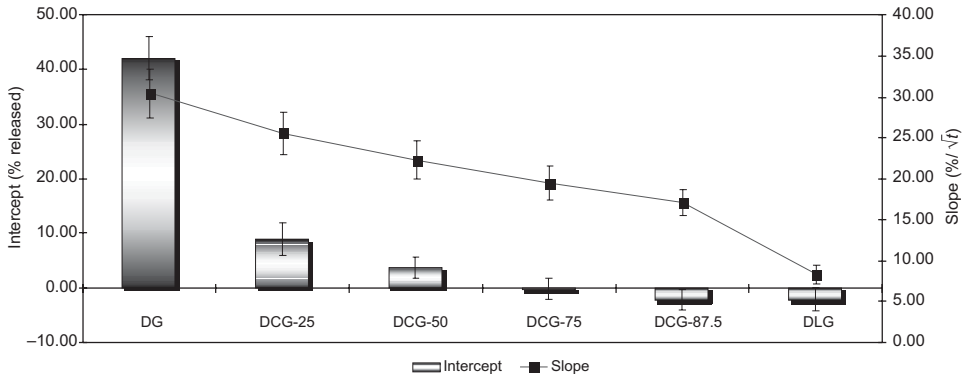


Fig. 6. Slopes and intercepts of Higuchi plots of release data from dibucaine gels in relation to gel composition.

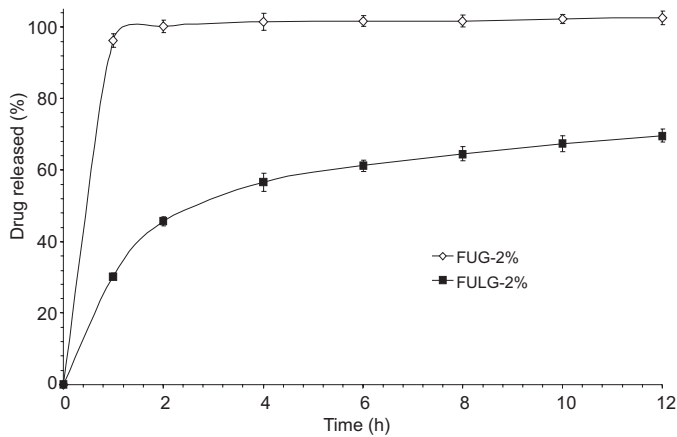


Fig. 7. Release profiles at 32 °C of different 5-FU liposomal gels (mean \pm SD, $n = 3$).

was a direct function of the gel composition (Fig. 6), again reflecting the potential to tailor initial release (burst effect) through varying the free drug content in the gels.

In an attempt to identify the rate limiting step in the release process of the drug from the liposomal gel, the release results for DLG were compared with the release profile generated for a similar liposomal system (D3), but as an aqueous concentrate and not a gel (Figs. 2 and 5). The similarity between the two liposome profiles suggests that the gelling agent in the concentration used had little effect on drug release and that the rate-limiting step was the diffusion of the drug out of the liposomes. A similar observation was reported in a study of lidocaine HCl release from topical liposomal formulations (18).

Release profile for the liposomal gel (FULG) is shown in Fig. 7. Incorporation of 5-FU SPLVs in 2% HPMC gel base reduced both the rate and extent of drug release over the twelve-hour study period; the drug release after twelve hours did not exceed 68%. The release profile for the plain drug gel (FUG) included for comparison indicated 100% drug release in one hour.

Judging by the cumulative percent released profiles (Fig. 7) approximately 45% of the drug was released from the 2% HPMC gels at a relatively rapid rate during the first 2 hours, followed by slower release rates over the next ten hours.

Obvious variations were observed within the *in vitro* release data of the relatively hydrophilic drug 5-fluorouracil and the totally hydrophobic drug dibucaine base from 2% HPMC liposomal gel. The *in vitro* release study of dibucaine base showed no burst effect, while the *in vitro* release study of 5-fluorouracil showed a clear burst effect with an initial fast release phase followed by a sustained release phase. This could be attributed to the relative hydrophilic nature of 5-fluorouracil when compared to the hydrophobic drug dibucaine base. Besides, 5-fluorouracil is a low molecular mass drug, easily leached out from the bilayer lipid structure of the liposomes to the external aqueous gel matrix. In contrast, dibucaine base is mainly associated within the bilayer lipid structure of the liposomes.

CONCLUSIONS

Evaluation of *in vitro* release profile of hydrophobic drugs from liposomal formulations could be problematic. But this could be manipulated through employment of a proper release medium that could provide sufficient sink conditions without affecting the stability of the liposomal formulation, agitation rate, well designed release device and adjusting various release technique parameters.

Besides, *in vitro* drug release could be used as a successful and efficient tool to examine stability parameters of liposomal formulations through providing a full release profile at different storage intervals. This could provide a full image of the drug release parameters over the storage period of various liposomal formulations.

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S A Ž E T A K

***In vitro* oslobađanje hidrofилnih i hidrofobnih ljekovitih tvari iz liposomskih disperzija i gelova**

MOHAMED MAHMOUD NOUNOU, LABIBA K. EL-KHORDAGUI, NAWAL A. KHALAFALLAH i SAID A. KHALIL

Opisana je metoda za određivanje brzine oslobađanja hidrofилnih i hidrofobnih ljekovitih tvari iz različitih vrsta liposomskih disperzija i gelova koristeći dijalizu. Dibukain baza i 5-fluorouracil uporabljeni su kao modeli hidrofobnog, odnosno hidrofилnog lijeka. Na brzinu oslobađanja utjecala je brzina rotacije lopatica u aparatu u kojem je pokus izvođen, temperatura i volumen medija za oslobađanje. Metoda je uporabljena za *in vitro* praćenje oslobađanja ljekovite tvari iz liposomskih disperzija i gelova. *In vitro* oslobađanje dibukain baze ne pokazuje učinak naglog oslobađanja, a 5-fluorouracila pokazuje, s brzim inicijalnim oslobađanjem iza kojeg slijedi usporeno oslobađanje.

Ključne riječi: dibukain, 5-fluorouracil, *in vitro* oslobađanje, stabilnost oslobađanja, liposomski gel, Higuchijev model difuzije

Department of Pharmaceutics, Faculty of Pharmacy, University of Alexandria, Egypt