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Derivative spectrophotometry as a tool for the determination of drug partition coefficients in water/dimyristoyl-L-α-phosphatidylglycerol (DMPG) liposomes

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Abstract

The partition coefficients (K_p) between lipid bilayers of dimyristoyl-L-α-phosphatidylglycerol (DMPG) unilamellar liposomes and water were determined using derivative spectrophotometry for chlordiazepoxide (benzodiazepine), isoniazid and rifampicin (tuberculostatic drugs) and dibucaine (local anaesthetic). A comparison of the K_p values in water/DMPG with those in water/DMPC (dimyristoyl-L-α-phosphatidylcholine) revealed that for chlordiazepoxide and isoniazid, neutral drugs at physiological pH, the partition coefficients are similar in anionic (DMPG) and zwitterionic (DMPC) liposomes. However, for ionised drugs at physiological pH, the electrostatic interactions are different with DMPG and DMPC, with the cationic dibucaine having a stronger interaction with DMPG, and the anionic rifampicin having a much larger K_p in zwitterionic DMPC. These results show that liposomes are a better model membrane than an isotropic two-phase solvent system, such as water–octanol, to predict drug–membrane partition coefficients, as they mimic better the hydrophobic part and the outer polar charged surface of the phospholipids of natural membranes.

Keywords: Partition coefficient; Unilamellar liposomes; Dimyristoyl-L-α-phosphatidylglycerol (DMPG); Derivative spectrophotometry; Hydrophobic/electrostatic interactions

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1. Introduction

The therapeutic and toxic effects of drugs are strongly influenced by their lipid affinity, and the study of drug–membrane interaction is of vital importance in drug absorption, distribution, metabolism and elimination phenomena, as well as in assessing toxic or therapeutic effects and bioaccumulation.

Liposomal membrane possess an ordered molecular arrangement, with structural characteristics varying from a liquid crystalline state to a rigid gel, and have the option of being able to exert electrostatic influences [1]. Liposomes are thus a better model membrane to predict drug/membrane partition coefficients than an isotropic two-phase solvent system, since they mimic better the hydrophobic part and the outer polar and negatively charged surface of the phospholipids of natural membranes [2].

Numerous studies on the spectrophotometric determination of liposome/water partition coefficients (K_p) for several drugs have appeared in the literature, since this parameter is important in predicting drug behaviour in biological environments. The determination of the partition coefficients by spectrophotometry requires that drug and vesicle mixture solutions must be separated into aqueous and lipid phases by centrifugation or filtration, since lipid vesicles cause intense background signals by light scattering [3]. However, when a measurable spectral change occurs due to drug/lipid interaction, derivative spectrophotometry may be used to obtain partition coefficients without phase separation, as the background signals due to liposome light scattering can normally be eliminated [4,5].

In a recent paper, we reported a comparative study using phase-separation and derivative spectrophotometry to determine the partition coefficients of some drugs, chlordiazepoxide (a benzodiazepine) and isoniazid and rifampicin (tuberculostatic drugs), between lipid bilayers of dimyristoyl-L-α-phosphatidylcholine (DMPC) unilamellar liposomes and water [6]. Both methods were shown to provide precise and accurate values for the partition coefficient of the drugs, despite their different hydro-lipophilic balance in a water (Hepes)/DMPC system.

As electrostatic/hydrophobic interactions across biological membranes are very important in predicting drug absorption and therapeutic effects, in this paper we report the partition coefficients of the same drugs between water and unilamellar liposomes of dimyristoyl-L-α-phosphatidylglycerol (DMPG), a negatively charged phospholipid.

Initially, we tried to separate non-encapsulated drugs from DMPG liposomes using ultracentrifugation, or centrifugal filter devices, a technique that has proved successful in the determination of partition coefficients of these drugs between DMPC vesicles and water. However, the DMPG liposomes were found to precipitate in centrifugal devices during the separation procedure, or to remain in the supernatant after ultracentrifugation. Thus, for DMPG/water vesicles, it was not possible to determine drug partition coefficients by separation procedures. These observations can explain why it was not possible to find K_p values for this system in the literature, since it is not possible to use separation methods to calculate this parameter, due to the characteristics of this lipid.

In this study, we demonstrate the usefulness of derivative spectrophotometry to determine the partition coefficients of chlordiazepoxide, isoniazid and rifampicin and of dibucaine (a local anaesthetic) in water/DMPG. A comparison of the K_p values obtained in water/DMPC with those obtained in water/DMPG clearly indicates that changes in the polar charged surface of phospholipids can turn out to be a key parameter in predicting drug–membrane interactions. Furthermore, this latter observation corroborates the fact that liposomes are a better model membrane to predict drug–membrane partition coefficients than an isotropic two-phase solvent system, such as water–octanol.

2. Experimental

Chlordiazepoxide was a gift from Hoffman La Roche. Isoniazid, rifampicin, dibucaine and *N*-

(2-hydroxyethyl)piperazine-*N'*-ethanesulfonic acid (Hepes) were from Sigma, dimyristoyl-L-α-phosphatidylglycerol (DMPG) was from Avanti and all other chemicals from Merck (pro analysi). All lipid suspensions were prepared with aqueous 10 mM Hepes buffer solution (I = 0.1 M NaCl, pH 7.4). Extruded liposomes were obtained by passing liposome suspensions through polycarbonate filters with a pore size of 100 nm (Corning) in a 10-ml stainless steel extruder (Lipex) attached to a circulating water bath.

Phase separation was attempted using either an ultracentrifuge (Sorvall Du Pont Model OTD 75B) or a Kubota 6900 centrifuge with a centrifugal filter unit (Centricon 30.000 units; Amicon).

Absorption spectra were recorded with a Unicam UV-300 spectrophotometer equipped with a constant-temperature cell holder. Absorption spectra of sample solutions (suspensions) were obtained by two methods. In one, they were measured against the corresponding reference solution (suspension), which had the same composition, but without drug, whereas in the other, spectra of sample solutions (suspensions) and of the corresponding reference solutions (suspensions) were measured against the buffer solution, and the latter was subtracted from the former. The final corrected absorption spectra were comparable in both procedures. All spectra were recorded at 37°C in 1-cm cuvettes with a slit width of 2 nm. The spectral window was from 225 to 400 nm for chlordiazepoxide, isoniazid and dibucaine, and from 375 to 600 nm for rifampicin. Derivative spectra were calculated using the Savitzky–Golay method [7], in which the second-order polynomial convolution of 13 points was employed.

Size distribution of extruded liposomes was determined by quasi-elastic light-scattering analysis using a Malvern Instruments ZetaSizer 5000. Lipid concentration in vesicle suspensions was determined by phosphate analysis, using a modified version of the Fiske and Subbarow method [8].

2.1. Liposome and drug / liposome preparation

2.1.1. Incubation method

Liposomes were prepared by evaporation to dryness of a lipid solution in chloroform under a stream of argon; the film was then left under vacuum overnight to remove all traces of the organic solvent. The resulting dried lipid film was dispersed with Hepes buffer and the mixture was vortexed above the phase transition temperature (37°C) to produce multilamellar liposomes (MLV). Frozen and thawed MLVs were obtained by repeating five times a cycle of freezing the vesicles in liquid nitrogen and then thawing in a water bath at 37°C. Lipid suspensions were equilibrated at 37°C for 30 min and extruded 10 times through polycarbonate filters, to form large unilamellar vesicles (LUV) [9].

Sample solutions were prepared by mixing a known volume of drug, a suitable aliquot of vesicle suspension and Hepes; the corresponding reference solutions were prepared identically, but without drug. All suspensions were then vortexed for 5 min and incubated at 37°C for 30 min. Typically, two sets of 10 vials (1.5 ml) were used in each experiment.

2.1.2. Encapsulation method

Two identical dried lipid films were prepared by the method described above. One of them was dispersed with 3 ml of drug Hepes buffer solution, while the other was dispersed with the same volume of Hepes buffer and used as reference. The MLVs were then freeze–thawed (five cycles) and extruded through 100-nm polycarbonate filters at 37°C in order to form LUVs.

Drugs concentration were as follows for all methods: rifampicin ≈ 30 μM; chlordiazepoxide ≈ 10 μM; isoniazid ≈ 170 μM; and dibucaine ≈ 160 μM.

3. Results

Attempts to separate the lipid and aqueous phases were not successful for DMPG liposomes, a possible consequence of their small size and negative charge. The mean particle size of DMPG liposomes was found to be 296 ± 1 nm for MLVs, a value much smaller than the typical values for DMPC, approximately 1000 nm and with a very large polydispersion. For LUVs, the mean particle size was 100 ± 0.3 nm, as expected.

Two methods were used to try to separate the phases; in one, the suspensions were centrifuged using an ultracentrifuge ($100\,000 \times g$), whereas in the other, the separations were performed using centrifugal filter devices. However, in the first method, a certain amount of DMPG liposome always remains in the supernatant, and in the latter method, liposomes are retained in the filter device during the separation procedure.

Thus, it is not possible to determine reproducible K_p values for the drugs using phase separation. It must be recalled that most determinations of K_p reported in the literature rely on phase separation, and that no study for drug partition in DMPG was found in the literature. In fact, for negatively charged lipids, the separation method has only been reported for chain lengths of C18 or longer [10,11].

Consequently, the partition coefficients (K_p) reported in the present work were calculated from spectrophotometric data, but without phase separation. The absorption spectra were recorded for liposome suspensions, and the values of K_p were calculated from derivative spectrophotometry. The concentration range over which Beer's law is obeyed, both for absorption and derivative spectrophotometry in aqueous solutions, was determined for each drug. The ranges observed were: chlordiazepoxide, 6.7–40.4; isoniazid, 105–211; rifampicin, 8.7–41.8; and dibucaine, 9.2–193.4 μM.

3.1. Determination of partition coefficients from spectrophotometric data

The molar partition coefficient of drugs between lipid bilayer vesicle suspensions and aqueous solution is defined as:

$$K_p = \frac{(C_m/C_t)/[\text{lipid}]}{(C_w/C_t)/[\text{water}]} \quad (1)$$

where C_t is the drug molar concentration, the subscripts m and w stand for drugs in lipid and in aqueous media, and [lipid] and [water] represent lipid and water molar concentrations, respectively. Partition coefficients can be determined from changes in drug absorbance caused by binding to vesicles. As absorbance is proportional to solute concentration, at a specific wavelength, $A = \varepsilon_m C_m + \varepsilon_w C_w$, where ε_m and ε_w are the drug extinction coefficients in lipid bilayer and water, respectively. The difference between absorption in the presence and absence of liposomes (ΔA) and can be related to K_p by the following equation:

$$\Delta A = \frac{K_p \varepsilon \; C_t[\text{lipid}]}{[\text{water}] + K_p[\text{lipid}]} \quad (2)$$

where $\varepsilon = \varepsilon_m - \varepsilon_w$.

Derivative intensity, like absorbance, is proportional to solute concentration, as long as the background signal caused by the liposomes is entirely eliminated in the derivative spectrum. Denoting ($d^nA/d^n\lambda$) by D and ($d^n\varepsilon/d^n\lambda$) by E, ΔD and K_p are related by an expression formally identical to Eq. (2), which can be written as:

$$\Delta E = \frac{K_p E C_t[\text{lipid}]}{[\text{water}] + K_p[\text{lipid}]} \quad (3)$$

The values of the partition coefficients, K_p, can be obtained by fitting Eq. (2) or Eq. (3) to the experimental data (ΔA or ΔD vs. [lipid]) for a given drug concentration, using a non-linear least-squares regression method [12].

3.2. Spectral changes induced by addition of liposomes

Analysis of absorption spectra reveals that background signals due to liposomes cannot always be completely balanced in the sample and reference suspensions, despite the fact that they were prepared with the same nominal vesicle concentration. This problem is particularly important at short wavelengths, for which light scattering is higher.

In the spectra of all drugs, it is noticeable that a decrease in absorption intensity occurs when the concentration of DMPG is increased. Besides this general trend, our data can be grouped into two sets based on other spectral features caused

by increasing amounts of added liposome. Absorption spectra of chlordiazepoxide, rifampicin and dibucaine exhibit isosbestic points and shifts in λ_{max}, an observation that provides a clear indication that these drugs exist in two forms: drugs in polar bulk water and in polar DMPG bilayers [13]. Furthermore, for the wavelengths used, the scattering is already completely eliminated in the first-derivative spectra.

In contrast, the spectra of isoniazid, for which the scattering could not be completely eliminated in the first-derivative spectra ($\lambda_{max} \approx 260$ nm), show only a small decrease in absorption intensity with increasing DMPG concentration. In addi-

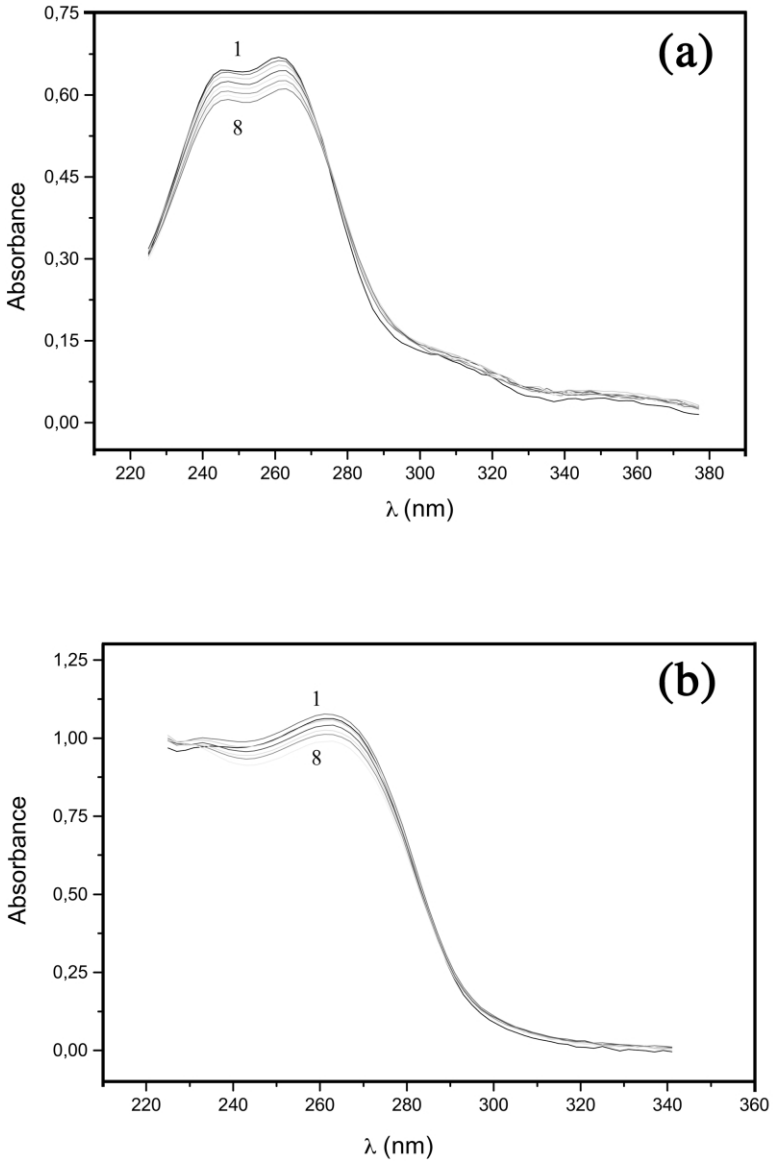

Fig. 1. Absorption spectra of (a) chlordiazepoxide and (b) isoniazid at different DMPG concentrations: (1) 0; (2) 175; (3) 250; (4) 375; (5) 500; (6) 675; (7) 810; and (8) 970 μM.

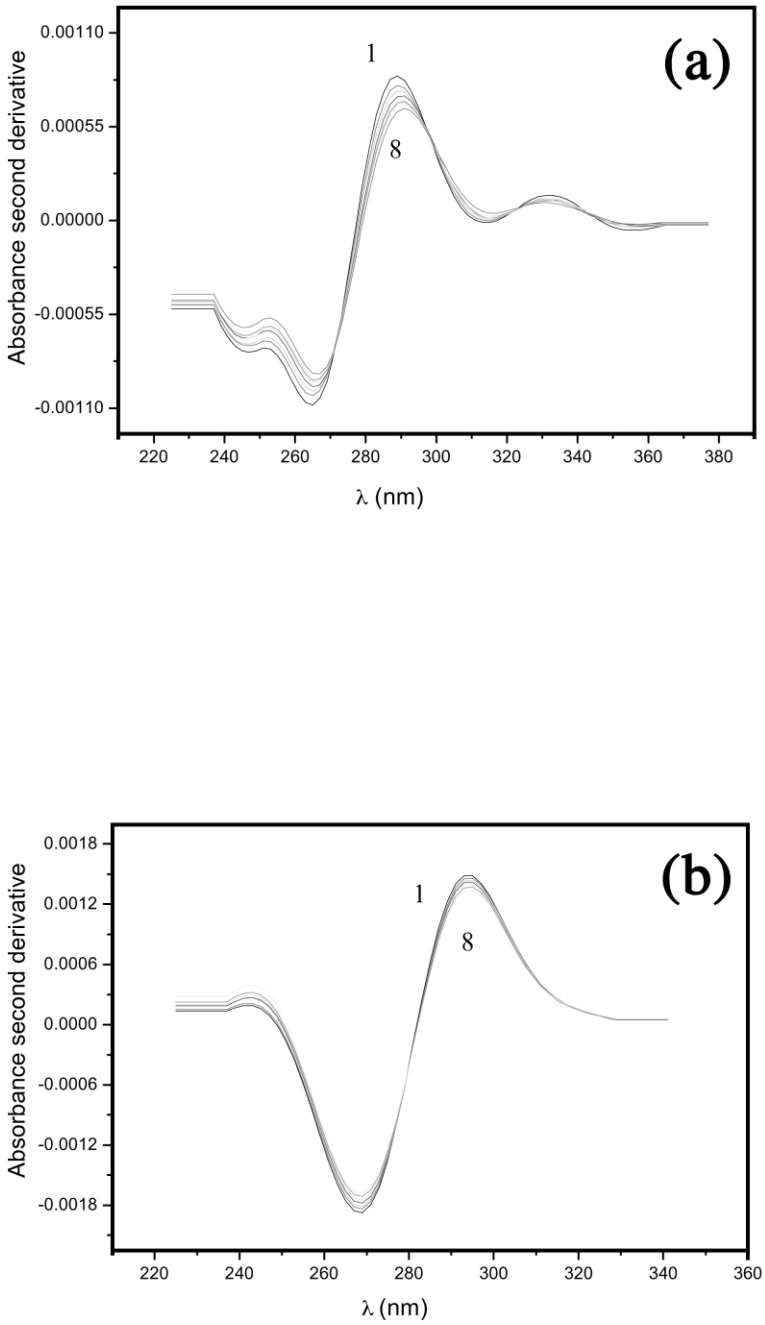

Fig. 2. Second-derivative spectra of (a) chlordiazepoxide and (b) isoniazid at different DMPG concentrations: (1) 0; (2) 175; (3) 250; (4) 375; (5) 500; (6) 675; (7) 810; and (8) 970 μM.

tion, the absorption spectra of isoniazid in the presence of DMPG are practically identical to those in aqueous solution (Fig. 1).

The scattering could be completely eliminated in the second-derivative spectra, and these are shown in Fig. 2. For chlordiazepoxide, rifampicin and dibucaine, similar to what is observed in the absorption spectra, an increase in DMPG concentration induces shifts in λ_{max} and a decrease in the intensity of the trough. The derivative spectra of isoniazid parallel the behaviour of the absorption spectra, and show only a slight decrease in the intensity of the trough with increasing DMPG concentration, but no shifts in λ_{max}.

3.3. Determination of partition coefficients

In non-separation methods, light scattering from liposomes must be eliminated before partition coefficients can be calculated, and the use of derivative spectrophotometry has been claimed to eliminate the effect of background signals, and also to improve resolution of overlapping signals by sharpening them [12,14–16].

In general, values of K_p can be calculated by fitting experimental data (ΔD vs. [lipid]) to Eq. (3). In principle, data at any wavelength can be used, but some care must be taken regarding their choice. To increase reproducibility and signal-to-noise ratio, the maximum-peak method is normally used for heterogeneous samples, in which the ΔD values are obtained at λ_{max} of the absorption spectra, as in their vicinity, small inaccuracies in wavelength reproducibility do not induce large variability in ΔD values [6,12,17].

The K_p values were obtained in the present work using data from first-, second- and third-derivative spectra, at a wavelength of approximately λ_{max} in the absorption spectrum. As a final comment, it must be stressed that light scattering as a source of additional noise in absorption measurements is more important at shorter wavelengths and will have a greater effect on weaker bands, so the ΔD values used were always measured for the highest λ_{max} of each drug. The use of data from at least two different order derivatives provides a check on the effective elimination of scattering.

The K_p values obtained by fitting Eq. (3) to data from at least two independent experiments, each with at least seven different lipid concentrations, are included in Table 1. The values of K_p determined for all the derivatives were identical, within experimental error, and only for isoniazid was it not possible to determine values from the first derivative.

4. Discussion

It was not possible to use phase separation to determine K_p values for our drugs, and it is interesting to note that no value for partition coefficients in the water/DMPG system could be found in the literature. However, the results obtained in this work show that it is possible to determine precise and reliable partition coefficients in water (Hepes)/DMPG using derivative spectrophotometry, with data directly collected from the liposome media. Furthermore, partition

Table 1
Partition coefficients for DMPG/water systems, of rifampicin, chlordiazepoxide, isoniazid, dibucaine calculated from derivative intensities

Intensity	$K_p/10^4$			
	Rifampicin	Chlordiazepoxide	Isoniazid	Dibucaine
First derivative	0.54 ± 0.02 (507)	2.53 ± 0.30 (277)	Not possible	6.47 ± 0.33 (338)
Second derivative	0.54 ± 0.07 (483)	2.56 ± 0.18 (289)	0.59 ± 0.13 (293)	6.68 ± 0.62 (326)
Third derivative	0.55 ± 0.10 (503)	2.54 ± 0.25 (277)	0.61 ± 0.23 (281)	6.67 ± 0.96 (338)

The values reported are the mean of two independent measurements; the error that affects each value is the standard deviation. The values in parentheses indicate the wavelengths used in the determination of K_p.

Table 2
Partition coefficients of rifampicin, chlordiazepoxide, isoniazid, dibucaine calculated from second-derivative intensity

	$K_p/10^4$			
	Rifampicin	Chlordiazepoxide	Isoniazid	Dibucaine
DMPG	0.54 ± 0.25	2.56 ± 0.18	0.59 ± 0.12	6.68 ± 0.62
DMPC	5.09 ± 0.49	2.40 ± 0.24	0.84 ± 0.12	1.48 ± 0.14

The values reported are the mean of at least three independent measurements; the error that affects each value is the standard deviation.

coefficients were found to be independent of drug and DMPG concentration, at least if the latter does not exceed 1000 μM.

The K_p values obtained in this study were compared with those obtained for the same drugs in water/DMPC media (Table 2) to assess the effect of the polar charged surface of the phospholipids on drug partition. For chlordiazepoxide and isoniazid, the K_p values were found to be practically independent of the liposome, whereas for rifampicin and dibucaine, the K_p values in water/DMPG are completely different from those in water/DMPC.

This difference in behaviour can be explained by the acid–base properties of the drugs. As chlorodiazepoxide and isoniazid are neutral species at physiological pH (7.4), the interactions between these drugs with the liposomes are mainly due to the hydrophilic–lipophillic balance of the medium, and the contribution from electrostatic interactions must be very small or null. On the other hand, rifampicin and dibucaine are partially ionised at pH 7.4, since their pK_a values are 7.9 and 8.3, respectively [18], and consequently the interactions between these drugs and the liposomes are not only due to the hydrophilic–lipophillic balance of the medium, but must also have a significant contribution from electrostatic interactions.

Furthermore, the K_p values of these two latter drugs underlie the caveats of using water/octanol partition coefficients, $K_{w/o}$, or solubility in water, S (mg/l), to predict the partition of charged species in phospholipid membranes, as can be gathered from data in Table 3. If for neutral drugs there is a general relationship between K_p and $K_{w/o}$, for charged drugs, no such a relation can be established. Thus, in the water/DMPG system, the K_p value of rifampicin is much smaller than that expected based on $K_{w/o}$, whereas that of dibucaine is much larger [19,20]. Contrastingly, in water/DMPC, the K_p value of dibucaine is much smaller than expected, and that of rifampicin much larger.

The large partition coefficient of dibucaine in DMPG parallels the observation that in water/phosphatidylserine, also a negatively charged phospholipid, this value is also much larger than in water/DMPC, a consequence of the electrostatic interaction of the negative charge of these lipids with the drug cationic form, which predominates ($\approx 75\%$) at pH 7.4 [10,11]. On the other hand, the anionic form of rifampicin at physiological pH is present at $\approx 30\%$ and a strong electrostatic repulsion must occur between this form and the negative charge of the DMPG lipids, which explains the smaller partition coefficient obtained in this medium when compared with water/DMPC.

Table 3
Comparison of the partition coefficients of rifampicin, chlordiazepoxide, isoniazid, dibucaine in water/DMPG and water/DMPC with their water/octanol partition coefficients, $K_{w/o}$, and their solubility in water, S

Compound	$K_p/10^4$		Log $K_{w/o}$ [a]	S (mg/l) [b]
	DMPG	DMPC		
Rifampicin	0.54	5.09	4.24	3.7×10^{-4}
Dibucaine	6.68	1.48	2.52	28.44
Chlordiazepoxide	2.56	2.40	2.44	10.35
Isoniazid	0.59	0.84	−0.70	1.7×10^4

[a] Values obtained by KOWWIN program and from [17].
[b] Values obtained by WSKOW program and from [18].

5. Conclusions

Partition coefficients in water/DMPG could not be determined by separation methods. In this paper, we show that derivative spectrophotometry can provide a precise and reliable alternative method to determine K_p in this medium.

A comparison of the K_p values determined in this medium and those determined in water/DMPC with $K_{w/o}$ values reveals that liposomes are, without doubt, a better membrane model than an isotropic two-phase solvent system, such as water–octanol. They allow for a better prediction of drug–membrane partition coefficients, since they mimic better the hydrophobic part and the outer, polar and negatively charged surface of the phospholipids in natural membranes.

The determination of partition coefficients of drugs in water/liposomes media with different head groups can be a very important tool at the molecular level, since it allows for discrimination between the ionised hydrophilic and hydrophobic contributions to the formation of drug–lipid interactions.

As a final remark, we can conclude that electrostatic/hydrophobic interactions across biological membranes are very important in predicting drug absorption and therapeutic effects, especially when they exist in ionised forms at physiological pH. For chlordiazepoxide and isoniazid, neutral drugs at physiological pH, partition coefficients are identical for charged or zwitterionic liposomes, but for rifampicin and dibucaine, ionised drugs at physiological pH, the different electrostatic interactions for charged or zwitterionic liposomes can significantly enhance or reduce partition coefficients, and therefore drug absorption and therapeutic effects.

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