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Pharmacokinetic Profile of Clonazepam in Rhesus Monkeys

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Received March 3, 1977, from the *Departments of Pharmaceutical Sciences and Neurological Surgery, Schools of Pharmacy and Medicine, University of Washington, Seattle, WA 98195.* Accepted for publication June 7, 1977.

Abstract □ The pharmacokinetic profile of clonazepam was studied in five chronically catheterized male rhesus monkeys using short-term intravenous infusion and oral administration in a Latin-square design. Clonazepam in a 40% polyethylene glycol 400 solution was infused at a constant rate over 25 hr into each monkey. Steady-state plasma levels in the ranges of 20, 40, 60, and 80 ng/ml were achieved with infusion rates of 15, 30, 45, and 60 μg/hr/kg, respectively. An oral dose of 0.5 mg of clonazepam also was administered to each monkey by intranasal gastric intubation. Plasma levels were assayed by electron-capture GLC. The intravenous and oral kinetics of clonazepam could be described in terms of a one-compartment open model with first-order elimination. The mean (±*SE*) parameters from short-term infusion studies were: total body clearance, 0.86 ± 0.03 liter/hr/kg; elimination half-life, 5.6 ± 0.6 hr; and volume of distribution, 6.8 ± 0.5 liters/kg. Intra- and interindividual variability in these parameters was prevalent, implying that dosing of monkeys during efficacy testing requires individual adjustments. Long-term (7–10-day) infusion studies revealed a small, but significant, decrease in clearance as well as diurnal oscillation in "steady-state" plasma levels.

Keyphrases □ Clonazepam—pharmacokinetics in rhesus monkeys □ Pharmacokinetics—clonazepam in rhesus monkeys □ Anticonvulsants—clonazepam, pharmacokinetics in rhesus monkeys

Clonazepam, 5-(2-chlorophenyl)-1,3-dihydro-7-nitro-2*H*-1,4-benzodiazepin-2-one, has shown anticonvulsant properties in several animal species (1). It was used for several years in Europe in the treatment of petit mal epilepsy prior to its recent approval for use in the United States. Clinically, the oral formulation is particularly effective in controlling petit mal at doses of 1–2 mg/day (2). In Europe and Australia, the intravenous formulation also has been found effective in suppressing status epilepticus (3, 4).

Clonazepam will be evaluated in an epileptic monkey model specific to motor seizures (5, 6). The present study was undertaken to determine the pharmacokinetic profile in the rhesus monkey and to design an optimum dosage regimen.

Earlier studies[1] in monkeys indicated that rapid intravenous injection of clonazepam (in 10% propylene glycol) in the femoral vein produced irregularities in the early time points of the plasma concentration–time curve. These irregularities were attributed to *in vivo* drug precipitation. As a result, intravenous bolus injections were replaced by short-term (1-day) constant-rate infusions in the present study design. The preliminary studies[1] also indicated that the clonazepam elimination half-life is short, precluding a once a day oral administration regimen. This situation suggested chronic intravenous administration (through indwelling catheters) for efficacy evaluation. Consequently, long-term (7–10-day) infusion studies also were included in the experimental design.

EXPERIMENTAL

Animal Preparation—Five male rhesus monkeys were chair adapted for 1 month prior to the study and maintained in a three-level restraining chair during each individual experiment. The monkeys were given cage rest at appropriate intervals. The jugular and femoral veins of each monkey were catheterized chronically to enable withdrawal of venous blood samples and drug infusion, respectively. Patency of catheters was assured by a slow, continuous saline infusion.

All monkeys were maintained on monkey chow and fresh fruit in the Vivarium at the University of Washington.

Protocol—Four short-term (25-hr) intravenous infusion studies and one oral study were planned for each monkey using a randomized 5 × 5 Latin-square design. For the short-term infusions, clonazepam in a sterile polyethylene glycol 400–water mixture (40:60 v/v) was infused at a constant rate[2] of 1 ml/hr over 25 hr. Steady-state levels of 20, 40, 60, and 80 ng/ml were achieved with infusion rates of approximately 15, 30, 45, and 60 μg/hr/kg. The oral dose was an aqueous suspension of a 0.5-mg clonazepam tablet[3]/monkey, administered by intranasal gastric intubation.

[1] R. H. Levy, J. S. Lockard, and H. J. Kupferberg, unpublished results.
[2] Holter infusion pump, Extracorporeal Medical Specialties, King of Prussia, Pa.
[3] Clonopin, Roche Laboratories, Nutley, N.J.

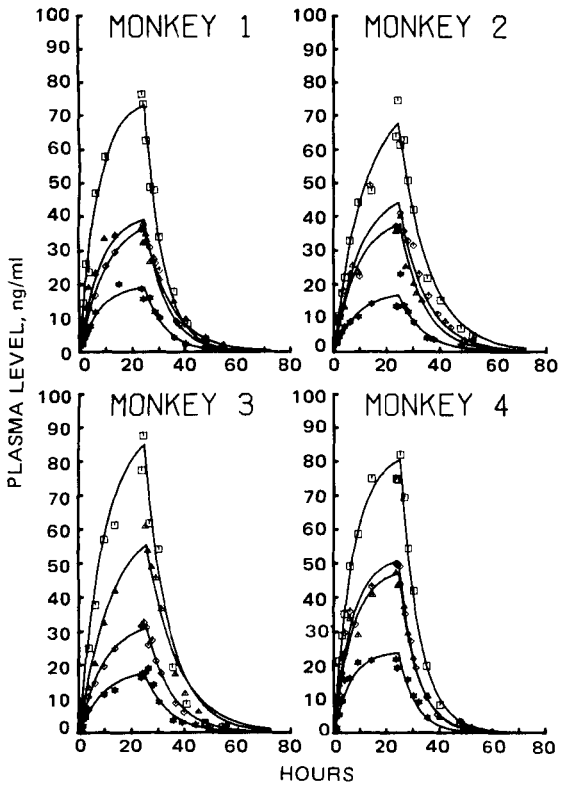

Figure 1—*Plots of plasma clonazepam concentrations* versus *time for the four 25-hr infusions in Monkeys 1–4. The continuous lines were obtained by a least-squares fit of experimental data points.*

Long-term (7–10-day) intravenous infusions, designed to achieve a steady-state plasma level of 40 ng/ml, also were performed (one per monkey).

Blood samples were collected at predetermined times into 3-ml tubes containing edetate potassium as an anticoagulant. The blood specimens were centrifuged immediately, and plasma samples were separated and frozen. Plasma samples generally were assayed on the day following the end of a study. All studies in the Latin-square design were completed, except for two short-term infusions and one oral study in Monkey 5 (technical difficulties with catheters). For the long-term infusion, Monkey 5 was replaced by Monkey 6.

Analytical Procedures—Plasma samples were analyzed for clonazepam using the electron-capture GLC assay of de Silva and Bekersky (7) with the following modifications. Since plasma was used in place of whole blood, the blood denaturation step was eliminated. Moreover, 80–100-mesh Chromosorb W-HP[4] replaced 60–80-mesh Gas Chrom Q. The gas chromatograph[5] was equipped with a 15-mCi ^{63}Ni-electron-capture detector. The operating conditions were as follows: injection port, 300°; oven, 260°; detector, 300°; and argon–methane (90:10) carrier gas flow, 20 ml/min. The reliability of the determinations was monitored by assaying one control sample with each run. The percent standard deviation of multiple assays over 1 year was 7.7%.

Data Analysis—Experimental data points were fitted to appropriate model equations using the BMDX85 nonlinear least-squares program on a digital computer[6]. All plasma concentrations were assigned equal weights. Experimental concentrations during the infusion and postinfusion periods were fitted to Eqs. 1 and 2, respectively. The computer output yielded two parameters, total body clearance(s) and elimination rate constant(s). Values for the volume of distribution were calculated from these parameters (ratio of total body clearance and elimination rate constant) as well as from area measurements (Eq. 3).

Bioavailability of the oral formulation was calculated as the ratio of the area under the plasma concentration–time curves after oral and intravenous administrations, with correction for dosage (Eq. 4) as well as intrasubject variation in half-life (Eq. 5).

[4] Supelco, Bellefonte, Pa.
[5] HP model 5710A, Hewlett-Packard, Avondale, Pa.
[6] CDC 6400, Control Data Corp., Minneapolis, Minn.

Table I—Pharmacokinetic Parameters of Clonazepam in Rhesus Monkeys Obtained from Short-Term (25-hr) Intravenous Infusion Studies

Monkey	Mean Elimination Half-Life, hr (*SD*)	Mean Volume of Distribution, liters/kg (*SD*)	Mean Total Body Clearance, liter/hr/kg (*SD*)
1	5.7 (1.0)	7.0 (1.0)	0.86 (0.18)
2	6.7 (0.7)	7.7 (0.7)	0.83 (0.16)
3	7.0 (0.8)	7.9 (1.1)	0.78 (0.16)
4	4.7 (0.3)	6.5 (0.3)	0.96 (0.05)
5	4.0 (0.2)	5.0 (0.8)	0.86 (0.10)
Mean (*SE*)	5.6 (0.6)	6.8 (0.5)	0.86 (0.03)

RESULTS AND DISCUSSION

Short-Term Infusion Kinetics—Figure 1 shows the plasma concentration–time curves obtained for the four 25-hr infusions in Monkeys 1–4. In most cases, steady-state plasma levels were nearly achieved by 25 hr; on the following day, plasma concentrations decayed to less than 10% of the 25-hr levels. The experimental concentrations during infusion (C_i) and postinfusion (C_p) were fitted to the following equations (8), respectively:

$$C_i = \frac{R}{Cl}(1 - e^{-K_E t}) \qquad \text{(Eq. 1)}$$

$$C_p = \frac{R}{Cl}(1 - e^{-K_E t_i})e^{-K_E(t - t_i)} \qquad \text{(Eq. 2)}$$

where R represents the zero-order infusion rate, t is the time since the beginning of the infusion, t_i is the time at which infusion is stopped, Cl is the total body clearance, and K_E is the first-order rate constant for elimination by all processes.

The least-squares fitting procedure involved a simultaneous fit of the data points to both equations, yielding a single set of parameters[7]. The results of curve fitting are shown by the continuous lines in Fig. 1. The agreement between data points and the least-squares lines suggests that, as a first approximation, a one-compartment open model with first-order elimination is appropriate to describe the short-term infusion kinetics of clonazepam in monkeys.

A summary of pharmacokinetic parameters obtained by least-squares regression analysis in all five monkeys is given in Table I. The uniqueness of the parameters obtained from the regression analysis of each set of experimental data can be assessed from the corresponding standard deviations. The mean percent standard deviations for half-life ($T_{1/2}$) and total body clearance in the 18 infusion studies were 8.1 and 3.3%, respectively.

The mean half-life in individual monkeys ranged from 4.0 to 7.0 hr. The overall mean half-life (±*SE*) was 5.6 ± 0.6 hr. A half-life of 4.8 hr reported for one squirrel monkey given an intravenous bolus dose is within the range of values found in this study (9). The clonazepam elimination half-life in humans is longer, ranging from 17 to 60 hr (9–12). In the present study, the intrasubject variability in half-life, expressed as percentage standard deviation, ranged from 5 to 18%. Data on intrasubject variability in the clonazepem biological half-life in humans are scarce. Knop *et al.* (9) found half-life values of 17 and 22 hr in the same volunteer given a 2-mg iv infusion 3 months apart.

Mean total body clearance in individual monkeys ranged between 0.78 and 0.96 liter/hr/kg with an overall mean (±*SE*) of 0.86 ± 0.03 liter/hr/kg. The intrasubject variability (5–21%) was of the same order of magnitude as that found with the half-life. The clearance values found in this study are larger than the values reported in one squirrel monkey (9) and the range of values (0.02–0.09 liter/hr/kg) found in humans (9, 10).

The volume of distribution (V_d) was calculated by two methods: from the ratio of the total body clearance and the elimination rate constant (Method I) and by Eq. 3 (Method II):

$$V_d = \frac{(D)_{iv}}{AUC\ K_E} \qquad \text{(Eq. 3)}$$

where AUC is the area under the plasma concentration–time curve (from $t = 0$ to $t = \infty$) calculated by the trapezoidal rule, $(D)_{iv}$ is the total dose infused, and K_E is as previously defined. The mean ratio (±*SD*) of 18 sets

[7] The infusion (C_i) and postinfusion (C_p) concentrations were fitted to Eqs. 1 and 2 separately and yielded almost identical sets of parameters, indicating that the simultaneous fitting approach is valid.

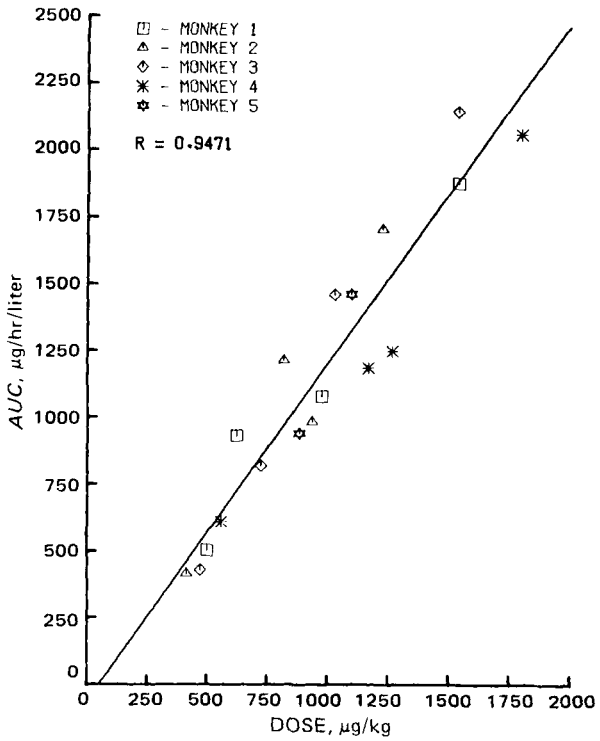

Figure 2—*Plots of* AUC versus *dose following 25-hr infusions in Monkeys 1–5.*

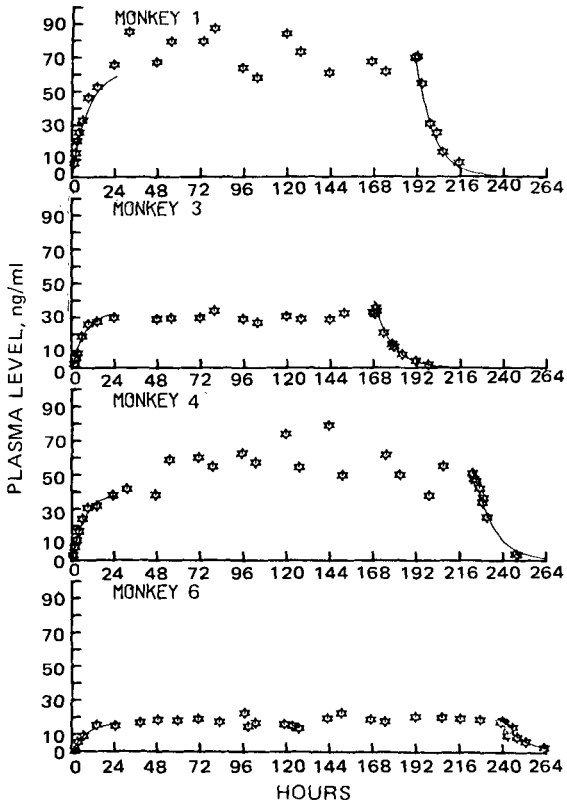

Figure 3—*Plots of plasma clonazepam concentrations* versus *time following a 7–10-day continuous zero-order intravenous infusion in Monkeys 1, 3, 4, and 6. The continuous lines were obtained by a least-squares fit of experimental data points.*

of values of volume of distribution calculated by these two methods was 1.02 ± 0.05, indicating that Methods I and II are equivalent.

The values listed in Table I (obtained by Method I) ranged from 5.0 to 7.9 liters/kg with an overall mean (±*SE*) of 6.8 (±0.5) liters/kg. These values are larger than those reported for any other species, suggesting that, in monkeys, clonazepam partitions extensively outside of the total body water compartment. Knop *et al.* (9) found a volume of distribution of 2.1 liters/kg in one squirrel monkey. The values of this parameter ranged from 1.5 to 4.4 liters/kg in humans (9–12) and from 2.4 to 3.5 liters/kg in dogs (9, 11). The intrasubject variability (5–21%) was of the same magnitude as that found for other parameters.

A plot of the area under the plasma concentration–time curve *versus* dose (Fig. 2) shows a linear relationship with a correlation coefficient of 0.94. This result suggests that the kinetics of clonazepam in monkeys are linear in the 400–1800-μg/kg range. The slope of this plot yields a total body clearance value very close to the overall mean of individual studies. The presence of kinetic linearity further supports the use of a one-compartment open model with first-order elimination to describe clonazepam kinetics in monkeys.

A urinary excretion study was performed in one monkey (Monkey 3) given a 15.39-mg dose of clonazepam by intravenous infusion over 48 hr. Urine samples were collected at 24-hr intervals for 4 days. Analysis of these samples yielded a total of 122.35 μg of clonazepam excreted unchanged, *i.e.*, 0.8% of the dose. This value is close to the values reported by Kaplan *et al.* (11) for dogs and humans, 0.1 and 0.5%, respectively. Thus, it appears that clonazepam is almost completely metabolized in all species investigated so far.

Long-Term Infusion Kinetics—Figure 3 shows experimental plasma concentrations as a function of time following a 7–10-day continuous zero-order infusion of clonazepam in Monkeys 1, 3, 4, and 6. The 0–25-hr data were fitted to Eq. 1, and the postinfusion data were fitted to Eq. 2; the resulting pharmacokinetic parameters (Table II) were subjected to statistical comparison (paired *t*-test). The results indicated that there is a significant difference in half-life and total body clearance between the 0–25-hr period and the postinfusion decay. The postinfusion clearances were, on the average, 14.4% smaller than the 0–25-hr values, and the postinfusion half-lives were 29.7% longer.

Table II—Pharmacokinetic Parameters of Clonazepam in Rhesus Monkeys Obtained from the 0–25-hr and Postinfusion Data during Long-Term (7–10-day) Intravenous Infusion Studies

	Elimination Half-Life, hr		Total Body Clearance, liters/hr/kg	
Monkey	0–25 hr	Post-infusion	0–25 hr	Post-infusion
1	6.3	6.3	0.67	0.60
2	—	11.6	—	0.42
3	5.4	6.6	1.06	0.91
4	5.0	7.7	0.98	0.72
6	6.1	8.7	1.19	1.13
	$p < 0.05$		$p < 0.05$	

The shorter apparent half-life obtained in the 0–25-hr period could be the result of simultaneous distribution and elimination if clonazepam exhibits a prolonged distribution phase. A lengthening of the tetracycline half-life when going from single to multiple dosing was reported by Doluisio and Dittert (13). Subsequent studies[8] suggested that the shorter initial tetracycline half-life is due to a prolonged distribution phase. An alternative explanation for the longer postinfusion clonazepam half-lives at the end of long-term infusions could be metabolite(s) inhibition of metabolism, as suggested for diazepam (14). A decrease in total body clearance and an increase in half-life after 6 days of administration of diazepam to seven volunteers were attributed to the accumulation of desmethyldiazepam, the major metabolite.

The clearances and half-lives obtained in the long-term infusions were compared to the corresponding values obtained in the short-term studies. There was no significant differences ($p \leq 0.05$) between the short-term infusion parameters and either the 0–25-hr or postinfusion values.

Fluctuations in steady-state plasma levels during long-term infusions (from Days 2 to 7–10) were present in all monkeys. The degree of oscillation within a 24-hr period was measured by the ratio C^*_{max}/C^*_{min}, where C^*_{max} and C^*_{min} represent the highest and lowest "steady-state" concentrations, respectively. These ratios ranged from 1.01 to 1.64 (mean ± *SD* = 1.21 ± 0.17) in five monkeys; consequently, the infusion data from Day 2 onward were not fitted to Eq. 1. Fluctuations in steady-state plasma levels could be caused by variations in experimental factors such as drug assay and/or infusion pump rotational speed. However, in the present study, appropriate steps were taken to ensure that these factors would not contribute to the observed oscillations.

[8] W. G. Crouthamel, School of Pharmacy, University of Maryland, Baltimore, MD 21201, personal communication.

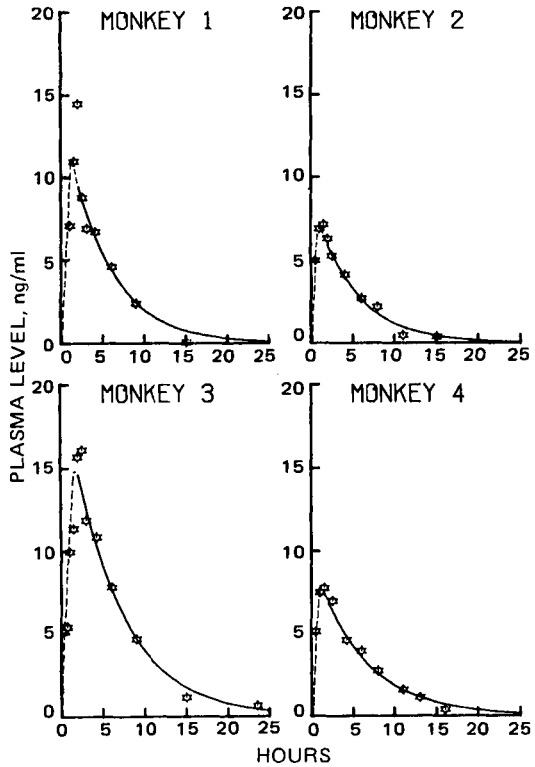

Figure 4—*Plots of plasma clonazepam concentrations* versus *time following oral administration of 0.5 mg of clonazepam to Monkeys 1–4. The continuous lines were obtained by a least-squares fit of postabsorption experimental data points.*

A similar type of time dependency was observed for two other anticonvulsants, ethosuximide (15) and valproic acid (16, 17), during chronic infusion in rhesus monkeys. Twenty-four-hour monitoring of plasma concentrations under controlled environmental conditions showed that the fluctuations in steady-state plasma levels of these two drugs could be attributed to diurnal oscillations in total body clearance (14–16). Such a phenomenon may also be present with clonazepam.

Oral Kinetics—Plasma concentration–time profiles following oral administration of 0.5 mg of clonazepam to Monkeys 1–4 are shown in Fig. 4. The peak time (t_{max}) was generally between 1 and 2 hr, suggesting that the broken tablet was rapidly absorbed. However, the absorption half-life could not be determined because of an insufficient number of data points during the absorption phase. The experimental data points at times larger than t_{max} were fitted to a monoexponential equation, and postabsorption half-lives were obtained (Table III). These values were much shorter than the corresponding mean intravenous half-lives. Statistical comparison (paired t-test) showed that the difference is significant ($p < 0.025$).

Apparent elimination half-lives obtained following oral administration can be longer than the corresponding intravenous half-lives in the presence of prolonged absorption. However, decreases in apparent half-lives following oral administration have not been reported. As discussed earlier, if a prolonged distribution phase is present, a shorter half-life would be expected from the decay curve starting at 2 hr after oral administration than after 25 hr of constant-rate infusion. Similarly, if metabolism is inhibited by metabolites, the latter would be expected to reach higher levels after 25 hr of constant-rate infusion than at 2 hr after a single oral dose. Another difference between the two experimental conditions is the presence of polyethylene glycol 400 in the intravenous studies, and its potential role in the observed phenomenon cannot be excluded.

Ronfeld and Benet (18) showed that the intravenous kinetics of phenytoin in rhesus monkeys were not altered following prolonged infusion of polyethylene glycol 400. The possible effect of polyethylene glycol on the oral kinetics of clonazepam is presently being investigated.

Bioavailability (F) was assessed using the following two equations:

$$F = \frac{(AUC)_{oral}(D)_{iv}}{(AUC)_{iv}(D)_{oral}} \quad \text{(Eq. 4)}$$

$$F = \frac{(AUC)_{oral}(D)_{iv}(K_E)_{oral}}{(AUC)_{iv}(D)_{oral}(K_E)_{iv}} \quad \text{(Eq. 5)}$$

Table III—Pharmacokinetic Parameters of Clonazepam in Rhesus Monkeys Obtained after Oral Administration

Monkey	Elimination Half-Life, hr	Bioavailability, % With Elimination Constant Adjustment	Bioavailability, % Without Adjustment
1	3.5	90	57
2	3.5	91	48
3	4.3	156	97
4	4.3	50	46
Mean (*SD*)	3.9 (0.5)	97 (44)	62 (24)

where F, AUC, K_E, and $(D)_{iv}$ are as previously defined; $(D)_{oral}$ is the oral dose; and subscripts iv and oral stand for the modes of administration. Equation 4 makes the assumption that total body clearance during the oral study was identical to the mean value obtained in the short-term infusion studies. This method gave values ranging between 46% (Monkey 4) and 97% (Monkey 3) with a mean of 62%. Calculation of bioavailability using Eq. 5 (19) makes the assumption that the volume of distribution of each animal during the oral study was the same as the mean value obtained in the short-term infusion studies. This method gave values ranging between 50% (Monkey 4) and 156% (Monkey 3) with a mean value of 97%.

In view of the substantial intrasubject variability in the volume of distribution observed in the intravenous studies (both short and long term), even the values obtained from Eq. 5 may not represent an accurate assessment of the fraction of dose absorbed. Other factors may contribute to the wide range of bioavailability values obtained. The possibility of a first-pass effect exists since clonazepam is almost totally metabolized and its total body clearance represents over 30% of the liver blood flow (20) in the monkey. Metabolism by microorganisms of the GI flora is another possibility (21) since a major pathway of clonazepam metabolism involves reduction of the aromatic nitro group to 7-aminoclonazepam.

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Effect of Vehicles and Other Active Ingredients on Stability of Hydrocortisone

V. DAS GUPTA

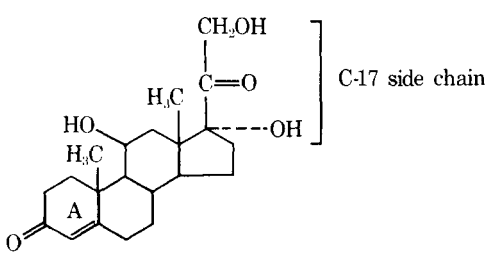
Received December 23, 1976, from the *College of Pharmacy, University of Houston, Houston, TX 77004.* Accepted for publication May 10, 1977.

Abstract □ The stability of hydrocortisone in various types of vehicles, aqueous, water-washable (polyethylene glycol ointment base), and oil in water or water in oil-type emulsified vehicles, and in the presence of other ingredients, iodochlorhydroxyquin, menthol, and phenol, was studied under normal conditions (room temperature and weakly acidic pH). The study was conducted using a stability-indicating assay method, high-pressure liquid chromatography. The hydrocortisone was very unstable in water and water-washable ointment base. The addition of alcohol and glycerin to water had a stabilizing effect. Under drastic conditions (very acidic or very basic pH), hydrocortisone proved to be unstable only on the basic side. The data at higher temperatures confirmed that the decomposition in water and polyethylene glycol was pseudo-first order. The decomposition process appears to be different in the highly basic solution *versus* weakly acidic media or in water *versus* polyethylene glycol ointment base.

Keyphrases □ Hydrocortisone—stability in various types of vehicles, effect of other active ingredients □ Stability—hydrocortisone in various types of vehicles, effect of other active ingredients □ Vehicles, various types—effect on stability of hydrocortisone, effect of other active ingredients □ Dosage forms—hydrocortisone in various types of vehicles, effect of other active ingredients on stability □ Glucocorticoids—hydrocortisone, stability in various types of vehicles, effect of other active ingredients

Hydrocortisone (I) is widely used in topical dosage forms that often contain other active ingredients such as iodochlorhydroxyquin, menthol, and phenol. Many different types of vehicles have been used to incorporate the active ingredients, *i.e.*, aqueous, water washable, water in oil or oil in water emulsion, and nonpolar vehicles. No comprehensive study on the stability of hydrocortisone in various vehicles and in the presence of other ingredients has been reported.

The degradation of the C-17 side chain (Structure I) of certain corticosteroids was studied (1–3) using base-catalyzed degradation, and it was suggested that the reaction was complex pseudo-first order. The degradation of hydrocortisone hemisuccinate at 70° was studied (4) over a narrow pH range and found to be a first-order reaction. Various factors influencing the stability of corticosteroids in aqueous suspensions and solutions were investigated (5), and it was concluded that prednisolone should not be exposed to materials capable of producing an elevated pH during formulation. Prednisolone in anhydrous form was considered to be stable (6) in liquid paraffin but not in water.



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The stability of cortisone and hydrocortisone was investigated (7, 8) using the UV spectrophotometric and phenylhydrazine methods to detect alterations in ring A (Structure I); the blue tetrazolium method was used to detect deterioration of the C-17 side chain. One such study (8) determined the shelflife of hydrocortisone in polyethylene glycol base to be approximately 6 months.

Only recently have better methods of analysis (from a stability point of view) become available (9, 10). The purpose of this study was to evaluate the effect of various vehicles and other active ingredients (iodochlorhydroxyquin, menthol, and phenol) on the stability of hydrocortisone. The other bases studied were water, polyethylene glycol ointment base USP (11), cold cream[1] USP (12), petrolatum[2], and three commercial bases[3]. The study was conducted using a stability-indicating assay method by high-pressure liquid chromatography (HPLC).

EXPERIMENTAL

Chemicals and Reagents—All chemicals and reagents were USP, NF, or ACS grade and were used without further purification.

Apparatus—A high-pressure liquid chromatograph[4] capable of operating at an inlet pressure of 6000 psig was used.

Column—The column consisted of a monomolecular layer of cyano-

[1] E. Fougera & Co., Hicksville, NY 11802.
[2] McKesson Laboratories, Bridgeport, Conn.
[3] Aquaphore, Duke Laboratories, Norwalk, Conn.; HEB, Barnes-Hind Pharmaceuticals, Sunnyvale, Calif.; and Dermovan, Texas Pharmacal Co., San Antonio, TX 78296.
[4] Waters ALC 202 equipped with a U6K universal liquid chromatograph injector and a UV detector (254-nm fixed wavelength), Waters Associates, Milford, Mass.