amount of sodium benzoate which disappeared from the perfusion solution equaled the amount of benzoic acid absorbed. With the single-pass perfusion experiments, a linear relationship between concentration of sodium benzoate and the rectal absorption was found, which may indicate passive diffusion possibly as benzoic acid.

REFERENCES

(1) N. Senior, "Advances in Pharmaceutical Sciences," vol. 4, Academic, London, 1974, p. 363.

(2) L. S. Schanker, D. J. Tocco, B. B. Brodie, and C. A. Hogben, *J. Pharmacol. Exp. Ther.*, **123**, 81 (1958).

(3) K. Kakemi, T. Arita, and S. Muranishi, *Chem. Pharm. Bull.*, **13**, 861 (1965).

(4) N. F. H. Ho and W. I. Higuchi, *J. Pharm. Sci.*, **60**, 537 (1971).

(5) S. Riegelman and W. J. Crowell, *J. Am. Pharm. Assoc., Sci. Ed.*, **47**, 115, 123, 127 (1958).

(6) R. E. Beck and S. Schultz, *Science*, **170**, 1302 (1970).

(7) K. Kakemi, T. Arita, R. Hori, R. Komishi, and K. Nishimura, *Chem. Pharm. Bull.*, **17**, 248 (1969).

(8) K. Thoma, "Arzneiformen zur Rectalen und Vaginalen Applikation," Werbe- und Vertriebsgesellschaft Deutscher Apotheker mbH, Frankfurt, West Germany, 1980, p. 17.

(9) F. Moolenaar and A. J. M. Schoonen, *Pharm. Int.*, **1**, 144 (1980).

(10) C. J. Devroede and S. F. Phillips, *Gut*, **11**, 438 (1970).

(11) E. Bechaard, *Acta Pharmacol. Toxicol.*, **33**, 123 (1973).

(12) N. I. McNeil, *Gut*, **20**, 400 (1979).

(13) C. J. Edmonds, *Gut*, **12**, 356 (1971).

(14) F. Moolenaar, A. G. G. Stuurman-Bieze, J. Visser, and T. Huizinga *Int. J. Pharm.*, **1**, 323 (1978).

(15) A. J. Quick, *Am. J. Med. Sci.*, **185**, 630 (1933).

(16) N. H. Nie and C. H. Hull *et al.*, "SPSS-6000," 2nd ed., McGraw-Hill, New York, N.Y., 1975, p. 4.

(17) D. J. A. Crommelin, J. Modderkolk, and C. J. De Blaey, *Int. J. Pharm.*, **3**, 299 (1979).

Quantitation of Norfloxacin, a New Antibacterial Agent in Human Plasma and Urine by Ion-Pair Reverse-Phase Chromatography

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Abstract □ A specific and sensitive high-performance liquid chromatographic method for the analysis of norfloxacin in human plasma and urine is described. Norfloxacin was extracted from the sample matrix using dichloromethane under neutral conditions, followed by back extraction into dilute phosphoric acid for chromatographic analysis on a reverse-phase column with a mobile phase consisting of methanol, phosphate buffer, and ion-pairing reagent (pH 3.0) at a flow rate of 2.0 mL/min. The ability of this method to distinguish intact norfloxacin from its metabolites was demonstrated. The method is linear, quantitative, and reproducible for both plasma analysis (0.05–2.5 μg/mL) and urinalysis (1.0–500 μg/mL) using peak area ratios (norfloxacin–internal standard) for quantitation. The stability of norfloxacin and its metabolites in dilute phosphoric acid was studied. To assess the presence of norfloxacin conjugates in the urine of dosed individuals, the effects of urine hydrolysis on drug quantitation were examined. Urine and plasma levels of norfloxacin at selected time points following the administration of single drug doses are presented.

Keyphrases □ Norfloxacin—quantitation, antibacterial agent, human plasma and urine, ion-pair reverse-phase, high-performance liquid chromatography □ High-performance liquid chromatography—norfloxacin, human plasma and urine, ion pairing □ Antibacterial agents—quantitation of norfloxacin, human plasma and urine, ion-pair reverse-phase high-performance liquid chromatography

Norfloxacin (1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid) (I) is a new quinolinecarboxylic acid antibiotic which exhibits a broad spectrum of antibacterial activity. *In vitro* studies have demonstrated its efficacy towards most Gram-positive and Gram-negative bacteria, including nalidixic acid-resistant pathogens (1, 2).

The development of a rapid, sensitive, and specific assay for norfloxacin quantitation was critical for the measurement of drug levels in clinical specimens. Although a high-performance liquid chromatographic (HPLC) method specific for norfloxacin has been described (3), this method proved unsuitable for use in clinical studies since it offers inadequate sensitivity and requires extensive sample preparation. The use of nonspecific microbiological methods[1] (4) in pharmacokinetic and bioavailability studies is also precluded due to the antibacterial nature of several norfloxacin metabolites (III–VIII) (3).

This report describes a reverse-phase ion-pair HPLC method developed for the quantitation of norfloxacin in plasma and urine, using pipemidic acid (II) as the internal standard. The assay was applied to the measurement of norfloxacin in the plasma and urine of human volunteers given increasing bolus doses of the drug.

EXPERIMENTAL

Reagents and Materials—All solvents[2] were distilled-in-glass and liquid chromatography grade, and purified water[3] was used throughout. All chemicals were ACS grade and used without further purification. Norfloxacin and its metabolites were used as received[4]. Pipemidic acid was extracted from 250-mg tablets[5].

Apparatus—The isocratic HPLC system consisted of a constant-flow pump[6], an autosampler[7], and variable-wavelength detectors; the assay for norfloxacin in plasma utilized a fluorescence detector[8] (excitation, λ = 280 nm; emission, λ = 445 nm; slits, 8 nm), while the urine assay used a UV absorbance detector[9] set at 280 nm. Data were recorded and reduced

[1] Dr. J. A. Bland, Merck Sharp & Dohme Research Laboratories, Rahway, N.J., personal communication.

[2] Burdick & Jackson Laboratories, Muskegon, Mich.

[3] Milli-Q System; Millipore Corp., Bedford, Mass.

[4] Kyorin Pharmaceutical Co., Ltd., Japan.

[5] Dolcol tabs from Dainippon Pharmaceutical Co., Japan.

[6] Model 6000A solvent delivery system; Waters Associates, Milford, Mass.

[7] WISP 710A autosampler; Waters Associates, Milford, Mass.

[8] 650S fluorescence detector equipped with 150B Xenon power supply; Perkin-Elmer, Norwalk, Conn.

[9] Schoeffel SF770 UV-spectroflow monitor; Kratos Analytical Instruments, Westwood, N.J.

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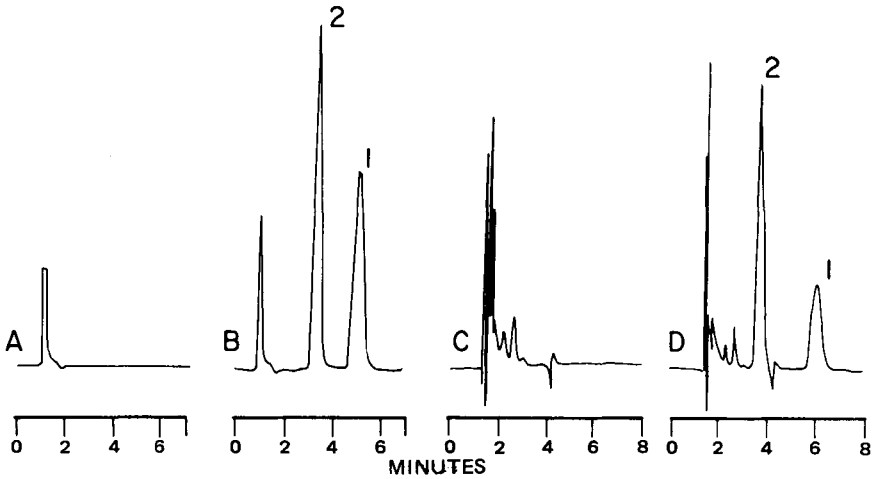


Figure 1—*Representative chromatograms; flow rate = 2.0 mL/min, chart speed = 0.5 cm/min. Key: (A) control plasma; (B) plasma spiked with 0.50 μg/mL of norfloxacin (1) and internal standard (2); (C) control urine; (D) urine spiked with 5.0 μg/mL of norfloxacin (1) and internal standard (2).*

by a computing integrator[10] set for peak area calculations at a chart speed of 0.5 cm/min and attenuation of 16 mV full scale.

Chromatographic Conditions—A 30-cm × 4-mm i.d. reverse-phase stainless steel column[11] was used for all assays. The mobile phase consisted of 300 mL of methanol and 700 mL of water, to which was added 1.74 g of monobasic potassium phosphate and 20 mg of ion-pairing reagent[12]. Prior to filtration through a 0.45-μm filter, the pH of the mobile phase was adjusted to a final value of pH 3.0 with ~1 mL of 85% phosphoric acid. All HPLC analyses were carried out at ambient temperature, using a 2-mL/min flow rate and a 30-μL sample injection volume.

Solution Preparation—Norfloxacin spiking solutions for use in the plasma assay were prepared by diluting a 100-μg/mL aqueous stock solution with water at 25.0, 10.0, 5.0, 2.5, 1.0, and 0.5 μg/mL. Similarly, for the urine assay a 5.0-mg/mL stock solution of norfloxacin in 0.05 M NaOH was diluted as necessary with water to obtain 5.0-, 2.5-, 1.0-mg/mL, 500-, 250-, 100-, 50-, and 10-μg/mL spiking solutions. All norfloxacin solutions were stored at 4°C.

The internal standard stock solution was prepared by dissolving pipemidic acid in 0.1 M NaOH and diluting with water to 100 μg/mL for the urine assay and to 25 μg/mL for the plasma assay. The acid was obtained by crushing a 250-mg tablet to a fine powder, dissolving the powder in 75 mL of 0.1 M NaOH, filtering through a 0.45-μm filter, and diluting the filtrate with water. Alternatively, the acid could also be continuously extracted from a crushed tablet with dichloromethane using a soxhlet apparatus. The solvent was removed from the extract *in vacuo* leaving a residue of pure pipemidic acid. The internal standard solutions were stored at 4°C.

Stock solutions (500 μg/mL) of norfloxacin metabolites M1, M2, M3, $M4_1$, $M4_2$, and M5 were prepared in dichloromethane using sonication and vortexed as needed to effect dissolution. These solutions were stored at −15°C. Aliquots of the stock solutions were diluted to 50 μg/mL with methanol for HPLC analysis.

Assay Procedure—Immediately following vigorous vortexing (or sonication), 100–1000 μL of plasma or urine was added to 100 μL of internal standard solution in a 40-mL centrifuge tube. This solution was then neutralized by adding 1.0 mL of 0.5 M phosphate buffer, pH 7.5. The resulting urine mixtures were extracted once, while the plasma mixtures were extracted twice with dichloromethane as follows. After addition of 10 mL of dichloromethane, each sample was vortexed 10 s, centrifuged[13] at 1000 rpm for 5 min, and the heavier organic layer was transferred to a second 40-mL centrifuge tube. Plasma samples were reextracted with 10 mL of dichloromethane, and this second extract was pooled with the first. The dichloromethane extracts were back-extracted into 0.085% phosphoric acid by adding 1.0 mL of the dilute acid to the organic extract and vortexing this mixture 10 s, followed by centrifugation at 1000 rpm for 5 min. Approximately 400 μL of the resulting acidic extracts were transferred into autosampler vials equipped with microvolume inserts.

[10] Model SP4100; Spectra Physics, Santa Clara, Calif.
[11] μBondapak C18; Waters Associates, Milford, Mass.
[12] Plasma assay: 1-hexanesulfonic acid, sodium salt; urine assay: 1-octanesulfonic acid, sodium salt.
[13] Model Centra 7; International Equipment Co., Needham Heights, Mass.

Preparation of the Standard Curve—Calibration standards were prepared by adding 100 μL of an appropriate spiking solution to 900 μL of urine or plasma and assaying as described above. The plasma assay used six calibration levels of norfloxacin for its standard curve (2.5, 1.0, 0.50, 0.25, and 0.10 μg/mL and 50 ng/mL), while the urine assay used eight calibrations (500, 250, 100, 50, 25, 10, 5.0, and 1.0 μg/mL). Concentrations of norfloxacin were calculated from calibration curves constructed by plotting the peak area ratio of norfloxacin to the internal standard *versus* the spiked concentration of norfloxacin. A freshly prepared set of calibration spikes was run daily, along with control blank extracts prepared both with and without the internal standard.

Urine Hydrolysis—To a 2.5-mL urine sample was added an equal volume of 2 M NaOH (hydrolysis pH 11.9) or 1 M HCl (hydrolysis pH 0.6–0.7). The mixture was vortexed briefly, and a 1.0-mL aliquot was removed immediately (t = 0 min), neutralized, and extracted according to the normal assay procedure. The remainder of the mixture was stoppered and placed in a constant temperature bath at 60°C. Additional 1.0-mL aliquots were removed, immediately neutralized, and assayed at t = 10, 20, and 30 min. Acid-hydrolyzed aliquots were neutralized by adding 0.5 mL of 2 M NaOH, while base-hydrolyzed aliquots were treated with 0.5 mL of 1 M HCl. The final pH of these mixtures after the addition of phosphate buffer (according to assay procedure) was in the range of pH 6.8–7.0.

Clinical Samples—The assay method was applied to both plasma and urine samples obtained from a clinical study in which five single doses of norfloxacin were orally administered to healthy male volunteers. All samples were stored at −20°C until the day of assay.

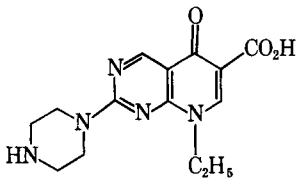
RESULTS AND DISCUSSION

A sensitive and specific HPLC method was developed utilizing the natural UV absorbing and fluorescing properties of norfloxacin. Using the chromatographic conditions described for this assay, norfloxacin (I) was completely resolved from each of its metabolites (III–VIII) and the internal standard (II), as is evident from the retention time data sum-

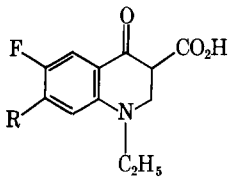
Table I—Chromatographic Properties of Norfloxacin, Pipemidic Acid, and Metabolites Under Urine and Plasma HPLC Assay Conditions

Compound	Retention Time, min	
	Plasma HPLC Conditions	Urine HPLC Conditions
Pipemidic acid (internal standard)	3.3	3.6
M2	4.0	4.4
Norfloxacin	5.1	5.6
M5	12.9	11.9
M3	19.0	17.8
M1	—[a]	21.3
$M4_2$	—[a]	30.9
$M4_1$	—[a]	42.6

[a] No fluorescence at 50 μg/mL.



II



I [Norfloxacin]: R = —N NH

III [M1]: R = —N NH (O)

IV [M2]: R = —$NH(CH_2)_2NH_2$

V [M3]: R = —$NH(CH_2)_2NHCCH_3$ (O)

VI [$M4_1$]: R = —N N—CCH_3 (O)

VII [$M4_2$]: R = —N N—CHO

VIII [M5]: R = —NH_2

marized in Table I. Representative chromatograms of control urine and plasma and urine and plasma spiked with norfloxacin and internal standard shown in Fig. 1. The chromatograms obtained by injection of drug-free urine or plasma show no interfering peaks at the retention times of norfloxacin and the internal standard. Sample preparation for HPLC analysis involved initial extraction of the drug from an aliquot of urine or plasma into dichloromethane, followed by back-extraction into 0.085% phosphoric acid.

Norfloxacin and metabolites M2, M3, and M5 were found to be stable for at least 24 h at ambient temperature in the final acidic extraction medium. However, M1 slowly reacted under these conditions to form a new product which was detected only after 15 h as a fluorescing species

Table II—Representative Standard Curve Data

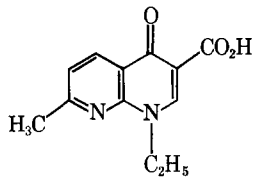
x: Standards, μg/mL	*y*: Area Ratio (norfloxacin/internal std.)
	Plasma Assay[a]
0.00	0.00
0.05	0.086
0.10	0.203
0.25	0.538
0.50	1.17
1.00	2.60
2.50	6.21
	Urine Assay[b]
0.00	0.00
1.0	0.091
5.0	0.467
10.0	1.19
25.0	2.90
50.0	6.16
100	12.45
250	32.83
500	63.65

[a] Least-mean-squares analysis: slope = 2.51, *y*-intercept = 0.03; correlation coefficient = 0.9996. [b] Least-mean-squares analysis: slope = 0.128; *y*-intercept = −0.103; correlation coefficient = 0.99986.

Table III—Norfloxacin Calibration Curve Replicate Analysis for the Plasma Assay

Plasma Concentration, μg/mL	*CV* (*n* = 6)
0.050	2.6%
0.10	6.5%
0.25	1.3%
0.50	4.3%
1.00	4.6%
2.50	2.2%

eluting ~2 min before norfloxacin. Similarly, $M4_1$ decomposed ($t_{1/2}$ = 23 h) in the dilute acid. In this case, however, no fluorescing products were observed for up to 27 h at ambient temperature. When dissolved in 0.085% phosphoric acid, $M4_2$ formed two fluorescing products. Both species were detected within 30 min of sample preparation. Their concentrations increased with time and reached maximum levels at 24–27 h. While one of these products (A) coeluted with $M4_2$, the retention time of the second degradation product (B) was coincident with that of norfloxacin. Since the amount of norfloxacin excreted in urine was 50 times that of $M4_2$ (3) and ~20% of $M4_2$ was converted to product B after 24 h in dilute acid at room temperature, the maximum norfloxacin quantitation error resulting from the appearance of this degradation product was only 0.4%. The $M4_2$ metabolite, as with the other metabolites and norfloxacin, attained a maximum excretion rate within 2.5–5 h. Later urinary excretion rates of the metabolites showed similar time-dependent variations to norfloxacin (3). Finally, when $M4_2$ was dissolved in 0.3 M NaOH, product B was again formed, along with two new products (C and D). No peak coeluting with $M4_2$ was detected at any time. As in acid, the concentration of product B increased with time, while the concentrations of C and D decreased during the same period. The identities of these degradation products were not pursued.



IX

The urine of subjects receiving nalidixic acid (IX) has been shown to contain conjugate derivatives of IX which were readily cleaved by hydrolysis (5). Because of the structural similarities between norfloxacin and nalidixic acid, the presence of analogous conjugates in the urine of norfloxacin-dosed individuals was assessed using conditions comparable with those described for the hydrolysis of nalidixic acid conjugates. It was found that even after 30 min of acid or base hydrolysis at 60°C, the apparent concentration of norfloxacin was unchanged. This suggests that norfloxacin conjugates are not formed in clinical urine specimens.

A structurally similar compound, pipemidic acid (II), was used as the internal standard, since it was found to exhibit favorable UV-absorbing, fluorescence extraction, and chromatographic properties. Using peak area ratios (norfloxacin–internal standard), the detector response was found to be linear in the 0.05–2.50-μg/mL range for plasma (fluorescence detection) and in the 1.0–500-μg/mL range of urine (UV absorbance). The mean recoveries of norfloxacin were 85.6 ± 16.7% in the plasma assay (double extraction), and 42.4 ± 6.0% in the urine assay (single extraction). The mean recoveries of the internal standard were 42.4 ± 6.0% for plasma and 21.7 ± 1.8% for urine. The recovery values are based on unextracted

Table IV—Norfloxacin Calibration Curve Replicate Analysis for the Urine Assay

Urine Concentration, μg/mL	*CV* (*n* = 6)
1.0	11.8
5.0	9.7
10.0	2.0
25.0	2.0
50.0	2.0
100.0	1.5
250.0	2.7
500.0	3.9

Table V—Norfloxacin in Plasma and Urine Following Single Oral Doses to an Individual

Dose, mg	Plasma Concentration, μg/mL Collection Time, h 0	0.5	1.0	1.5	2.0	3.0	4.0	6.0	8.0	12.0
200	0	0.63	1.22	0.81	0.60	0.28	0.47	0.33	0.21	0.14
400	0	0.51	1.42	1.21	0.91	0.74	0.52	0.42	0.28	0.22
800	0	2.91	3.22	2.43	2.20	1.88	1.31	0.83	0.73	0.41
1200	0	3.48	5.69	4.77	5.10	1.60	2.20	1.49	1.19	0.85
1600	0	0.73	2.73	4.61	5.15	4.61	3.54	2.26	1.63	1.13

Dose, mg	Urine Concentration[a] μg/mL Collection Interval, h 0–1	1–2	2–3	3–4	4–6	6–8	8–12	12–24	24–48
200	245	155	44	34	37	42	13	3	16
	(17.9)	(16.1)	(11.0)	(2.1)	(7.4)	(8.8)	(5.7)	(2.0)	(12.4)
400	271	69	41	129	102	109	32	6	4
	(14.4)	(35.9)	(16.8)	(9.8)	(11.6)	(10.0)	(14.4)	(1.1)	(3.0)
800	339	95	111	120	208	243	150	40	5
	(35.6)	(58.9)	(24.0)	(30.6)	(37.0)	(21.6)	(21.5)	(31.6)	(5.5)
1200	1125	1902	653	697	474	76	163	41	18
	(37.1)	(144.6)	(13.7)	(82.9)	(56.4)	(44.8)	(32.8)	(20.1)	(22.5)
1600	432	1414	505	161	524	583	134	70	10
	(34.6)	(60.8)	(72.2)	(67.6)	(74.4)	(43.1)	(56.3)	(39.9)	(30.4)

[a] Values in parentheses indicate total milligrams excreted.

standards. Representative standard curve data are shown in Table II. Reproducibility of this method was validated by the construction of calibration curves over the appropriate concentration ranges for plasma and urine analysis. The coefficients of variation so obtained are summarized in Tables III and IV. The day-to-day reproducibility of this method was demonstrated by assaying replicate aliquots of a spiked plasma sample. Thus, the interassay coefficient of variation was 8.7% (n = 8) for a 0.50-μg/mL plasma standard.

The assay method described in this report was used to quantitate norfloxacin levels in plasma and urine specimens from human subjects participating in an increasing-dose study of the drug. Representative data obtained for one of the volunteers is presented in Table V.

REFERENCES

(1) Y. Kumasaka, H. Nakahata, K. Imamura, and K. Takebe, *Chemotherapy*, **29(S-4)**, 56 (1981).

(2) K. Hirai, A. Ito, Y. Abe, S. Suzue, T. Irikura, M. Inoue, and S. Mitsuhashi, *Antimicrob. Agents Chemother.*, **19**, 188 (1981).

(3) T. Ozaki, H. Uchida, and T. Irikura, *Chemotherapy*, **29(S-4)**, 128 (1981).

(4) Y. Oomori, S. Murayama, Y. Abe, and T. Irikura, *Chemotherapy*, **29(S-4)**, 91 (1981).

(5) E. W. McChesney, E. J. Froelich, G. Y. Lesher, A. V. R. Crain, and D. Rosi, *Toxicol. Appl. Pharmacol.*, **6**, 292 (1964).

Application of Diffusion Theory to the Relationship Between Partition Coefficient and Biological Response

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Abstract □ A diffusion model for transport through multilaminates is studied as a possible way to predict the optimal biological response of a set of congeners with respect to the oil–water partition coefficient, P. The model predicts the bilinear form typical of biological response data and, unlike the earlier kinetic model, also relates the results to physical processes, predicts the structure with the optimal response in terms of diffusion constants, and shows such an optimum prior to steady state for an infinite dose. Diffusion through an oil–water multilaminate causes extraordinary separation of permeating species based on partition coefficient and diffusion constant for times shorter than the lag time.

Keyphrases □ Multilaminates—oil–water, diffusion model, optimal biological response with respect to partition coefficient □ Partition coefficient—oil–water multilaminates, diffusion model, optimal biological response □ Optimal biological response—diffusion model, oil–water multilaminates, partition coefficient

In an earlier paper (1), we reviewed the kinetic (2) and equilibrium (3) models for predicting the optimal biological response of a set of congeners with respect to the partition coefficient, P. The kinetic models (1, 2) which treat this problem in terms of rate constants, can be a useful approximation for steady-state transport across a series of unstirred layers of the lipid–water interfaces. However, this kinetic model predicted a large degree of asymmetry (1) in the biological response curve with respect to P, and this asymmetry in transport is not satisfying from a fundamental physical viewpoint. Furthermore, the kinetic treatment neglects differences in diffusion between the lipid and aqueous phases. To understand the role of diffusion in transport across biological tissue of alternating physical properties, it is useful to apply classical diffusion theory to oil–water multilaminates.

Like the kinetic and equilibrium models, the diffusion model developed in this paper predicts a peak in the transport with respect to the partition coefficient for a multilaminated membrane. For a plot of the logarithm of

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