Rotating-Disk Method for Determining Cutaneous Metabolism

L. C. LEUNG and H. Y. ANDO ˣ

Received September 13, 1978, from the *Department of Pharmacy, Philadelphia College of Pharmacy and Science, Philadelphia, PA 19104.* Accepted for publication October 19, 1978.

Abstract □ Since design and evaluation of topical dosage forms should account for both skin permeation and cutaneous metabolism, an *in vitro* system was formulated to determine the metabolic component of viable guinea pig skin utilizing the diffusion layer property of the rotating disk. The drug investigated was vidarabine, an antiviral agent, which was rapidly metabolized to 9-β-D-arabinofuranosylhypoxanthine. The aqueous diffusion coefficient of the drug was determined by the capillary cell method. The rotating-disk system was standardized using benzoic acid. The dorsal skin of a guinea pig was removed after shaving, and the epidermal section was excised by a keratome. After the section was mounted on the stainless steel disk with a tissue adhesive, the preparation was immersed in a 10-ml beaker containing 5 ml of drug solution at 37°. At suitable intervals, samples were withdrawn, separated by TLC, and assayed by liquid scintillation. The enzyme rate constant was 1.54×10^{-1} sec^{-1}.

Keyphrases □ Rotating disk—analysis, cutaneous metabolism of vidarabine, guinea pig, *in vitro* study □ Skin—metabolism of vidarabine, rotating-disk method, guinea pig, *in vitro* study □ Antiviral agents—vidarabine, cutaneous metabolism, rotating-disk method, guinea pig, *in vitro* study □ Vidarabine—cutaneous metabolism, analysis by rotating-disk method, guinea pig, *in vitro* study

Recently (1, 2), an *in vitro* model was proposed for determining the simultaneous transport and metabolism of vidarabine (I) into 9-β-D-arabinofuranosylhypoxanthine (II) in hairless mouse skin. However, some experimental parameters required for the metabolic rate constant calculation were not determined, namely, the aqueous diffusion coefficient of I, D_{aqA}, and the diffusion layer thickness, h.

The objectives of the present investigation were to assess these parameters and to determine the enzyme metabolic rate constant. A capillary cell method (3) was used for the determination of D_{aqA}. By using the rotating-disk theory (4) and benzoic acid standardization (5), h was calculated. These parameters were then used for the calculation of the cutaneous metabolic enzyme rate constant.

EXPERIMENTAL

Apparatus—A constant-speed synchronized motor[1], a 50-μl syringe[2], a liquid scintillation counter[3], a laboratory press[4], two variable-speed synchronized motors[5], a light tachometer[6], 10-ml micropipets[7], a membrane filter[8], an electric shaver[9], a keratome[10], and silica GF plates[11] were used.

Reagents and Materials—The following were used: vidarabine[12], ^{3}H-vidarabine[13], 9-β-D-arabinofuranosylhypoxanthine[12], benzoic acid (primary standard)[14], ^{14}C-benzoic acid, a commercially prepared liquid scintillation cocktail[15], 0.01 *N* HCl[14], female albino guinea pigs (500–600 g), and pentobarbital sodium.

Aqueous Diffusion Coefficient, D_{aqA}, Determination—The upper reservoir of the diffusion cell (3) was nylon, and the capillary portion was a 50-μl gas-tight syringe[2] ground flat to the zero calibration mark. The value of D_{aqA} was determined by Saraf's method (3) using ^{3}H-vidarabine and liquid scintillation.

Estimation of Aqueous Diffusion Layer, h—The stainless steel rotating disk consisted of an outer cone-shaped manifold and an inner cylindrical disk (Fig. 1). Experimental deviations from the Levich (4) theory due to geometry and hydrodynamics were assessed using a benzoic acid standardization (5, 6).

By recrystallizing 0.1 mCi (0.9 mg) of ^{14}C-labeled and 9 g of primary standard benzoic acid from alcohol, radioactive benzoic acid crystals were obtained. Pellets of this material were prepared on a laboratory press by directly compressing about 500 mg of the crystals in a flat surface die (1.27 cm i.d.) with an average force of 4086 kg (9000 lb).

For each dissolution run, 5 ml of 0.01 *N* HCl, preequilibrated at 37°, was pipetted into a 10-ml beaker in a 37° controlled bath. The benzoic acid pellet was firmly seated in the outer cone-shaped manifold, which was connected to a synchronized motor. The rotation speed was determined with a light tachometer and adjusted to 300 rpm. After the disk was immersed in the hydrochloric acid solution, 10-μl samples were withdrawn with a micropipet at 5-min intervals and were assayed by liquid scintillation counting.

Metabolism Study of Vidarabine (I)—*Preparation of Radioactive Solution*—A 600 μ*M* I solution was prepared by dissolving 16.0 mg of I in 100 ml of Delbecco buffer (7) and filtering the solution through a 0.22-μm filter. By successive serial dilutions, stock solutions of 0.25, 0.5, 0.75, 1.0, and 6.0 μ*M* were obtained. Five-milliliter samples of these solutions were spiked with 10 μCi of ^{3}H-vidarabine (I) prior to each experiment.

Preparation of Skin—A female albino guinea pig, 500–600 g, was anesthetized with pentobarbital sodium (100 mg/kg ip). The dorsal skin was shaved first with an animal shaver and then with a manual razor. The area was washed with distilled water and dried with a stream of warm air. After removal from the animal, the dorsal skin was stapled onto a wooden board. The animal was then sacrificed with pentobarbital sodium.

A keratome set at 0.2 mm removed a thin layer of the epidermis plus some underlying dermis from the skin. The inner disk was removed from the outer manifold and placed over the shaved surface; tissue adhesive[16] was used to secure the skin to the side of the disk. To expose a constant surface area of the skin, polytef tape was wrapped around the skin on the cylindrical surface and secured with tissue adhesive. The outer cone-shaped manifold was then replaced on the inner disk (Fig. 1). This preparation was connected to a synchronized motor and immediately immersed in the 5 ml of radioactive I solution prepared prior to each experiment and maintained at 37°.

Five microliters of this solution was spotted with 1.5 μg of nonradioactive I and II onto prescored silica GF plates under a nitrogen stream. The plates were immediately developed in the solvent system described previously (8). After separation, I and II were scraped into vials and assayed by liquid scintillation. As a control, the experiment was duplicated twice without the skin.

RESULTS AND DISCUSSION

Aqueous Diffusion Coefficient Determination—The aqueous diffusion coefficient for Compound *A*, D_{aqA}, can be determined from (9):

$$D_{aqA} = \frac{4L^2}{\pi^2 T} \ln\left(\frac{8C_0}{\pi^2 C_a}\right) \qquad \text{(Eq. 1)}$$

[1] Hurst Manufacturing Co., Princeton, Ind.
[2] Hamilton Co., Whittier, Calif.
[3] Packard Tri-Carb model 3385, Packard Instrument Co., Downers Grove, Ill.
[4] Fred Carver Inc., Summit, N.J.
[5] GT210 laboratory stirrer, Gerald K. Hiller Co.
[6] Power Instruments Inc., Skokie, Ill.
[7] Bio-Rad Laboratories, Richmond, Calif.
[8] Millipore Corp., Boston, Mass.
[9] Oster Co., Milwaukee, Wis.
[10] Storz Surgical Instruments, St. Louis, Mo.
[11] Altech, Inc., Newark, Del.
[12] PL-Biochemicals, Milwaukee, Wis.
[13] New England Nuclear, Boston, Mass.
[14] Fisher Co., Pittsburgh, Pa.
[15] Aquasol, New England Nuclear, Boston, Mass.
[16] Eastman-910, Kodak, Rochester, N.Y.

0022-3549/79/0500-0571$01.00/0

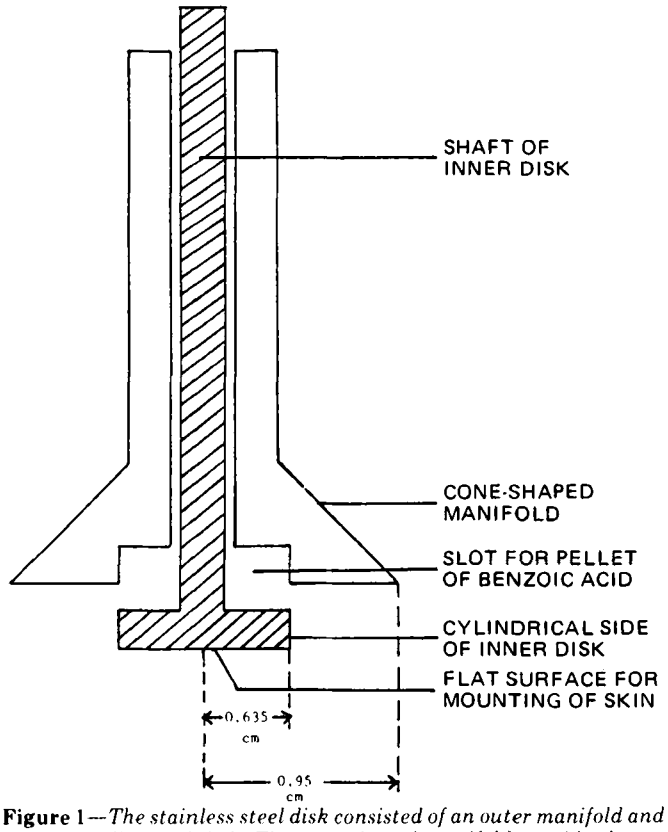

Figure 1—*The stainless steel disk consisted of an outer manifold and an inner cylindrical disk. The cone-shaped manifold provides better hydrodynamic conditions and serves as a slot for the benzoic acid pellet. The inner cylindrical disk is for the anchorage of skin in the metabolism experiments.*

where C_a and C_0 are the solute concentrations of A at time T and time zero, respectively, and L is the capillary length in centimeters. Compound I was found to have an average D_{aqA} of $(1.05 \pm 0.01) \times 10^{-5}$ cm^2/sec at 37° (Table I).

This value appears to be a little high compared with that of cyclic adenosine monophosphate (III), which was reported as 4.44×10^{-6} cm^2/sec at 20° (10). This difference may be due to the difficulty in the measurement and in the accurate reproduction of the capillary length. However, this higher value may be justified partly by the Stoke-Einstein equation (10). First, I was at a higher temperature, so the viscosity of the solvent was lower. Second, I has a smaller molecular weight and a lesser degree of solvation than III due to the absence of the phosphate group.

Estimation of Aqueous Diffusion Layer Thickness—When Compound A diffuses out of a rotating disk into an aqueous liquid, a theoretical diffusion layer thickness, h_{th}, which retards the loss of A, is described by the Levich (4) relationship:

$$h_{th} = (1.612)(D_{aqA}^{1/3})(\nu^{1/6})(\omega^{-1/2}) \qquad \text{(Eq. 2)}$$

where D_{aqA} is the aqueous diffusion coefficient (square centimeters per second), ν is the kinematic viscosity (centimeters per second), and ω is the angular velocity (radians per second). This equation requires that the disk have an infinite diameter and that it rotate with a constant velocity in a liquid of infinite volume. Therefore, the finite size and shape

Table I—Parameters in Determination of Aqueous Diffusion Coefficient of I by Capillary Cell Method [a]

Time[b], sec	L, cm	C_0, cpm	C_a, cpm	D_{aqA}, (cm^2/sec) $\times 10^5$
87,900	2.300	24,731.1	13,037.4	1.051
86,400	2.415	20,779.7	11,528.0	1.307
86,460	2.415	20,900.2	11,459.1	1.069
86,400	2.725	6,314.7	3,665.2	1.043
86,580	2.350	4,608.2	2,474.6	1.064

[a] The average value of the coefficient is $(1.05 \pm 0.01) \times 10^{-5}$ cm^2/sec. [b] About 24 hr.

Table II—Dissolution Profile of Benzoic Acid Pellets (A = 1.27 cm^2) in 0.01 N HCl at 300 rpm

Minutes	Concentration, mg/ml	ΔC[a], mg/ml
5	1.21	0.87
10	2.08	0.62
15	2.70	0.46
20	3.16	0.34
25	3.50	0.25
30	3.75	

[a] Concentration differential between successive time periods.

of the disk and the hydrodynamic condition within a real system may cause h to be considerably different from h_{th}. If h is the effective diffusion layer thickness in the experimental system, then let:

$$R = \frac{h}{h_{th}} \qquad \text{(Eq. 3)}$$

To determine R, consider the Levich relationship for the flux from the disk (4), assuming sink conditions:

$$J_{th} = (0.62)(D_{aqA}^{2/3})(\nu^{-1/6})(\omega^{1/2})(C_s) \qquad \text{(Eq. 4)}$$

where C_s is the saturation concentration of A.

Combining Eqs. 2 and 4 gives:

$$J_{th} = \left(\frac{D_{aqA}}{h_{th}}\right)(C_s) \qquad \text{(Eq. 5)}$$

which is Fick's first law. Since this law must hold for the experimental system as well:

$$J = \left(\frac{D_{aqA}}{h}\right)(C_s) \qquad \text{(Eq. 6)}$$

Then R can be determined by combining Eqs. 5 and 6 to give:

$$R = \frac{J_{th}}{J} \qquad \text{(Eq. 7)}$$

The dissolution of benzoic acid was used to standardize the disk for the determination of J_{th} since its aqueous diffusion coefficient is well known.

Data Treatment—Three dissolution experiments of benzoic acid pellets rotating at 300 rpm in 0.01 N HCl (Table II) showed the relation between the concentration and the successive concentration differential and time. The dissolution rate constant, K, was determined from these data (11) (Fig. 2) assuming a first-order equation. This constant was 6.17 $\pm$ 1.19 min^{-1}. The experimental flux was determined from:

$$J_{exp} = (K)(C_s)\left(\frac{V}{A}\right) \qquad \text{(Eq. 8)}$$

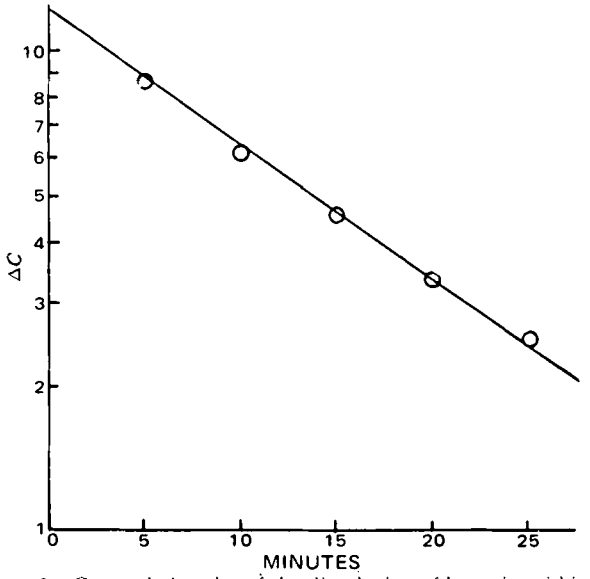

Figure 2—*Guggenheim plot of the dissolution of benzoic acid in 0.01 N HCl;* $C_t = C_{(t+i)} - C_t$, *where* i *is the sampling interval (5 min).*

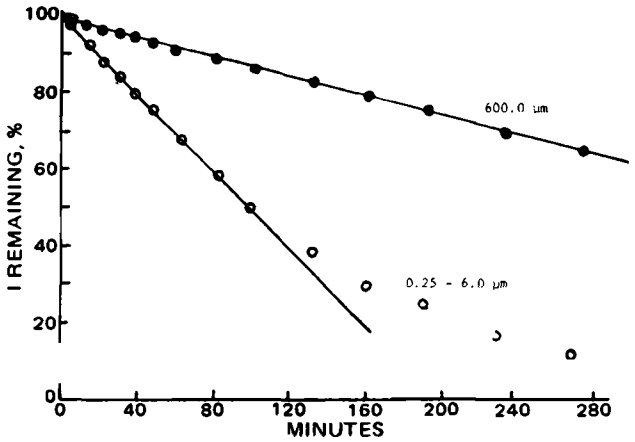

Figure 3—*Metabolism of I by guinea pig skin rotating at 300 rpm and 37°. Plots for concentrations of 0.25–6.0 μM were similar.*

where (V/A) is the volume to surface area ratio (3.95 cm in this system). The flux J_{exp} was 0.244 ± 0.047 C_s.

By using reported values of the aqueous diffusion coefficient of benzoic acid (1.40×10^{-5} cm²/sec) (12) and the aqueous kinematic viscosity (0.00699 cm²/sec) (13), the theoretical flux and the diffusion layer thickness, under the same experimental conditions, were computed to be 0.277 C_s and 27.57 μm, respectively. From Eq. 3, R was estimated to be 1.135. This value of R predicts an experimental aqueous diffusion layer thickness, h, of 31.3 μm, which differs from h_{th} by 13.5%.

This high discrepancy came as a surprise since the cone-shaped design is accepted as the best configuration for a rotating-disk system (14). However, this deviation can be explained by the fact that within the rotating system, the solvent does not have an infinite volume, nor does the disk have an infinite diameter, as required by the Levich (4) equation. Also, as benzoic acid dissolution continued, surface erosion occurred, disturbing the flow pattern. All of these nonideal hydrodynamic conditions, as well as the continual solvent evaporation, contributed to the 13.5% deviation, which is similar to that previously reported (5).

Metabolism Study of Vidarabine—Vidarabine (I) was metabolized to 9-β-D-arabinofuranosylhypoxanthine by the cutaneous enzyme system (Fig. 3). The initial slope was derived from the regression analysis, which included all data points up to 100 min. These regression coefficients were all greater than 0.995.

The flux of I, F_A, was then calculated from:

$$F_A = \left(\frac{V}{A}\right)\left(\frac{dC}{dt}\right) \qquad \text{(Eq. 9)}$$

where V is the solution volume in milliliters, A is the area of the exposed skin (square centimeters), and (dC/dt) is the initial slope of metabolism (percent per minute) (Table III). Statistical analysis of the fluxes showed a significant difference between 600.0 μM and the other concentrations. The mean flux was 1.75 ± 0.09%/min.

Determination of Cutaneous Metabolic Rate Constant—As shown in Eq. 10, the enzyme metabolic rate constant, k_m, can be expressed in terms of the initial substrate concentration, $C_A(h)$; the flux of the substrate in the system, F_A; the aqueous diffusion coefficient, D_{aqA}; the epidermal layer thickness, M; and the diffusion layer thickness, h:

$$k_m = \left(1 \Big/ \left[\frac{C_A(h)}{-F_A} - \frac{h}{D_{aqA}}\right]\right)(M) \qquad \text{(Eq. 10)}$$

This equation is derived by using Eq. 25 of previous work (2) and taking the limit of $\theta \to 0$, such that $\tanh \theta \to 0$. By substituting into Eq. 10 the reported epidermal thickness of 20.85 μm (15) and the other experimentally determined parameters, the enzyme metabolic rate constant was found to be 1.56×10^{-1} sec^{-1}.

In the controlled metabolism study, I remained unchanged at the initial concentration of 98.1% over 6 hr in the presence of the stainless steel rotating disk. The stability of I during the storage period was also investigated. The stock solution had been sterilized by being passed through a 0.22-μm filter and was then stored in an enclosed sterile vial and refrigerated. There was no apparent adsorption of I onto the filter. At the end of the 40th day, when all of the stock solutions were used up, the I purity was determined to be 97.9%. These findings show that the degradation of I into II was the sole result of the enzyme system existing in the viable guinea pig epidermal slice.

Table III—Fluxes of I at Different Concentrations

Concentration, μM	Flux, %/cm²/min	Mean Flux, %/cm²/min
0.25	1.630	1.826
	1.956	
	1.892	
0.50	1.888	1.283
	0.797	
	1.164	
0.75	1.662	1.679
	1.713	
	1.717	
1.0	1.691	1.616
	1.921	
	1.230	
6.0	1.568	1.839
	1.787	
	2.144	
600.0	0.388	0.378
	0.399	
	0.346	

The enzyme that converts I into II is adenosine deaminase, which metabolizes adenosine and various adenine nucleoside drug analogs, rendering them pharmacologically inactive. Calf intestinal mucosa adenosine deaminase has a metabolic rate constant of 0.8×10^{-1} sec^{-1} mg^{-1} (16). Although no direct comparison can be drawn, it can be concluded that adenosine deaminase is present in abundance in guinea pig skin.

In the final calculation of the enzyme rate constant from Eq. 10, the I flux and the epidermal thickness were the two most important parameters.

Since the flux depends on the concentration data of I and II, the assumption of mass balance used in their determination was evaluated. The criterion for mass balance is that the sum of the activities of I and II on the TLC plate is equal to the total activities of the sample. This assumption holds if II is the only major metabolite of I and if I and II bind identically to the tissue.

Several 6th-hr samples of the metabolism experiments were examined. The entire plate was scraped into scintillation vials in strips of 2 cm. It was found that I and II accounted for over 98% of the total activities.

Possible binding of I and II to the tissue was investigated statistically. From the raw data from five experiments at 0.5 μM, the corresponding activities of I and II were plotted *versus* time. The slope for the disappearance of I was 31.44 cpm/min, while the appearance rate of II was 31.48 cpm/min. Since the appearance rate of II was accompanied by an equivalent amount of disappearance of I, and since I and II accounted for over 98% of all activities, the assumption of mass balance was valid.

The average flux was obtained from the results of all concentrations except those of 600.0 and 0.5 μM. The former was rejected because it was significantly different from the others. The latter was excluded on the ground that two of the values were obtained from animals that did not meet the protocol.

The epidermal thickness is another important parameter in the determination of the rate constant. The model calls for the use of only the epidermal thickness on the assumption that the dermis is less active enzymatically than the epidermis. However, the dermis does contribute to the overall metabolism (17). Variation of hand pressure and speed of dermatoming caused variation in skin thickness, which was usually about 0.3 mm microscopically. Future studies may investigate the possibility of preparing epidermal slices of reproducible thickness using a motor-driven constant-pressure dermatome device. A method for accurate measurement of skin thickness must be developed also.

Future studies may determine the epidermal diffusion coefficient of the drug. With this parameter and the present data, one can estimate how metabolism and, possibly, formulation adjuvants can modify the bioavailability of the drug as it is administered in a topical dosage form.

REFERENCES

(1) H. Y. Ando, N. F. H. Ho, and W. I. Higuchi, *J. Pharm. Sci.*, **66**, 755 (1977).

(2) *Ibid.*, **66**, 1525 (1977).

(3) D. N. Saraf, A. A. Witherspoon, and L. H. Cohen, *Science*, **142**, 955 (1963).

(4) V. G. Levich, "Physiochemical Hydrodynamics," Prentice Hall,

Englewood Cliffs, N.J., 1962.
(5) S. Prakongpan, W. I. Higuchi, K. H. Kwan, and A. M. Molokhia, *J. Pharm. Sci.*, **65**, 685 (1976).
(6) C. V. King and S. S. Brodie, *J. Chem. Soc.*, **59**, 1375 (1937).
(7) D. J. Merchant, R. H. Kant, and W. H. Murphy, Jr., "Handbook of Cell and Organ Culture," Burgess, Minneapolis, Minn., 1969.
(8) J. C. Drack and J. M. Novack, *Anal. Biochem.*, **52**, 633 (1973).
(9) J. H. Wang, *J. Chem. Soc.*, **74**, 1182 (1952).
(10) D. Dworkin and K. Keller, *J. Biol. Chem.*, **252**, 864 (1977).
(11) P. J. Niebergall and E. T. Sugita, *J. Pharm. Sci.*, **57**, 1805 (1968).
(12) S. Prakongpan, Ph.D. thesis, University of Michigan, Ann Arbor, Mich., 1974, p. 89.
(13) H. Nogami, T. Nagai, and A. Suzuki, *Chem. Pharm. Bull.*, **14**, 329 (1966).
(14) A. Riddiford, in "Advances in Electrochemistry and Electrochemical Engineering," vol. 4, P. Delahay, Ed., Interscience, New York, N.Y., 1966.
(15) I. Sarkany and P. M. Gaylarde, *Trans. St. John's Hosp. Dermatol. Soc.*, **59**, 241 (1973).
(16) J. Cory and R. Suhadolnik, *Biochemistry*, **4**, 1733 (1965).
(17) O. D. Laerum, *J. Invest. Dermatol.*, **52**, 204 (1969).

GLC Assay of Verapamil in Plasma: Identification of Fluorescent Metabolites after Oral Drug Administration

R. G. McALLISTER, Jr. *, T. G. TAN *, and D. W. A. BOURNE ‡x

Received August 21, 1978, from the **Department of Medicine, Veterans Administration Hospital, and the School of Medicine and the* ‡*College of Pharmacy, University of Kentucky, Lexington, KY 40506.* Accepted for publication October 20, 1978.

Abstract □ The fluorometric assay for verapamil in plasma is not useful after oral drug administration because of interference by inactive, but fluorescent, metabolites extracted along with the parent drug. A GLC method using a flame-ionization detector after silylation allows the separation of unchanged active verapamil from the metabolites and quantitation to a sensitivity of 0.025 μg/ml. After a single oral dose of drug in dogs, up to 80% of "fluorescent verapamil" represented inactive metabolites.

Keyphrases □ Verapamil—GLC analysis, plasma fluorometric analysis, metabolite interference □ GLC—analysis, verapamil in plasma □ Vasodilators—verapamil, GLC analysis in plasma

Verapamil, 5-[(3,4-dimethoxyphenethyl)methylamino]-2-(3,4-dimethoxyphenyl)-2-isopropylvaleronitrile (I), is an experimental antiarrhythmic and antianginal agent (1–5) and is currently undergoing clinical evaluation in the United States. Its pharmacological activity is related to suppression of transmembrane calcium fluxes in cardiac and other tissues (6, 7). These "slow" calcium currents may be involved in the genesis of fatal arrhythmias during myocardial ischemia and associated with sudden cardiac death (8).

BACKGROUND

A fluorometric assay for verapamil in plasma (9) has been used to determine its elimination kinetics in dogs after intravenous injection (10) and to demonstrate a linear correlation between plasma drug concentrations and both electrophysiologic and hemodynamic effects in dogs following systemic dosing (11). However, after oral administration of verapamil to dogs, no relationship between plasma level and effect was found (12), raising the possibility that metabolites generated during the first passage through the liver interfere with the fluorometric assay.

Verapamil is known to be metabolized extensively in rats and dogs, primarily to *O*- and *N*-dealkylated derivatives through loss of the 3,4-dimethoxyphenylethyl moiety (13). Schomerus *et al.* (14) used mass fragmentography to confirm the extensive metabolism of the drug in three normal human subjects after a single oral dose but did not identify the specific metabolites produced.

Since verapamil may become an important addition to the drugs available for treatment of cardiovascular disorders, the assay validity must be defined before further kinetic and pharmacological studies can be carried out. Two major metabolites of verapamil, 5-amino-2-(3,4-dimethoxyphenyl)-2-isopropylvaleronitrile (D620) (II) and 5-methylamino-2-(3,4-dimethoxyphenyl)-2-isopropylvaleronitrile (D617) (III)[1], were studied to determine possible interference with the fluorometric assay for parent verapamil. Furthermore, a GLC procedure specific for unchanged verapamil in the presence of II or III was developed and is described.

EXPERIMENTAL

Fluorometric Assay—The fluorometric assay for verapamil in plasma (9) involves extraction of drug from alkaline plasma with heptane and back-extraction into acid. Fresh plasma from normal drug-free human subjects was pooled. To one group of aliquots, verapamil was added to achieve concentrations of 0.1, 0.5, and 1.0 μg/ml; to two other aliquots, II or III were added similarly. All samples were prepared in triplicate.

The fluorometric assay was performed on each sample by an experienced technician under blind conditions, and the resulting fluorescence at an excitation/emission spectra of 275/310 nm was recorded[2]. The calculated recovery from these standards ranged from 85 to 94%.

To additional aliquots of plasma from the same pool, combinations of verapamil and II and III were added, each in a concentration of 0.5 μg/ml. The assay was carried out as previously described, and total fluorescence was determined[2].

GLC Assay—All reagents and solvents were of analytical grade, and glassware was cleaned by superheating after a distilled water rinse (15). Fresh plasma was collected from normal human volunteers as well as from drug-free mongrel dogs; no differences were found between the two for the work described here, and the term "plasma" used here may apply equally to either species.

Known amounts of verapamil and/or II and III were added to 2-ml plasma samples. After addition of 1.0 ml of 1 *N* NaOH and 5.0 ml of heptane, the mixture was shaken for 15 min and centrifuged at 1800 rpm for 10 min. Four milliliters of the heptane layer was transferred to a 15-ml centrifuge tube containing 2.0 ml of 0.1 *N* HCl; each tube was shaken and centrifuged as before. The heptane layer was aspirated and discarded, and the aqueous phase was washed twice with 2.0 ml of heptane. One milliliter of 1.0 *N* NaOH was added, and the acid layer was extracted with 4.0 ml of heptane, with agitation and centrifugation as before.

Three milliliters of the heptane layer was then transferred to another tube and evaporated under a nitrogen stream during heating to 60° on

[1] Kindly supplied by Prof. K. Hahn, Knoll AG, Ludwigshafen, West Germany.

[2] Aminco-Bowman spectrophotofluorometer with ratio photometer unit, American Instrument Co.

0022-3549/79/0500-0574$01.00/0