Reversed-Phase High-Pressure Liquid Chromatographic Determination of Serum Methotrexate and 7-Hydroxymethotrexate

ROBERT G. BUICE [x] **and PARAMJEET SIDHU**

Received February 2, 1981, from the *College of Pharmacy, University of Tennessee Center for the Health Sciences, Memphis, TN 38163.* Accepted for publication May 29, 1981.

Abstract □ A reversed-phase high-pressure liquid chromatographic method for determining methotrexate and 7-hydroxymethotrexate in human serum is presented. A mobile phase of acetate buffer (0.2 *M*, pH 5.5 with 0.03 *M* ethylenediaminetetraacetate), methanol, and acetonitrile (85.3:8.4:6.3), passed through a μBondapak C_{18} column at 1.5 ml/min produced excellent resolution of sharp, symmetrical bands. An improved extraction process, using a sample preparation cartridge, resulted in analytical recoveries in excess of 90% for methotrexate and 70% for 7-hydroxymethotrexate, permitting the determination of serum concentrations of 2.20 and 2.13 × 10^{-7} *M*, respectively, using only 200 μl of serum. UV detection at 313 nm provided adequate sensitivity for each component. While the reproducibility for 7-hydroxymethotrexate was approximately equal to previous methods, that for methotrexate was greatly improved. Serum methotrexate data at selected time points following high dose methotrexate therapy are presented.

Keyphrases □ Methotrexate—reversed-phase high-pressure liquid chromatographic determination □ 7-Hydroxymethotrexate—reversed-phase high-pressure liquid chromatographic determination □ High-pressure liquid chromatography—determination of methotrexate and 7-hydroxymethotrexate.

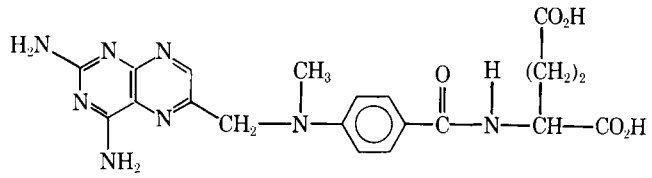
The effectiveness of high dose methotrexate therapy followed by leucovorin rescue was greatly enhanced by the observation that patients at high risk of serious toxicity might be detected by monitoring serum methotrexate (I) concentrations (1). Numerous analytical methods of I have been reported, including a spectrophotofluorometric method (2), several radioimmunoassays (3–7), a competitive protein binding method (8), an enzyme inhibition method (9), and a ligand-binding procedure (10). While recent work (11, 12) established the specificity of several such methods within certain concentration limits, potential cross-reactivity with I metabolites still raises doubt about these and other similar assay methods.

BACKGROUND

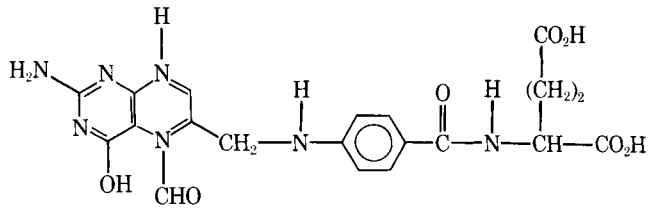
The major potentially-interfering metabolite of I, 7-hydroxymethotrexate (IV), has been reported in high concentrations in patient urine (13) and serum (14). Moreover, since the relatively low aqueous solubility of IV has caused some concern that this metabolite might be responsible for nephrotoxicity (13), it also should be monitored. More recently (11), in high dose methotrexate patients, a minor metabolite, 2,4-diamino-10-methylpteroic acid (III), was detected in later (48 hr) serum samples in concentrations that might impede quantitation of low I concentrations.

This potential cross-reactivity from metabolites, coupled with the possible need to quantitate certain metabolites, initiated the development of several high-pressure liquid chromatography (HPLC) methods (13–20). While these methods provide reasonable sensitivities, all require relatively large biological fluid samples (1–3 ml) and only one (19) reports an acceptable reproducibility for I (±3% at 10^{-5} *M*).

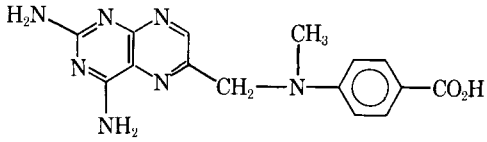
Moreover, most of these methods employ lengthy extractions with analytical recoveries varying from 40% (18) to 70% (15). Donehower *et al.* (11) used preparative HPLC and reported a 90% recovery of I, although a large serum sample (3 ml) was required. Lankelma and Poppe (17) used on-column concentration and reported a 70.2% analytical recovery of I, although again, a large serum sample (3 ml) was required.



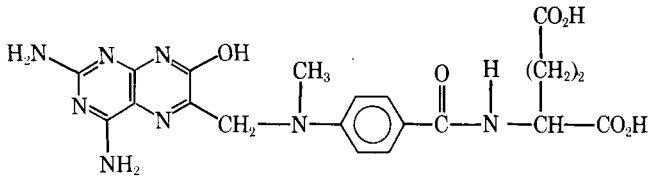
I



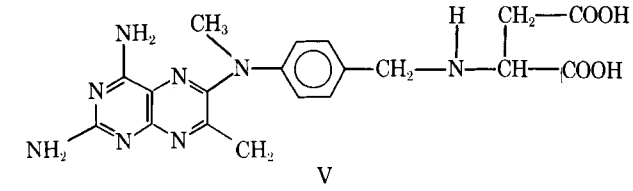
II



III



IV



V

Another study (15), included no internal standard, but reported a day-to-day variation as high as 13.4%. Finally, other reported HPLC chromatograms (14, 16, 20) show poor resolution of I and IV. The present report describes an improved and relatively simple HPLC method for the quantitation of I and IV in the serum of patients receiving methotrexate therapy.

EXPERIMENTAL

Materials and Methods—Methanol and acetonitrile, used for the mobile phase, were HPLC grade solvents. Acetate buffer for the mobile phase was prepared using deionized water and reagent grade sodium acetate. All other chemicals were reagent grade. Analytical samples of I[1], leucovorin calcium[1] (II), III[1], IV[2], and the internal standard, *N*-[4-

[1] Lederle Laboratories, Pearl River, N.Y.
[2] Dr. David Johns, National Cancer Institute, Bethesda, Md.

0022-3549/82/0100-0074$01.00/0

Table I—HPLC Conditions and Correlation Coefficients for each Calibration Curve

Curve	Sample Time, hr	Concentration Range[a], μg/ml	Internal Standard[b], μg/ml	Reconstitution Volume / Injection Volume, μl
a	0 (I)	10–100	100.0	200/80
b	0 (IV)	1–10	5.0	200/40
c	12	1–10	5.0	200/100
d	24	0.1–1	0.5	100/80

Curve	Sample Time, hr	Detector Sensitivity	Correlation Coefficients ± *SEM* I	Correlation Coefficients ± *SEM* IV
a	0 (I)	0.500	0.99993 $n = 1$	
b	0 (IV)	0.010		0.98842 $n = 1$
c	12	0.050	0.99372 ± 0.00562 $n = 3$	0.98794 ± 0.00173 $n = 3$
d	24	0.005	0.99955 ± 0.00032 $n = 3$	0.99392 ± 0.00391 $n = 3$

[a] Molar concentration of I = (μg/ml) × (2.2×10^{-6}); molar concentration of IV = (μg/ml) × (2.13×10^{-6}). [b] Prior to Sep Pak treatment, 200 μl of this solution was added to each serum sample.

[(2,4-diamino-6-quinazolinyl)methylamino]benzoyl]aspartic acid[3] (V), were used as received.

Blood samples were obtained from patients at selected time points (0, 12, and 24 hr) following completion of 6-hr high dose methotrexate infusions (3500–5000 mg/m^2). Although it was not possible to obtain a sample from every patient at each preselected time point, a total of five samples were obtained at postinfusion time (0 hr), 11 at 12 hr, and six at 24 hr. Samples were centrifuged and the serum was stored at −5° until required for assay.

Serum concentrations of I and IV were determined using an HPLC system equipped with a 30-cm × 4-mm i.d. reversed-phase column[4]. Separation was followed by UV absorbance detection at 313 nm. A mobile phase consisting of buffer A (0.2 *M* acetate buffer, pH 5.5 with 0.03 *M* ethylenediaminetetraacetate), methanol, and acetonitrile (85.3:8.4:6.3) was passed through the column at a flow rate of 1.5 ml/min. Prior to analysis, the serum samples were processed by a reversed-phase sample preparation cartridge[5].

To each 200-μl serum sample was added 200 μl of internal standard (Table I), 400 μl of tromethamine (0.01 *M*, pH 9), and 1 ml of water. This mixture was vortexed 60 sec and passed through a sample preparation cartridge which had been pretreated with 10 ml of methanol and 10 ml of buffer B (0.2 *M* acetate buffer, pH 5.5). The cartridge was then treated with an additional 5 ml of buffer B, 1 ml of 0.1 *N* NaOH, and another 1 ml of buffer B. The cartridge was vacuum dried and the compounds of interest were eluted with 2 ml of methanol. The methanol was evaporated to dryness under nitrogen and the residue reconstituted in the mobile phase for injection. Analytical recoveries exceeded 90% for I and 70% for IV in the 10^{-7} *M* concentration range.

Four separate calibration curves were required to cover a concentration range of 2.20×10^{-7}–2.20×10^{-4} *M*. Conditions needed for each curve are presented in Table I. The large differences in I and IV concentrations in 0-hr samples required that each compound be determined from separate 200-μl portions of serum (calibration curves a and b). All other I and IV concentrations (12 and 24 hr) were determined simultaneously on single 200-μl serum samples. All samples were assayed in duplicate. Zero and 12-hr samples were quantitated using ratios of peak areas determined by a calculating integrator[6]. Ratios of peak heights were used to quantitate each compound in the 24-hr samples. Fresh calibration curves for I and IV were prepared daily in serum. Correlation coefficients of these curves are presented in Table I.

RESULTS

While most previous methods employed either methanol or acetonitrile as the organic portion of the mobile phase (11, 14, 15, 20), the present system attained maximum resolution of I, IV, and internal standard by combining these solvents. A flow rate of 1.5 ml/min resulted in a symmetrical band for each compound. Faster flow rates produced slightly sharper bands but reduced resolution. Representative HPLC chromatograms resulting from the quantitation of I and IV in 12- and 24-hr patient samples are presented in Fig. 1. Data from all sample determinations, together with percent deviations of duplicates, are presented in Table II.

Although metabolite III was previously reported in later (48 hr) samples, none was detected in any samples of the present study. Moreover, injections of analytical samples clearly show that III, due to its long retention time on this system (28.1 min), cannot interfere with the present assay. Ordinarily, no interference from II would be anticipated since rescue therapy is normally initiated 24-hr postinfusion (1). Furthermore, the retention of II on this system (4.3 min) was much shorter than either of the compounds of interest.

As illustrated in Fig. 1, no interfering bands appeared in the serum blanks of 12-hr samples (calibration curve c). As anticipated, quantitation of samples containing higher concentrations of I and IV (calibration

Table II—Data Resulting from Duplicate Determinations of Each Serum Sample and Percent Difference

I Duplicate Determinations A	I Duplicate Determinations B	Percent Difference	IV Duplicate Determinations A	IV Duplicate Determinations B	Percent Difference
0 hr Molar Concentration × 10^4					
3.04	3.00	1	0.22	0.25	12
2.89	2.97	3	0.32	0.35	9
2.38	2.34	2	0.46	0.61	25
4.69	4.72	1	0.28	0.29	3
3.26	3.28	1	0.31	0.34	9
12 hr Molar Concentration × 10^6					
7.97	8.10	2	13.7	16.6	17
3.78	3.78	0	4.8	4.5	6
6.93	6.86	1	15.8	18.7	16
5.35	5.35	0	6.8	6.1	10
2.02	2.22	9	3.9	4.2	7
2.42	2.51	4	11.0	8.5	23
2.49	2.49	0	7.1	7.2	1
2.22	2.49	11	8.0	7.4	8
7.74	8.10	4	21.3	20.7	3
3.21	3.59	11	7.5	12.7	41
8.10	8.18	1	9.2	9.4	2
24 hr Molar Concentration × 10^7					
7.04	7.39	5	41.5	43.7	5
9.68	9.46	2	40.5	30.5	25
1.09	1.08	1	30.2	21.7	28
4.62	4.18	10	24.3	23.0	5
9.68	9.90	2	31.1	26.2	16
2.64	2.64	0	20.9	19.0	9

[3] Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md.

[4] Model U6K injector, Model 6000A solvent delivery system, μBondapak C_{18} column with guard column, and model 440 UV absorbance detector, Waters Associates, Milford, Mass.

[5] C_{18} Sep Pak cartridge, Waters Associates, Milford, Mass.

[6] Hewlett-Packard 3352B laboratory data system.

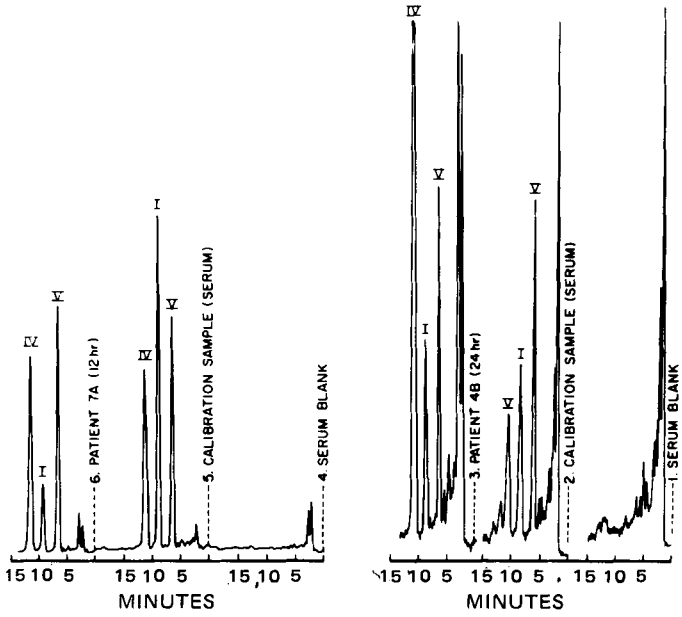


Figure 1—*Representative HPLC chromatograms of curve d serum blank (1), curve d calibration sample (2), 24-hr patient serum sample containing I (9.68 × 10^{-7} M) and IV (5.26 × 10^{-6} M) (3), curve c serum blank (4), curve c calibration sample (5), and 12-hr patient serum sample containing I (2.42 × 10^{-6} M) and IV (1.75 × 10^{-5} M) (6).*

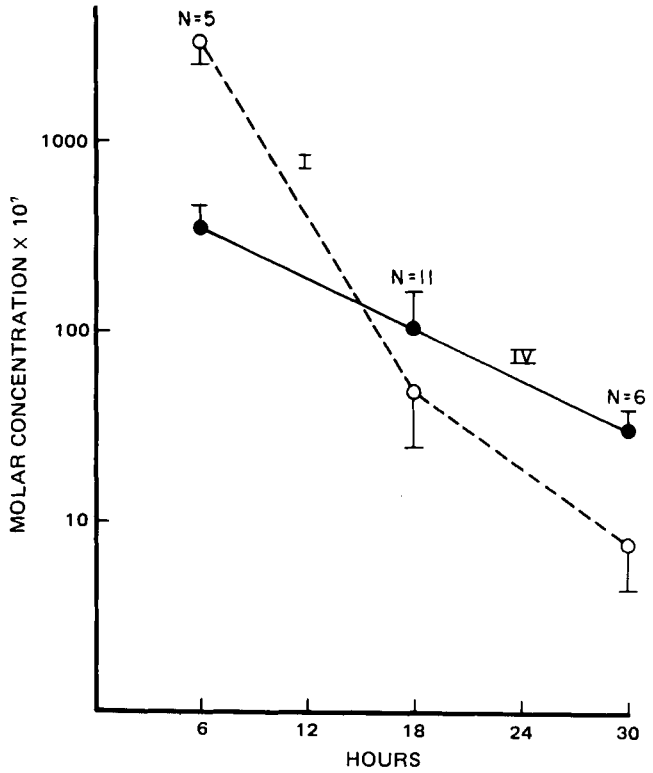


Figure 2—*Serum I (○) and IV (●) concentrations (mean ±SD) at selected time points following initiation of 6-hr high dose infusions (3500–5000 mg/m²).*

curves a and b) involved no interference from serum. Several low concentration (10^{-7}–10^{-6} *M*) samples, while showing no serum interference with I, did reveal a minute serum band slightly overlapping IV. Since the overlap was extremely slight, contribution to the peak height of IV was considered insignificant (Fig. 1).

Attempts to quantitate 24-hr serum samples using ratios of peak areas consistently resulted in poor reproducibility for I and IV. This appears to be characteristic of this calculating integrator in the absence of a completely horizontal baseline. Therefore, all 24-hr samples were quantitated using ratios of peak heights. Patient serum concentration data (mean ±*SD*) determined by this method are presented in Fig. 2.

The reproducibility of the method and the durability of the sample preparation cartridge were simultaneously evaluated by conducting multiple determinations on a single, spiked serum sample (10^{-6} *M* I) using the same cartridge. In duplicate studies, the first six consecutive determinations consistently produced a coefficient of variation which was less than 3%, although over the six repetitions all peak heights were reduced ~10%, suggesting a slightly declining cartridge efficiency. However, in the present study all patient samples were assayed in duplicate with each repetition using a new cartridge. No cartridge was reused.

DISCUSSION

The described method separates all three compounds of interest and produces sharp, symmetrical bands. UV detection at 313 nm, instead of at 254 nm, results in a twofold increase in assay sensitivity to IV, while sensitivity to I is approximately equal at either wavelength. This is consistent with previous reports (14, 20).

The high extraction efficiency resulting from this sample preparation procedure permitted the quantitation of serum I and IV concentrations as low as 2.20 and 2.13 × 10^{-7} *M*, respectively, using only 200 μl of serum. Previously reported methods require 1 ml of serum or more. These data suggest that the present method is readily adaptable to small animal pharmacokinetic studies. Moreover, the small volume of serum required makes the method highly useful in patient pharmacokinetic studies that require large numbers of data points, often from patients who are weakened by disease.

Data presented in Table II show excellent reproducibility for I, although that for IV shows no improvement over previously reported work. This is further evidenced by the calibration curve correlation coefficients given in Table I. The small contaminant which slightly overlapped the IV band in a few 10^{-7} *M* patient samples did not appear to influence reproducibility, suggesting that reproducibility differences in I and IV result from the extraction process.

This procedure results in only minimal column stress since ~800 injections of serum extracts may be made before the column begins to degenerate. Since a sample preparation cartridge can be used up to four times with no significant difference in assay results, the expense of this extraction is relatively small.

REFERENCES

(1) W. E. Evans, C. D. Pratt, R. H. Taylor, L. F. Barker, and W. R. Crom, *Cancer Chemother. Pharmacol.*, **3,** 161 (1979).

(2) J. M. Kinkade, Jr., W. R. Vogler, and P. G. Dayton, *Biochem. Med.*, **10,** 337 (1974).

(3) C. Bohuon, F. Dubrey, and C. Boudene, *Clin. Chim. Acta* **57,** 263 (1974).

(4) J. Hendel, L. J. Sarek, and E. F. Hvidberg, *Clin. Chem.*, **22,** 813 (1976).

(5) L. Levine and E. Powers, *Res. Commun. Chem. Pathol. Pharmacol.*, **9,** 543 (1974).

(6) V. Raso, *Cancer Treat. Rep.*, **61,** 585 (1977).

(7) V. Raso and R. Schreiber, *Cancer Res.*, **35,** 1407 (1975).

(8) C. E. Myers, M. E. Lippman, H. M. Eliot, and B. A. Chabner, *Proc. Natl. Acad. Sci. USA*, **72,** 3683 (1975).

(9) L. C. Falk, D. R. Clark, S. M. Kalman, and T. F. Long, *Clin. Chem.*, **22,** 785 (1976).

(10) S. P. Rothenberg, M. da Costa, and M. P. Igbal, *Cancer Treat. Rep.*, **61,** 575 (1977).

(11) R. C. Donehower, K. R. Hande, J. C. Drake, and B. A. Chabner, *Clin. Pharmacol. Ther.*, **26,** 63 (1979).

(12) R. G. Buice, W. E. Evans, J. Karas, C. A. Nicholas, P. Sidhu, A. B. Straughn, M. C. Meyer, and W. R. Crom, *Clin. Chem.*, **26,** 1902 (1980).

(13) J. L. Wisnicki, W. P. Tong, and D. B. Ludlum, *Cancer Treat. Rep.*, **62,** 529 (1978).

(14) J. L. Cohen, G. H. Hisayasu, A. R. Barrientos, M. S. B. Nayer, and K. K. Chan, *J. Chromatogr.*, **181,** 478 (1980).

(15) C. Canfell and W. Sadee, *Cancer Treat. Rep.*, **64,** 165 (1980).

(16) S. K. Howell, Y. M. Wang, R. Hosoya, and W. W. Sutow, *Clin.*

Chem., **26**, 734 (1980).
(17) J. Lankelma and H. Poppe, *J. Chromatogr.*, **149**, 587 (1978).
(18) J. A. Nelson, B. A. Harris, W. J. Decker, and D. Farquhar, *Cancer Res.*, **37**, 3970 (1977).
(19) E. Watson, J. L. Cohen, and K. K. Chan, *Cancer Treat. Rep.*, **62**, 381 (1978).
(20) Y. M. Wang, S. K. Howell, and J. A. Benvenuto, *J. Liq. Chromatogr.*, **3**, 1071 (1980).

ACKNOWLEDGMENTS

Supported by NCI Grant CA-16518-06.

Theoretical and Experimental Studies of Transport of Micelle-Solubilized Solutes

GREGORY E. AMIDON *x, WILLIAM I. HIGUCHI, and NORMAN F. H. HO

Received February 9, 1981, from the *University of Michigan, Ann Arbor, MI 48109.* Accepted for publication April 27, 1981.
*Present address: Pharmacy Research, The Upjohn Company, Kalamazoo, MI 49001.

Abstract □ A physical model describing the simultaneous diffusion of free solute and micelle-solubilized solute across the aqueous boundary layer, coupled with partitioning and diffusion of free solute through a lipoidal membrane, is derived. *In vitro* experiments utilizing progesterone and polysorbate 80 showed excellent agreement between theoretical predictions based on independently determined parameters and experimental results. The physical model predicts that micelles can assist the transport of solubilized solute across the aqueous diffusion layer, resulting in a higher solute concentration at the membrane surface than would be predicted if micelle diffusion is neglected. At high surfactant concentrations, the aqueous diffusion layer resistance can be eliminated and the activity of the solute at the membrane can approach the bulk solute activity. This mechanism could explain observed enhanced absorption rates *in vivo* when both micelle solubilization occurs and the aqueous diffusion layer is an important transport barrier. The importance of determining and defining the thermodynamic activity of the diffusing solute is emphasized.

Keyphrase □ Diffusion—transport of micelle-solubilized solutes, theoretical and experimental □ Solutes—micelle solubilized, transport, theoretical and experimental □ Micelles—theoretical and experimental transport, solutes

The effects of micelle solubilization on the solubility and intestinal absorption of nonpolar solutes are well documented (1–8). Investigations have been performed to delineate the role of surfactants in diffusional transport. As a result of these studies, it is clear that several factors must be considered, such as the thermodynamic activity of the solute, diffusivities of the free solute and micelles, membrane permeability, and the importance of the aqueous diffusion layer in determining the overall transport rate.

BACKGROUND

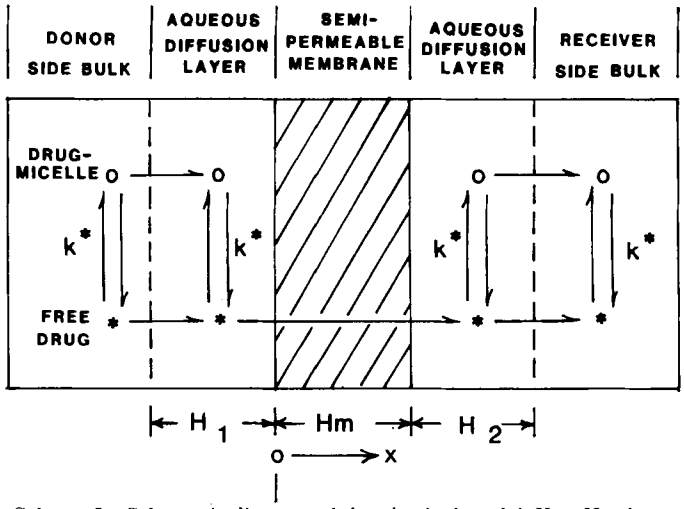
In a diffusional process (*e.g.*, intestinal absorption), a difference in the thermodynamic activity determines the direction of and driving force for the net transport of mass. Therefore, when micelle solubilization occurs and the thermodynamic activity of solute is lowered, a decreased diffusional rate is expected. On this basis, decreased absorption rates of salicylic acid in the presence of polysorbate 60 from rat intestinal segments were explained (1).

However there are numerous examples of increased absorption rates *in vivo* when solubilizing agents are present (2, 3). For example, the serum blood levels of indoxole when administered in a polysorbate 80 solution to humans were three to four times higher than comparable doses with an aqueous suspension or hard capsule (4). It was shown (5, 6), however, that membrane permeabilities may change in the presence of surfactant, possibly resulting in a net increase in the absorption rate even with a decreased thermodynamic activity of solute in bulk aqueous solution.

Another reason for enhanced absorption in the presence of solubilizing agents is associated with the diffusion of solute in the aqueous phase (*i.e.*, within the aqueous diffusion layer). Westergaard and Dietschy (7) pointed out that, in addition to the importance of bulk solute concentration and membrane permeability in determining absorption rates, the aqueous boundary layer is an important barrier to transport *in vivo* and should be evaluated. They concluded that the apparent functions of the micelle were to overcome the diffusion layer resistance *in vivo* and to deliver a maximum solute concentration to the membrane surface. It was also suggested (8) that the diffusion coefficient of the free solute–micelle complex is important in quantitatively assessing absorption in the presence of micelles.

Thus, it is clear that an evaluation of the aqueous diffusion layer and the physicochemical events occurring within it is necessary in the development of a realistic physical model. Furthermore, micellar diffusion, membrane permeation, and the thermodynamic activity of the solute in the surfactant solution need to be included in any complete analysis. This report presents a comprehensive physical model incorporating all of the principles just discussed. *In vitro* experiments along with independent determinations of all physicochemical parameters defined in the physical model were carried out utilizing progesterone and the nonionic surfactant polysorbate 80. Therefore, theoretical predictions based on independent estimates of the important parameters and experimentally determined fluxes can be compared.

The physical model is defined for two hydrodynamic conditions: the



Scheme I—Schematic diagram of the physical model. Key: H_1, *donor side aqueous diffusion layer thickness;* H_m, *semipermeable membrane thickness;* H_2, *receiver side diffusion layer thickness; and* k*, *micelle-free solute equilibrium distribution coefficient.*

[1] Dow Corning, Midland, Mich.

0022-3549/82/0100-0077$01.00/0