Improved Microdetermination for Diphenylhydantoin in Blood by UV Spectrophotometry

YUKIYA SAITOH*, KAZUYO NISHIHARA*, FUJIO NAKAGAWA*, and TOKUJI SUZUKI†▲

Abstract □ A modification of the UV spectrophotometric method proposed by Wallace for the determination of diphenylhydantoin in blood samples is described. Improvement of the Wallace method was made possible by changing various reaction conditions, including oxidation time, and by determining the diphenylhydantoin oxidation product using an appropriately expanded scale of a recording spectrophotometer. The improved method is simple, reproducible, and capable of screening large numbers of samples in a short time, since the tedious procedure of collecting the oxidation product by continuous extraction (as in the original method) is not required to oxidize diphenylhydantoin. The improved method can be used to determine blood and plasma levels after a single injection of 50 mg. of sodium diphenylhydantoin in a human subject. Plasma levels of diphenylhydantoin and phenobarbital were determined after a simultaneous administration of 5 mg./kg. of these compounds in rabbits using a combination of the present method with the Williams method for analysis of barbiturates.

Keyphrases □ Diphenylhydantoin in blood—UV spectrophotometric analysis, in presence of phenobarbital □ Blood levels, diphenylhydantoin—UV spectrophotometric analysis, in presence of phenobarbital □ UV spectrophotometry—analysis, diphenylhydantoin in blood

In the clinical management of patients during long-term treatment with diphenylhydantoin, it is desirable to check frequently its blood levels so that the adequacy of dosage and the possibility of toxicity may be appraised. For such purposes, a rapid, simple, and accurate method for determining diphenylhydantoin in small amounts of blood is necessary. It has been found that the frequent therapeutic use of diphenylhydantoin and phenobarbital in combination presents a considerable problem in the analysis of the two drugs, since they interfere with each other in most colorimetric (1–3) and spectrophotometric methods (4–8). The presence of interfering substances, particularly phenobarbital, in biological specimens requires preliminary tedious separation by extraction procedures (1, 4–6) or TLC (2, 3, 7, 8). Recently, GLC methods for the analysis of diphenylhydantoin became available (9–17), but they are time consuming and technically difficult for routine clinical situations.

The UV spectrophotometric assay described by Wallace and his coworkers (18–21) is based on permanganate oxidation of diphenylhydantoin to benzophenone, which is subsequently extracted into *n*-heptane. The reaction product, benzophenone, which has a well-defined UV absorption spectrum and high molar absorptivity, is determined spectrophotometrically at 247 nm. in *n*-heptane. This method does not require the preliminary separation of diphenylhydantoin from other drugs. However, the procedure requires a relatively large volume (5–10 ml.) of whole blood or plasma for a single determination. Morselli (22) and Bock and Sherwin (23) employed improved equipment and a modified extraction method to reduce the blood samples to 1–2 ml. Lee and Bass (24) described a micromodification of the Wallace method, in which a micro-Kjeldahl steam distillation apparatus and microcells were used. However, variations in the blood sample blanks in the modified methods as well as in the original Wallace method contribute to the inaccuracy in determining diphenylhydantoin at relatively low concentrations.

The present study describes a modification that improves the diphenylhydantoin assay reported by Wallace. The improvement was made possible by changing various reaction conditions, including oxidation time, in the oxidation of diphenylhydantoin with alkaline permanganate and by determining the oxidation product on an appropriately expanded scale of a recording spectrophotometer using microcells. The conversion of diphenylhydantoin to benzophenone was found to be quantitative, and the blank value was negligibly small. This modified determination, employing only 0.1–0.4 ml. of whole blood or plasma, is simple, reproducible, and capable of determining large numbers of diphenylhydantoin samples in a short time, since the refluxing of the reaction mixture with stirring has been eliminated.

EXPERIMENTAL

Reagents and Apparatus—A phosphate buffer solution at pH 6.8 was prepared by mixing equal volumes of 1 *M* Na_2HPO_4 and 1 *M* KH_2PO_4. All chemicals used in this study were reagent grade except *n*-heptane which was spectrophotometric grade. A double-beam recording spectrophotometer[1] was used for UV absorption measurements. Absorbances were recorded on an appropriately expanded scale (×5 or ×10) using standard cells (10 mm. in path length and 5-ml. capacity) or microcells (10 mm. in path length and 0.8-ml. capacity).

Examination of Blank Absorbance—In the procedure described by Wallace (20), 20 ml. of 1% $KMnO_4$ in 7 *N* NaOH and 5 ml. of *n*-heptane are added to diphenylhydantoin extracted from biological specimens, and the mixture is then refluxed for 30 min. with constant magnetic stirring. After cooling, the *n*-heptane layer is separated from the aqueous permanganate layer and its absorbance is determined at 247 nm. against pure *n*-heptane. The blank values (less than 0.04 in absorbance) from 10 ml. of oxalated whole blood containing no diphenylhydantoin were shown to be less than 0.5 mcg./ml. diphenylhydantoin (20). Reexaminations of the blank values from 5 ml. of plasma and water showed them to be about 0.020 and 0.010 in absorbance, respectively.

However, small deviations in the blank absorbance may be responsible for inaccuracy in estimating diphenylhydantoin using an expanded scale of the recording spectrophotometer. Therefore, the reaction conditions in the oxidation of diphenylhydantoin with permanganate were examined to minimize the blank value. Ten milliliters of the alkaline permanganate solution was added to 5 ml. of *n*-heptane and to 5 ml. of 3.97×10^{-6} *M* benzophenone[2] in *n*-heptane in separate centrifuge tubes. The mixtures were refluxed for 30 min. with constant magnetic stirring. After cooling and cen-

[1] Hitachi model 124.

[2] If converted quantitatively, 5 mcg. of diphenylhydantoin corresponds to the amount of benzophenone in 5 ml. of this solution.

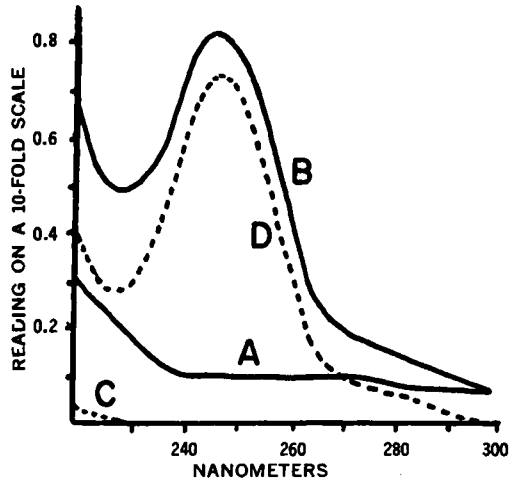

Figure 1—*UV absorption spectra of* n-*heptane and* n-*heptane containing benzophenone with and without the permanganate oxidation procedure. Solid lines A and B are UV absorption spectra of* n-*heptane and* n-*heptane containing benzophenone after the permanganate oxidation, respectively. Dotted lines C and D are UV absorption spectra of* n-*heptane and* n-*heptane containing benzophenone shaken with the alkaline permanganate solution, respectively. The benzophenone concentration in* n-*heptane is 3.97 × 10⁻⁶* M.

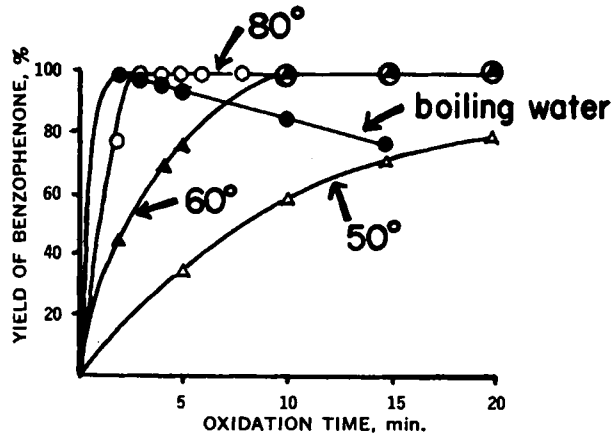

Figure 2—*Relationship between oxidation time and benzophenone yield. Curves show benzophenone yields against time in a constant-temperature bath (50, 60, and 80°) and in boiling water. Curves at 70 and 90° are omitted from the figure, since they almost coincide with the curve at 80°, with the exception of a gradual decrease in the yield at 90° after 10 min.*

trifuging, the *n*-heptane layers were separated from the aqueous permanganate layers, and the absorbance values were recorded on a 10-fold scale of the recording spectrophotometer from 220 to 300 nm. against pure *n*-heptane. The UV absorption spectra of *n*-heptane and *n*-heptane containing benzophenone after the permanganate oxidation are shown in Fig. 1. The spectrum of the *n*-heptane containing no benzophenone exhibits a considerably high absorption at 247 nm. On the other hand, no absorption of the *n*-heptane layer at 247 nm. was observed (Fig. 1) when a mixture of 5 ml. of the alkaline permanganate solution and *n*-heptane containing no benzophenone was shaken mechanically for 10 min. at room temperature. From these results it can be concluded that the blank reading from plasma containing no diphenylhydantoin was attributed to unknown reaction products derived from *n*-heptane during heating with the alkaline permanganate.

Effect of Reaction Conditions upon Yield of Benzophenone—The procedure of oxidation under refluxing and stirring in the Wallace (20) method makes it difficult to determine diphenylhydantoin in a number of samples during a short time. Ten milliliters of the alkaline permanganate solution was added to 1 ml. of 10 mcg./ml. diphenylhydantoin in 0.1 *N* NaOH to examine the effect of reaction conditions upon yields of benzophenone from diphenylhydantoin. The reaction mixtures in centrifuge tubes, closed with ground-glass stoppers, were immersed in a constant-temperature bath (50, 60, 70, 80, and 90°) and in boiling water. The centrifuge tubes were withdrawn at various time intervals, and 5 ml. of *n*-heptane was added to the reaction mixtures after cooling. After the mixtures were shaken for 10 min., the *n*-heptane layer was separated from the aqueous layer, and its absorbance was recorded on a fivefold scale from 220 to 300 nm. against pure *n*-heptane. Figure 2 shows the yield of benzophenone as a function of immersing time. At higher temperatures, the reading (fivefold absorbance) rapidly reached a value[3] of 0.750 based on the quantitative yield of benzophenone. The readings obtained at temperatures lower than 80° remained constant, while the readings obtained at 90° and in boiling water decreased gradually after 10 and 3 min., respectively. Therefore, the oxidation of diphenylhydantoin with alkaline permanganate should be performed at temperatures lower than 80°. The decrease in the readings was presumed to be caused by leakage of the benzophenone formed by oxidation through minute openings between the centrifuge tube and its ground-glass stopper. The results described here show that the refluxing for 30 min. with constant magnetic stirring proposed by Wallace (20) is unnecessary and that the benzophenone formed quantitatively by the permanganate oxidation at 80° for 5 min. is completely extracted by *n*-heptane.

Improved Analytical Method—*Procedure A*—In the absence of phenobarbital, the following procedure was used for determining diphenylhydantoin. Ten milliliters of water is added to 0.1 ml. of plasma or blood in a centrifuge tube, followed by 2 ml. of 1 *M* phosphate buffer solution (pH 6.8) and 30 ml. of chloroform. The mixture is shaken for 10 min. and centrifuged at 3000 r.p.m. for 5 min. After removal of the aqueous layer by aspirating, 27 ml. of the chloroform layer is transferred into another centrifuge tube containing 10 ml. of 1 *N* NaOH. This mixture is shaken for 10 min. and also centrifuged. Nine milliliters of the sodium hydroxide layer is then transferred into a third centrifuge tube, which is connected to a vacuum rotary evaporator assembly, and this tube is immersed in a water bath of 50–60°. The tube is rotated until the volume within it is reduced to approximately 0.5 ml. It is removed from the assembly, 10 ml. of 1% $KMnO_4$ in 7 *N* NaOH is added, and the tube is immersed in a water bath of 80° for 5 min. After cooling, 2 ml. of *n*-heptane is added to the solution, and the mixture is shaken for 10 min. and centrifuged for 5 min. The *n*-heptane layer is then separated from the aqueous permanganate layer and transferred into a microcell. The absorbance is recorded on a 10-fold scale of the recording spectrophotometer over the range of 220–270 nm. against pure *n*-heptane. The peak reading at 247 nm. is read on the recording paper[4].

Procedure B—Wallace (21) demonstrated that high levels of diphenylhydantoin do not affect the determination of phenobarbital by the Williams and Zak (25) method, nor do large amounts of phenobarbital influence the absorption curve of the diphenylhydantoin oxidation product. In the presence of phenobarbital, the following combination procedure with the Williams and Zak (25) method for analyzing barbiturates was used for determining diphenylhydantoin and phenobarbital.

Ten milliliters of water is added to 0.4 ml. of plasma or blood in a centrifuge tube, followed by 2 ml. of 1 *M* phosphate buffer solution (pH 6.8) and 30 ml. of chloroform. The mixture is shaken for 10 min. and centrifuged for 30 min. After removal of the aqueous layer, 27 ml. of the chloroform layer is transferred into another centrifuge tube containing 2 ml. of 0.45 *N* NaOH. This mixture is shaken for 10 min. and centrifuged for 5 min. An upper layer of 0.4 ml. of the sodium hydroxide solution is transferred into a microcell, and its absorbance is recorded on a 10-fold scale of the recording spectrophotometer over the 230–280-nm. range against 0.45 *N* NaOH. To the sodium hydroxide solutions in the microcells is added 0.1 ml. of 16% NH_4Cl, and again the absorbance is recorded over the 230–280-nm. range. The difference (ΔA) in absorbance at 260 nm. is used for

[3] Calculated from the amount of benzophenone corresponding to 10 mcg. of diphenylhydantoin and the molar absorptivity (1.985×10^4) of benzophenone at 247 nm.

[4] Analysis using an absorption spectrum is desirable, since interfering absorption can be checked.

Table I—Standard Curve Data of Diphenylhydantoin Reaction Product by Procedure A

Diphenylhydantoin Added, mcg./ml.	Reading[a] of Reaction Product, Average ± SD[b]	Reading[a] / Concentration	Percent Recovery[c], Average ± SD[b]
5	0.153 ± 0.003	0.031	100.9 ± 2.1
10	0.303 ± 0.002	0.030	99.7 ± 0.7
15	0.456 ± 0.005	0.030	100.1 ± 1.1
20	0.611 ± 0.001	0.031	100.6 ± 0.2
25	0.760 ± 0.005	0.030	99.8 ± 0.6
30	0.909 ± 0.002	0.030	99.6 ± 0.3
Plasma blank	0.006 ± 0.002	—	—

[a] Absorbance determined on a 10-fold expanded scale of the recording spectrophotometer. [b] Standard deviation calculated from three determinations at each plasma level. [c] Recovery (%) = $100(A \times B)/(C \times D \times E \times F \times G)$, where A = expanded reading determined by Procedure A; B = volume (2/1000 l.) of *n*-heptane; C = molar absorptivity of benzophenone (1.895×10^4); D = molar quantity of diphenylhydantoin added to the sample; E and F = fractions (27/30 and 9/10) of $CHCl_3$ layer and 1 *N* NaOH layer used for the subsequent procedure, respectively; and G = expanded scale (10) of the spectrophotometer.

determining phenobarbital and is given by:

$$\Delta A = A_1 - 1.25A_2 \qquad \text{(Eq. 1)}$$

where A_1 and A_2 are the first and second readings at 260 nm.

One milliliter of the remaining sodium hydroxide layer is transferred into a centrifuge tube, which is connected to a vacuum rotary evaporator assembly, and the tube is immersed in a water bath of 50–60°. The tube is rotated until the volume is reduced to approximately 0.5 ml. Procedure A is then carried out using a fivefold scale.

Efficiency of Extraction of Diphenylhydantoin—Ten milliliters of water, 2 ml. of 1 *M* phosphate buffer solution (pH 6.8), and 30 ml. of chloroform were successively added to 0.4 ml. of plasma at a diphenylhydantoin concentration of 20 mcg./ml. The mixture was centrifuged after shaking, and 15 ml. of the chloroform layer was evaporated at 50–60°. The diphenylhydantoin in the residue was determined by Procedure A using a fivefold scale, and the average of six determinations was found to be 0.738 ± 0.002 (*SD*). If diphenylhydantoin is completely extracted from plasma with 30 ml. of chloroform, the extracted amount of diphenylhydantoin in 15 ml. of the chloroform layer would be 4 mcg. Fifteen milliliters of chloroform containing 4 mcg. of diphenylhydantoin was evaporated at 50–60° to obtain a standard value, and the diphenylhydantoin in the residue was determined by the same method. The average of six determinations was found to be 0.739 ± 0.003. Furthermore, 2 ml. of 0.45 *N* NaOH was added to 30 ml. of chloroform containing 8 mcg. of diphenylhydantoin, and the mixture was centrifuged after shaking. One milliliter of the 0.45 *N* NaOH layer was evaporated at 50–60°. If diphenylhydantoin is completely extracted from the chloroform layer with 2 ml. of 0.45 *N* NaOH, the extracted amount of diphenylhydantoin in 1 ml. of the 0.45 *N* NaOH layer would be 4 mcg. The diphenylhydantoin in the residue was determined by the same method, and the average of six determinations was found to be 0.735 ± 0.006. These results show that the extraction of diphenylhydantoin from plasma with chloroform and the extraction of diphenylhydantoin from the chloroform layer with 0.45 *N* NaOH are quantitatively performed, since there was no significant difference between the values determined.

Clinical Study—A healthy male subject was given a single intravenous dose of 50 mg. of sodium diphenylhydantoin[5] dissolved in 1 ml. of a vehicle consisting of 40% propylene glycol and 10.5% ethanol in water. The drug was injected into an antecubital vein at a rate of 50 mg./min. Heparinized blood samples (about 1.5 ml.) from the opposite arm were taken 1, 15, 30, and 45 min. and 1, 2, 3, 6, 8, and 10 hr. after administration. The hematocrit values were determined by centrifuging at 3000 r.p.m. for 10 min. and the resulting plasma samples were assayed for diphenylhydantoin using Procedure A. A blood sample was taken as the blank before injection.

Animal Study—Plasma concentrations of diphenylhydantoin and phenobarbital at various times after intravenous administration were determined in three male white rabbits (2.5–3.0 kg.). Sodium diphenylhydantoin was dissolved in 0.9% NaCl to contain 50 mg. of diphenylhydantoin/ml., and phenobarbital was dissolved in an appropriately small volume of equimolar sodium hydroxide and diluted with 0.9% NaCl to contain 50 mg./ml. Both solutions were mixed in equal volume. Diphenylhydantoin and phenobarbital at a dose of 5 mg./kg. were simultaneously administered to rabbits through an ear vein. Heparinized blood samples (about 1 ml.) from the opposite ear were taken 1, 15, and 30 min. and 1, 2, 3, 4, 5, 6, 7, 8, 9, 24, and 32 hr. after administration. A blood sample was taken as the blank before injection. The plasma samples were assayed for diphenylhydantoin and phenobarbital using Procedure B.

[5] Aleviatin sodium for injection, Dainippon Pharmaceutical Co. Ltd., Osaka, Japan.

RESULTS AND DISCUSSION

In the determination of diphenylhydantoin (Procedure A) there is a linear relationship between the absorbance readings and the concentrations up to 30 mcg./ml. (Table I). A linear capability of the analysis for diphenylhydantoin in the presence of phenobarbital (Procedure B) over the 0–20-mcg./ml. range is illustrated in Table II. The standard curve data of phenobarbital in combination with diphenylhydantoin are also shown in Table II. The averages of blank values were 0.006 and 0.003 for 0.1 ml. (Procedure A) and 0.4 ml. (Procedure B) of plasma or whole blood, which were equivalent to 0.2 and 0.1 mcg./ml. diphenylhydantoin, respectively. The standard curves of diphenylhydantoin and phenobarbital in whole blood were the same as those in plasma. It has been shown (see *Experimental*) that diphenylhydantoin is quantitatively converted to benzophenone in permanganate reagent at 80° within 5 min. The recoveries calculated from the molar absorptivity of benzophenone and the amounts of diphenylhydantoin added to plasma are shown in Tables I and II. The average recoveries (percent) of all the determinations by Procedures A and B were 100.1 ± 1.0 and 101.9 ± 1.6, respectively.

The higher sensitivity and reliability of the proposed method can be illustrated by comparing the recoveries of diphenylhydantoin associated with several published procedures. Wallace and coworkers (18–21) reported that the average recovery of diphenylhydantoin added in known amounts to whole blood was 87.5–95.9% by reference to a standard curve prepared from aqueous solutions of diphenylhydantoin (oxidized with the permanganate reagent in the presence of *n*-heptane). Morselli (22) obtained an average recovery of 86% for the determinations of diphenylhydantoin added to plasma.

It was observed in the *Experimental* section that the reading of the diphenylhydantoin reaction product decreased with an increase in the oxidation time at temperatures higher than 90°. One milliliter of 25 mcg./ml. benzophenone solution, which was prepared by diluting

Table II—Standard Curve Data of Diphenylhydantoin Reaction Product and Phenobarbital by Procedure B

Diphenylhydantoin and Phenobarbital Added, mcg./ml.	Reading[a] of Reaction Product, Average ± SD[b]	Reading[a] / Concentration	Percent Recovery[c], Average ± SD[b]
4	0.139 ± 0.004 (0.167 ± 0.002)[d]	0.035[e] (0.042)[d]	103.0 ± 2.7
8	0.277 ± 0.003 (0.326 ± 0.004)	0.035 (0.041)	102.7 ± 1.0
12	0.409 ± 0.005 (0.494 ± 0.004)	0.034 (0.041)	100.8 ± 1.1
16	0.553 ± 0.005 (0.651 ± 0.002)	0.035 (0.041)	102.1 ± 0.9
20	0.685 ± 0.007 (0.818 ± 0.005)	0.034 (0.041)	101.0 ± 1.0
Plasma blank	0.003 ± 0.003 (−0.001 ± 0.002)	— —	— —

[a] Absorbance determined on a fivefold (diphenylhydantoin) or a 10-fold (phenobarbital) expanded scale of the recording spectrophotometer. [b] Standard deviation calculated from three determinations at each plasma level. [c] Calculated in the same way as in Table I, using $F = 1/2$ and $G = 5$. [d] Calculated from differential absorption of phenobarbital at 260 nm. based on Eq. 1.

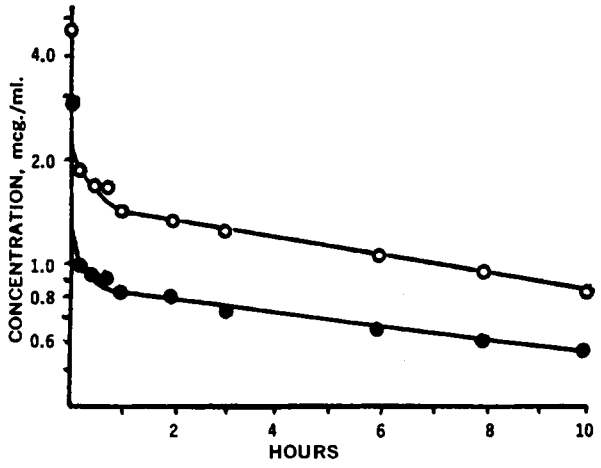

Figure 3—*Diphenylhydantoin concentration as a function of time after intravenous administration of 50 mg. of sodium diphenylhydantoin in a human subject. Key: ●, whole blood concentration; and ○, plasma concentration.*

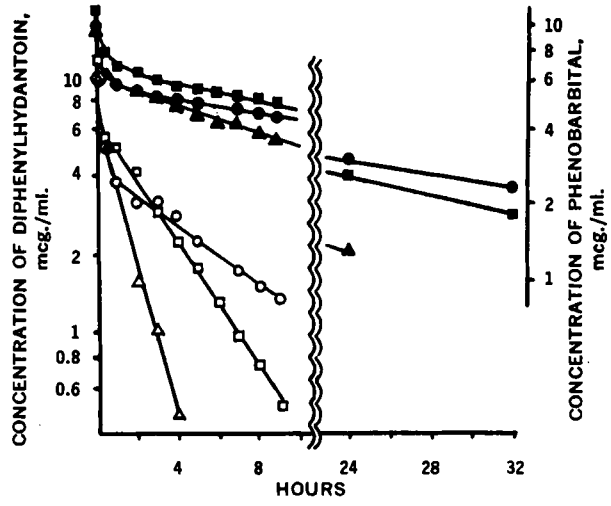

Figure 4—*Semilogarithmic plots of diphenylhydantoin and phenobarbital plasma levels after simultaneous intravenous administration of diphenylhydantoin (5 mg./kg.) and phenobarbital (5 mg./kg.) in rabbits. Open and closed symbols show the concentrations of diphenylhydantoin and phenobarbital, respectively. Key: ○,●, Rabbit B; □,■, Rabbit D; and △,▲, Rabbit E.*

1 mg./ml. benzophenone in ethanol with water, and 9 ml. of 7 *N* NaOH were mixed in a centrifuge tube. The centrifuge tube, closed with a ground-glass stopper, was immersed in a constant-temperature bath (70, 80, and 90°) and in boiling water for various lengths of time. After cooling, 2 ml. of *n*-heptane was added, and the mixture was shaken for 10 min. The reading of the *n*-heptane layer, separated from the aqueous layer by centrifugation, was recorded on a fivefold scale from 220 to 270 nm. against pure *n*-heptane. The peak reading at 247 nm. gradually decreased at 90° and in boiling water, while it remained constant over 60 min. at temperatures below 80°. A 20% decrease in the reading was observed at 90° and in boiling water after 30 min., and 25 and 34% decreases were observed at 90° and in boiling water after 1 hr., respectively. These results show that a decrease in benzophenone concentration results from vaporization through minute openings between the centrifuge tube and its ground-glass stopper during heating. The oxidation of diphenylhydantoin must be performed at temperatures below 80° to suppress vaporization of volatile benzophenone.

Various volumes (0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 ml.) of a plasma sample at a diphenylhydantoin concentration of 2 mcg./ml. were used to examine the effect of appropriate alterations of a sample volume upon the determination of diphenylhydantoin at relatively lower concentrations by Procedure A. These same volumes of a plasma sample at an equal concentration (8 mcg./ml.) of diphenylhydantoin and phenobarbital were also used for the determination of diphenylhydantoin and phenobarbital by Procedure B. The results were plotted using the amount of diphenylhydantoin or phenobarbital as the abscissa and the absorbance reading as the ordinate. The slopes of the straight lines so obtained were found to be equal to those of standard curves prepared from the data in Tables I and II, using the amount of diphenylhydantoin or phenobarbital in the sample solution as the abscissa. Consequently, appropriate sample volumes were used to obtain a reading higher than 0.2 in the following determination of diphenylhydantoin in a human subject and rabbits.

A normal subject was given a single intravenous dose of 50 mg. of sodium diphenylhydantoin. The diphenylhydantoin concentrations in whole blood and plasma after intravenous administration are shown in Fig. 3. The average of apparent diphenylhydantoin concentrations (micrograms per milliliter)[6] in the red cells was calculated to be 0.30 ± 0.05 over a concentration range in plasma diphenylhydantoin of 1.87–0.86 mcg./ml. except immediately after injection. However, higher apparent concentrations (4–11 mcg./ml.) were obtained in this laboratory for 12–20 mcg./ml. in plasma diphenylhydantoin level in patients during long-term treatment. The average ratio of whole blood concentration to plasma concentration for the samples shown in Fig. 3 was 0.58 ± 0.06. This was shown to be 0.50 ± 0.11 for 20 samples with an average plasma concentration of 13.8 mcg./ml. by Bock and Sherwin (23). The diphenylhydantoin concentration in plasma as a function of time could be described by $C_p = 3.6e^{-10.4t} + 1.7e^{-0.06t}$, yielding a half-life of 10.5 hr. for the terminal exponential phase. This value is comparable to 9.5 hr. (125 mg.) and 11.2 hr. (250 mg.) reported by Suzuki *et al.* (26) for higher doses. The concentrations extrapolated to time zero were proportional to the doses. The plasma levels of diphenylhydantoin and phenobarbital after simultaneous intravenous administration of diphenylhydantoin (5 mg./kg.) and phenobarbital (5 mg./kg.) in rabbits were determined by Procedure B. The plots of the logarithms of the plasma concentrations of diphenylhydantoin and phenobarbital against time are shown in Fig. 4. Rabbits exhibiting faster elimination rates for phenobarbital were found to possess increased rates of loss for diphenylhydantoin.

The method utilizing the UV absorption of benzophenone formed by permanganate oxidation is simple and specific. No interference with the assay occurred in the presence of many commonly used drugs (18–20, 22, 24). The present study adds acetylpheneturide, 4-amino-3-hydroxybutyric acid, carbamazepine, chlordiazepoxide, chlorpromazine hydrochloride, cyclandelate, diazepam, ethosuximide, mephobarbital, metharbital, nitrazepam, perphenazine, and thioridazine hydrochloride to the list of noninterfering substances. The primary metabolite, 5-(*p*-hydroxy)-5-phenylhydantoin, also did not interfere as reported by Wallace (20). The oxidation product of this metabolite was identified as benzoic acid by its UV and IR absorption spectra and by the results of the elemental analysis.

[6] Calculated from the whole blood concentration (C_w), plasma concentration (C_p), and hematocrit value (Ht) using the formula: $[C_w - C_p(1 - Ht)]/Ht$.

REFERENCES

(1) W. A. Dill, A. Kazenko, L. M. Wolf, and A. J. Glazko, *J. Pharmacol. Exp. Ther.*, **118**, 270(1953).

(2) J. W. Huisman, *Clin. Chim. Acta*, **13**, 323(1966).

(3) G. E. Simon, P. I. Jatlow, H. T. Seligson, and D. Seligson, *Amer. J. Clin. Pathol.*, **55**, 145(1971).

(4) G. L. Plaa and C. H. Hine, *J. Lab. Clin. Med.*, **47**, 649(1956).

(5) O. Svensmark and P. Kristensen, *ibid.*, **61**, 501(1963).

(6) O. V. Olesen, *Scand. J. Clin. Lab. Invest.*, **20**, 63(1967).

(7) O. V. Olesen, *Acta Pharm. Toxicol.*, **23**, 41(1965).

(8) S. Vedsö, C. Rud, and J. F. Place, *Scand. J. Clin. Lab. Invest.*, **23**, 175(1969).

(9) D. H. Sandberg, G. L. Resnik, and C. Z. Bacallao, *Anal. Chem.*, **40**, 736(1968).

(10) K. Sabih and K. Sabih, *ibid.*, **41**, 1452(1969).

(11) Von G. Grimmer, J. Jacob, and H. Schfer, *Arzneim.-Forsch.*, **19**, 1287(1969).

(12) M. A. Evenson, P. Jones, and B. Darcey, *Clin. Chem.*, **16**, 107(1970).

(13) J. MacGee, *Anal. Chem.*, **42**, 421(1970).

(14) H. J. Kupferberg, *Clin. Chim. Acta*, **29**, 283(1970).
(15) T. Chang and A. J. Glazko, *J. Lab. Clin. Med.*, **75**, 145 (1970).
(16) R. H. Hammer, B. J. Wilder, R. R. Streiff, and A. Mayersdorf, *J. Pharm. Sci.*, **60**, 327(1971).
(17) D. Sampson, I. Harasymiv, and W. J. Hensley, *Clin. Chem.*, **17**, 382(1971).
(18) J. E. Wallace, J. Biggs, and E. V. Dahl, *Anal. Chem.*, **37**, 410(1965).
(19) J. E. Wallace, *J. Forensic Sci.*, **11**, 552(1966).
(20) J. E. Wallace, *Anal. Chem.*, **40**, 978(1968).
(21) J. E. Wallace, *Clin. Chem.*, **15**, 323(1969).
(22) P. L. Morselli, *Clin. Chim. Acta*, **28**, 37(1970).
(23) G. W. Bock and A. L. Sherwin, *ibid.*, **34**, 97(1971).
(24) S. I. Lee and N. H. Bass, *Neurology*, **20**, 115(1970).
(25) L. A. Williams and B. Zak, *Clin. Chim. Acta*, **4**, 170(1959).
(26) T. Suzuki, Y. Saitoh, and K. Nishihara, *Chem. Pharm. Bull.*, **18**, 405(1970).

ACKNOWLEDGMENTS AND ADDRESSES

Received January 3, 1972, from the *Research Laboratory, Hospital Pharmacy, Tokyo University Hospital, Hongo, Bunkyo-ku, Tokyo, Japan, and the †Faculty of Pharmaceutical Sciences, University of Chiba, Yayoi-cho, Chiba, Japan.

Accepted for publication August 17, 1972.

The authors thank Dr. R. E. Notari for his help in the preparation of this manuscript.

▲ To whom inquiries should be directed.

Transport of Alkyl Homologs across Synthetic and Biological Membranes: A New Model for Chain Length-Activity Relationships

S. H. YALKOWSKY▲ and G. L. FLYNN

Abstract □ The ability of each member of a biologically active homologous series to reach the receptor site can often be equated with its relative ability to permeate biological barriers. This paper presents a structure–activity model based entirely on firmly established diffusional theories as well as solubility relationships generally applicable to aqueous systems. The equations derived and presented here were previously experimentally verified using synthetic membranes. These equations are generally applicable for transport across membranes and can be of use in describing a variety of passive absorption or permeation phenomena. Since true equilibrium is rarely attained in biological systems, a kinetic model provides an appropriate description of the physiological situation. Furthermore, a kinetic model offers considerable practical utility, because it can readily be incorporated into the overall pharmacokinetic treatment.

Keyphrases □ Membrane permeability, alkyl homologs—model for chain length–activity relationships, equations □ Transport across synthetic and biological membranes—alkyl homologs, model for chain length–activity relationships, equations □ Structure–activity relationships—model for transport of alkyl homologs across synthetic and biological membranes based on diffusion theories □ Chain length–activity relationships—alkyl homolog transport across membranes

Many theories have been advanced to explain relative biological activity of the members of a series of structurally related compounds (1–5). These theories usually relate the affinity of a particular congener for the receptor site to some property such as its molecular weight, its solubility, its surface tension, its partition coefficient between water and an organic solvent, or some other related physicochemical parameter. The structure–activity models can be categorized operationally as being either of kinetic or equilibrium nature. Current mathematical models for the former are generally of empirical design; quantitative relationships for the latter are complex and difficult to apply to actual data.

The present paper is concerned with a new kinetic approach applicable to many structure–activity profiles. The ability of each member of a homologous series to reach the receptor site is equated with its ability to permeate biological barriers, including the biomembrane(s) and contiguous aqueous layers. This is accomplished by modification of firmly established diffusional theories. Virtually all aspects of the theory and equations derived and presented here were experimentally verified (6–11) using synthetic membranes. It will be shown that much of the current biological data are interpretable based on these simple diffusional relationships.

These equations are general for transport across any membrane and can be of use in describing a variety of passive absorption or permeation phenomena. Furthermore, since true equilibrium is rarely attained in biological systems, a kinetic model may provide a more appropriate description for the general physiological situation, facilitating incorporation of passive transport processes into the total pharmacokinetic picture.

THEORETICAL

Importance of Diffusion Layers in Membrane Transport—The resistance to the transport of a solute across a membrane, which separates two aqueous compartments, lies not only within the membrane but also within the regions of unstirred water adjacent to its surfaces (6–11). The resistances of these aqueous regions of diffusion layers can be treated mathematically as additive resistances in series with the membrane (8–10). The flux of a solute across a membrane system (membrane and diffusion layers) is proportional to the concentration difference, C, across the barrier and is inversely proportional to the sum of the resistances of the laminates. Thus:

$$F^c = \frac{C}{R_{aq} + (R_m/PC)} \qquad \text{(Eq. 1)}$$