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Journal of Pharmaceutical and Biomedical Analysis
25 (2001) 319–324

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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Determination of partition coefficients of diazepam and flurazepam between phosphatidylcholine bilayer vesicles and water by second derivative spectrophotometric method

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Received 8 July 2000; received in revised form 3 September 2000; accepted 15 October 2000

Abstract

Second derivative spectrophotometry allowed the establishment of a simple and accurate method for the determination of partition coefficients of benzodiazepine drugs in a liposome/water system. The absorption spectra of diazepam (DZ) and flurazepam (FZ) in phosphatidylcholine (egg yolk) bilayer vesicle suspensions showed small spectral changes depending on the concentration of phosphatidylcholine vesicles. However, the intense background signals caused by the light scattering of the phosphatidylcholine vesicles made it difficult to yield a correct base line, thus the quantitative spectral data could not be obtained. In the second derivative spectra, the spectral changes were enhanced and three derivative isosbestic points were observed for each drug indicating the entire elimination of the residual background signal effects. The derivative intensity change of each drug (ΔD) induced by its interaction with phosphatidylcholine bilayers was measured at a specific wavelength. From the relationship between the ΔD value and the lipid concentration, the molar partition coefficients (K_ps) of DZ and FZ were calculated and obtained with a good precision of R.S.D below 10%. The fractions of the partitioned DZ and FZ calculated by using the obtained K_p values agreed well with the experimental values. The results prove that the derivative method can be usefully and easily applied to the determination of partition coefficients of benzodiazepines in the liposomes/water system without any separation procedures. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Partition coefficient; Diazepam; Flurazepam; Liposome; Second derivative spectrophotometry

1. Introduction

Benzodiazepine derivatives belong to a class of neuroactive drugs that enjoy a wide administration because of their muscle relaxant, hypnotic, anticonvulsant and aniolytic characteristics [1].

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PII: S0731-7085(00)00503-3

Benzodiazepines are able to partition into biological membranes [2] and different approaches have been suggested to investigate the partition of benzodiazepines into natural [3,4] and artificial [5,6] membranes.

Recently, the interest has focused on the determination of the partition coefficients of drugs between lipid bilayer vesicles (liposomes) and the aqueous phase in order to investigate the behavior of drugs towards biomembranes [5–7]. Also, the partition coefficients obtained with liposome/buffer systems are more useful than that derived with the traditional octanol/water system in the structure activity relationships (QSARs) of drugs [8,9]. Thus, it is of importance to develop a simple and accurate method to determine the partition coefficients of benzodiazepines in liposome/buffer systems.

Since the lipid vesicles cause intense background signals by light scattering, the spectrophotometric determinations of the partition coefficients of drugs to phosphatidylcholine bilayers have usually been accompanied by separation procedures such as centrifugation [10], filtration (hygroscopic desorption) [11] or equilibrium dialysis [5] to measure the concentrations of free drugs or the amount of bound drugs. However, the separation procedures are troublesome and may disturb the equilibrium states of the samples and also lead to errors arising from the turbidity of solutions with membrane fragments or non-specific matrix adsorption of drugs onto membranes.

The recognized high-resolution potential of the derivative spectrophotometry has been used advantageously in the elimination of spectral background interference and increasing the resolution of the overlapped signals [7,12–14].

In this work, the second derivative spectrophotometry is employed to develop a simple and accurate method for the determination of the partition coefficients of benzodiazepine drugs, diazepam (DZ) and flurazepam (FZ), between phosphatidylcholine bilayer vesicles and water (buffer).

2. Experimental

2.1. Calculation method

The molar partition coefficient of benzodiazepine between lipid bilayer vesicles and water is defined as [15]

$$K_p = \frac{([B_m]/[B_t])/[L]}{([B_w]/[B_t])/[W]} \quad (1)$$

where $[B_m]$ and $[B_w]$ represent the concentrations of benzodiazepine in the lipid bilayers and water, respectively, and $[B_t]$ equals to the total amount of benzodiazepine added ($[B_t] = [B_m] + [B_w]$). $[L]$ and $[W]$ are the molar concentrations of lipid and water (55.3 M at 37°C), respectively.

The derivative intensity is proportional to the solute concentration, so if the flat and zero-level base line can be obtained in the derivative spectrum, the derivative intensity (D) of benzodiazepine at a specific wavelength is represented as follows,

$$D = E_m[B_m] + E_w[B_w]$$

where E_m and E_w are the molar derivative intensities for $[B_m]$ and $[B_w]$, respectively. With a definition $E = E_m - E_w$, D can be written as.

$$D = E_w[B_t] + E[B_m] \quad (2)$$

The difference between D and E_w $[B_t]$, denoted as ΔD, is given as

$$\Delta D = D - E_w[B_t] \quad (3)$$

From Eq. (2).

$$\Delta D = E[B_m] \quad (4)$$

Thus, the ΔD value is proportional to the concentration of benzodiazepine in phosphatidylcholine bilayers.

Finally, from Eqs. (1) and (4),

$$\Delta D = \frac{\Delta D_{max} K_p[L]}{[W] + K_p[L]} \quad (5)$$

where $\Delta D_{max} = E\,[B_t]$. The values of K_p and ΔD_{max} can be calculated from the experimental values of $[L]$ and ΔD by applying a non-linear least-squares method (accompanying a Taylor expansion) to (Eq. (5)). The calculation was performed by a BASIC program [7].

2.2. Reagents and apparatus

DZ and FZ dihydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and used without further purification but good purity was confirmed by ^{1}H-NMR, thin layer chromatography (TLC) and melting point measurements. The buffer used was Hepes buffer (50 mM NaCl, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4). L-α-phosphatidylcholine (egg yolk) was supplied as a 5% (w/v) chloroform solution from Avanti Polar-Lipids Inc. (USA) and stored at −30°C. Sonication was performed by an ultrasonicator UD-200 (Tomy Seiko Co. Ltd., Tokyo, Japan). A model U-3210 spectrophotometer (Hitachi, Japan) equipped with a thermostatic cell holder was used to measure the absorption spectra. The spectrophotometer was connected with a personal computer (NEC PC-9801 VX) through an RS-232 interface. Calculations to obtain derivative spectra and K_p values were performed by the same personal computer.

2.3. Vesicle preparation

An appropriate volume of the phosphatidylcholine stock solution was evaporated and the residual was dried by using a vacuum pump for more than 4 h. The dried phosphatidylcholine was suspended in 5 ml buffer using a vortex mixer. The suspension was then sonicated by the ultrasonicator (at power level 5.0) under nitrogen stream in an ice-bath to produce small unilamellar vesicles. Ten consecutive cycles of 3 min sonication with 3 min interval were repeated, i.e. the net sonication time was 30 min. To eliminate the sediments from the sonication tip, the sonicated suspension was centrifuged at $2000 \times g$ (3500 rpm) for 20 min.

2.4. Lipid concentration

The exact phosphatidylcholine concentration in the vesicle suspension was calculated from phosphorus determination by using, the method of, open tube digestion with a perchloric–sulfuric acid mixture followed by spectrophotometric measurement of the phosphovanadomolybdate complex [16].

2.5. Procedure for determining K_p and ΔD_{max}

The sample solutions were prepared in 10-ml volumetric flasks by adding a suitable aliquot (50–150 μl) of a stock solution of 3.0 mM DZ or FZ to 8 ml of buffer solutions containing various amounts of phosphatidylcholine vesicle suspension and the buffer was further added to volume. The reference solutions were buffer solutions containing the same amount of the phosphatidylcholine vesicle suspension as the sample solutions. Each flask was shaken for a short time and incubated at 37°C for 30 min. Then the absorption spectrum of the sample solution was measured against the reference solution at 37°C with a slit width 2 nm and a wavelength interval of 0.1 nm using 10 mm light-path cuvette for 20 and 30 μM drug concentrations and 5 mm for 45 μM, respectively.

The second derivative spectra were calculated using a BASIC program [17] based on the Savitzky–Golay method [18]. The wavelength interval ($\Delta\lambda$) of 0.8 nm was used in the calculation.

3. Results and discussion

3.1. Absorption and second derivative spectra

The absorption spectra of DZ and FZ in the presence of various amounts of phosphatidylcholine vesicles are depicted in Fig. 1A and B, respectively. Both A and B show small spectral changes according to the amount of phosphatidylcholine vesicles. It is also obvious that the counterbalance of the sample and reference beams was incomplete, even though the solutions in the sample and reference cuvettes were prepared to contain the same amount of phosphatidylcholine vesicles. With the strong background signals, it is usually difficult to cancel their effects completely to obtain a flat and zero-level base line. Thus, further spectral data for calculating the partition coefficients could not be obtained from these absorption spectra.

Fig. 2A and B illustrate the second derivative spectra calculated from the absorption spectra in Fig. 1A and B, respectively. The spectral changes of both drugs are largely enhanced in the second derivative spectra showing a decrease in the derivative intensities according to the increase in the phosphatidylcholine vesicle concentration. Three derivative isosbestic points are clearly observed for both drugs, indicating that the residual background signal effects are entirely eliminated in the second derivative spectra, and that DZ (or FZ) exists in two states that have different derivative spectra [19], i.e. the drug exists in polar bulk water and non-polar phosphatidylcholine bilayer phases.

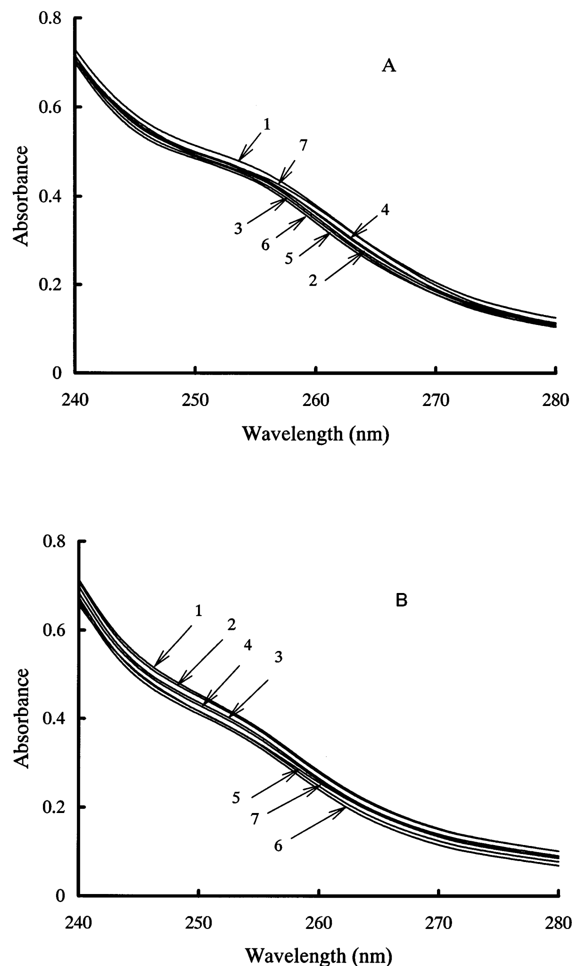

Fig. 1. Absorption spectra of 30 μM (A) DZ and (B) FZ in Hepes buffer solutions (pH 7.4, 37°C) containing various amounts of phosphatidylcholine vesicles. Phosphatidylcholine vesicles (mM), (A) (1) 0; (2) 0.098; (3) 0.196; (4) 0.295; (5) 0.491; (6) 0.786; (7) 1.182. (B) (1) 0; (2) 0.119; (3) 0.237; (4) 0.356; (5) 0.593; (6) 0.948; (7) 1.422.

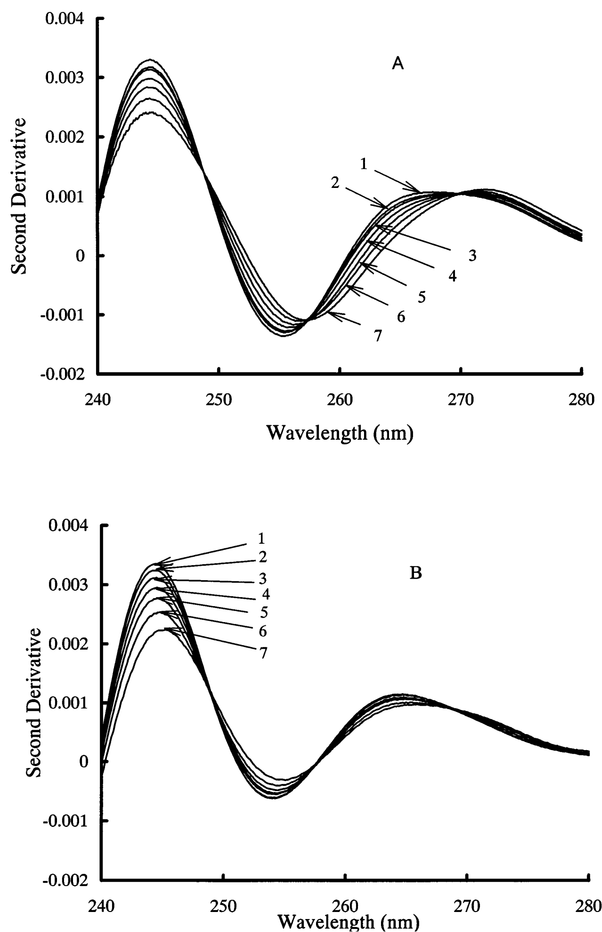

Fig. 2. Second derivative spectra of (A) DZ and (B) FZ calculated from the absorption spectra of Fig. 1A and B, respectively. The numbers in the figures are the same as in Fig. 1.

3.2. Calculation of K_p and ΔD_{max}

The ΔD values for DZ or FZ were measured at the wavelength 263 and 245 nm, respectively, since large ΔD values (as seen in Fig. 2) could be obtained with a good reproducibility at these wavelengths. Using the experimentally obtained ΔD values, the K_{p} and ΔD_{max} values were calcu-

lated. As summarized in Table 1, the K_p values of DZ and FZ were obtained with R.S.D. below 10% indicating a good precision of the derivative method. The results also show that the drug concentration has no significant effect on the K_p values. The difference of K_p values between DZ and FZ clearly indicates the difference in their lipophilicity.

Table 1
Molar partition coefficients (K_p values) of DZ and FZ at several concentrations

Concentration (μM)	$K_p \times (10^{-3})$[a]	
	DZ	FZ
20	31.5 ± 3.0	15.6 ± 1.0
30	30.8 ± 2.5	14.1 ± 0.6
45	31.6 ± 2.5	14.7 ± 0.7

[a] Mean ± S.D. (number of determinations = 5).

Table 2
K_p values of DZ and FZ calculated from the ΔD values at different wavelengths

DZ		FZ	
Wavelength (nm)	K_p ($\times 10^{-3}$)[a]	Wavelength (nm)	K_p ($\times 10^{-3}$)[a]
262	27.9 ± 2.6	244	13.6 ± 1.2
263	30.8 ± 2.5	245	14.1 ± 0.6
264	29.0 ± 3.7	246	13.8 ± 1.4

[a] Mean ± S.D. ($n = 5$).

Table 3
Effect of $\Delta\lambda$ on the K_p values

$\Delta\lambda$ (nm)	$K_p \times (10^{-3})$[a]	
	DZ	FZ
0.8	30.8 ± 2.5	14.1 ± 0.6
0.7	30.8 ± 3.1	14.8 ± 1.2
0.6	34.1 ± 5.7	14.8 ± 1.2
0.5	34.4 ± 7.9	15.9 ± 1.3

[a] Mean ± S.D. (number of determinations = 5).

Table 4
Comparison of the determined partition coefficient of DZ with literature values at pH 7.4

Method	$K_p \times (10^{-3})$[a]	Literature
Derivative spectrometry	30.8 ± 2.5	This study
Membrane dialysis	41.3 ± 0.4[b,d]	[5]
HPLC	26.3[c,d]	[6]

[a] Mean ± S.D.
[b] Using ^{14}C DZ.
[c] Using membrane stationary phase produced with a phosphatidylcholine analogue.
[d] The values of molar partition coefficient were obtained by multiplying the original values (at pH 7.4) by a factor 42 [15].

To which extent the K_p values are affected by a small variation in the wavelength at which the ΔD values are measured was examined by calculating the K_p values with the ΔD values measured at 263 ± 1 nm for DZ and 245 ± 1 nm for FZ, respectively. The results showed in Table 2 indicate that the small difference in these wavelengths does not affect the K_p values.

The effect of the $\Delta\lambda$ value on the K_p value was confirmed since the $\Delta\lambda$ value affects the results of second derivative spectrum calculation (17). The K_p values of both drugs were calculated by using the ΔD values measured from the second derivative spectra obtained with different four $\Delta\lambda$ values (0.5, 0.6, 0.7 and 0.8 nm). The results listed in Table 3 apparently show that a change in the $\Delta\lambda$ value does not affect the calculated K_p values. The working $\Delta\lambda$ value was chosen to be 0.8 nm because of its lowest R.S.D.

The reported partition coefficient values of DZ between phospholipid membrane or phosphatidylcholine analogue and water measured by membrane dialysis [5] and high-performance liquid chromatography (HPLC) [6], respectively, are listed in Table 4. Despite the differences in the experimental conditions, the K_p value obtained by the second derivative method is reasonably within the range of these two values.

The value of $\Delta D/\Delta D_{max}$, which corresponds to a fraction of the drug in phosphatidylcholine bilayer, was calculated from (Eq. (5)) with the obtained K_p and ΔD_{max} values and the results are

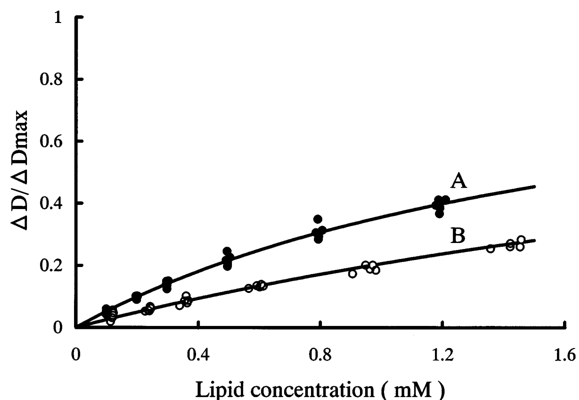

Fig. 3. Fraction ($\Delta D/\Delta D_{max}$) of 30 μM (A) DZ and (B) FZ in phosphatidylcholine vesicle bilayers at various phosphatidylcholine vesicle concentrations. The solid lines show the theoretical curves calculated from (Eq. (5)) using the obtained K_p and ΔD_{max} values. The circles are the experimental values (●) DZ and (○) FZ.

shown in Fig. 3 as the curves A and B for 30 μM DZ and FZ, respectively. The experimental $\Delta D/\Delta D_{max}$ values also plotted in Fig. 3 show a good correlation with the calculated curves.

4. Conclusions

In summary, the most important attribute of the proposed second derivative method is that it can eliminate the background signal effects caused by the phosphatidylcholine vesicles and enhance the net spectral changes to offer the accurate data to calculate the K_p values. Thus, the proposed method is a simple and accurate way that can be used for rapid and reliable determination of the partition coefficients of benzodiazepine derivatives without disturbing the equilibrium states of samples by separation procedures.

Acknowledgements

The authors thank Ms. Megumi Yoshida for her experimental assistance.

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