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AN EXPERIMENTAL BASIS FOR THE STUDY OF HYDROCORTISONE BIOAVAILABILITY IN HUMANS

by

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A thesis submitted in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY
(Pharmacy)

at the

UNIVERSITY OF WISCONSIN-MADISON

1981

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This thesis is dedicated to

Dean, Esther, and Cindy

three integral parts of my life.

ACKNOWLEDGEMENTS

I am forever indebted to Dr. Peter G. Welling for the superior training, guidance, encouragement, and opportunities he has unselfishly provided me throughout my graduate training. I will try to practice the same honesty and sincerity in all of my scientific endeavors that he has demonstrated in his contacts with me and with others.

Although to thank everyone involved with this work would be almost impossible, I do extend my sincere appreciation to D. Michael Walter for his excellent assistance with blood sampling and the sample assays, Dr. Thomas J. Goehl for his kind suggestions and assistance, Dr. William A. Craig for providing physical examinations and medical support, Dr. Michael Shanahan for time and advice given me through his role as my minor professor, and the Pharmaceutics faculty for their generosity in answering my many questions.

Financial support provided by the American Foundation for Pharmaceutical Education and by the United States Food and Drug Administration is deeply appreciated.

Production of this thesis would have been severly impeded without the advice, support, understanding, and marvelous typing skills of Cynthia A. Honer. Her contributions will be remembered forever.

And, of course, if Dean and Esther Toothaker had not instilled in their son such a respect for knowledge and an obsession to THINK, this thesis, and possibly this scientist, would not exist.

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AN EXPERIMENTAL BASIS FOR THE STUDY OF HYDROCORTISONE BIOAVAILABILITY IN HUMANS

Roger D. Toothaker

Under the supervision of Professor Peter G. Welling

Hydrocortisone (HC) dosage titration, to minimize possible side effects, can result in therapeutic failure due to changes in drug absorption (bioavailability) of oral HC tablets. The Food and Drug Administration has issued a bioequivalence requirement for HC tablets, pending the development of suitable *in vitro* or *in vivo* bioavailability testing methodology. At least three factors have impeded this development. First, assay methodology must detect plasma HC concentrations of 5 ng/ml, and must be free from interference from HC metabolites and other endogenous steroids. Second, exogenous plasma HC concentrations cannot be corrected for endogenous concentrations which vary from 40 to 200 ng/ml in an erratic diurnal pattern. Third, the concentration-dependent plasma protein binding of HC may result in dose-dependent pharmacokinetics.

In this study, a plasma HC assay has been developed which uses high-pressure liquid chromatography (HPLC) with ultraviolet absorbance. Three dexamethasone dosage regimens were examined for length, degree, and constancy of endogenous HC suppression in healthy male volunteers. The assay method and the most suitable dexamethasone regimen were used to study single dose pharmacokinetics of HC as oral tablets (3 subjects receiving 10, 30, and 50 mg), an intravenous

bolus (6 subjects receiving 5, 10, 20, and 40 mg as HC sodium succinate), and an oral suspension (8 subjects receiving 5, 10, 20, and 40 mg). Results were examined to determine the feasibility of performing bioavailability studies of oral HC tablets.

The assay method exhibited linear response for plasma HC concentrations from 5 to 3000 ng/ml, with 82% recovery of HC from plasma. Between-day variability (n=6) was less than 10%. Equivalent results were obtained for samples analyzed by this method and by a published HPLC method using fluorescence detection. Interference from other steroids was not observed.

HC was suppressed to constant concentrations (6 to 14 ng/ml) from 8 am to at least 8 pm by 2 mg of dexamethasone at 11 pm (prior) and by 1 mg at 11 pm and 6 am (prior), but only from 8 am to 6 pm by 1 mg of dexamethasone at 11 pm (prior). The first regimen (2 mg of dexamethasone at 11 pm) was chosen for use in subsequent studies.

Intravenous HC plasma concentrations were adequately described by a 2-compartment open model. The increase in the apparent steady state volume of drug distribution from 21 l at 5 mg to 37 l at 40 mg was highly correlated to the observed increase in overall elimination half-life (+0.771). Mean plasma clearance increased from 190 ml/min at 5 mg to 270 ml/min at 40 mg. Areas under plasma HC concentration versus time curves (AUC) were directly proportional to dose, except at 40 mg.

Concentrations and pharmacokinetics for tablets and suspension were essentially identical. Maximum plasma HC concentrations were obtained within 1 h post-dosing which made a high frequency of early blood samples necessary in order to measure absorption rates. Elimination half-life increased from 1.2 h at 5 mg to 1.7 h at 40 mg. AUC values and maximum concentrations increased linearly but not in direct proportion to increasing dose. Absolute bioavailability decreased from 90% at 5 mg to 60% at 10, 20, and 40 mg.

Results indicate that the assay method and suppression regimen (2 mg of dexamethasone at 11 pm) are suitable to study oral HC pharmacokinetics at doses of 5 mg or greater. The changes in apparent body distribution of HC for 40 mg doses, and in absolute bioavailability below 10 mg doses, indicate that bioavailability studies should be performed at doses of 20 mg.

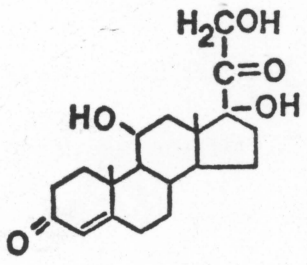
APPROVED: _____
Peter G. Welling, Professor

DATE: _____

INTRODUCTION

Background

Hydrocortisone (I) is an endogenous glucocorticoid formed



I

in the zona fasciculata and zona reticularis of the human adrenal gland (1). Hydrocortisone is not stored in the adrenals; rather, its synthesis and immediate release is controlled by plasma concentrations of adrenocorticotropic hormone (ACTH). ACTH is thought to trigger hydrocortisone synthesis by causing an influx of plasma cholesterol into the adrenocortical cell (2) where a sequence of at least six enzymatic reactions converts cholesterol into hydrocortisone (3). ACTH may also exert a cyclic-AMP mediated enhancement of the first enzymatic conversion of cholesterol to progesterone (4). Pituitary secretion of ACTH is largely controlled by the release of corticotrophin-releasing factor (CRF) from the hypothalamus.

The three main factors thought to regulate hydrocortisone secretion are plasma hydrocortisone concentrations, environmental

light, and stress. Circulating hydrocortisone exerts a homeostatic negative feedback inhibition on the release of ACTH from the pituitary, and may also inhibit release of CRF from the hypothalamus (5). The plasma hydrocortisone concentration required to inhibit ACTH secretion changes during the day, resulting in circadian fluctuations in ACTH secretion (6). This causes plasma hydrocortisone concentrations to follow a circadian rhythm (7, 8, 9). A simplified representation of this rhythm (9) in Figure 1 shows that concentrations are highest in the late morning, decline during the day to about 50 ng/ml by late afternoon, and remain low during the night, until early morning when a rapid concentration increase is seen. This diurnal rhythm appears to follow environmental changes between light and dark. It is not altered in blind persons or in persons working night shifts (10), but can be reversed if subjects are kept in an environment of complete day-night reversal (11). Superimposed on this diurnal rhythm are fluctuations caused by stress. Fever, acute illness, pregnacy, surgery, acute severe depression and other stresses can cause an increase in plasma ACTH which leads to increased hydrocortisone secretion (5). Thus, hydrocortisone is normally present in human plasma in significant and highly variable amounts.

Exogenous hydrocortisone has achieved widespread use as a therapeutic agent. The first clinical use was in 1951 as an intravenous treatment for rheumatoid arthritis (12). Since then, uses have extended to the treatment of other inflammatory diseases, allergies, connective tissue diseases, adrenal disorders, intestinal disease, shock, skin diseases, and many other indications. The great therapeutic success has been countered, however, by many complications and side effects associated with steroid therapy, some of which include adrenal atrophy and steroid dependence, increased risk of infection, bruising, cataracts, and osteoporosis (13). To minimize possible side effects, hydrocortisone dosage is normally titrated to the minimum amount of drug required for successful therapy. Dose titration assumes, however, that the amount of hydrocortisone delivered to the systemic circulation will not differ between production lots of a given drug formulation, or between formulations of the same drug from different manufacturers. Normally, this assumption may be assumed valid for parenteral drug formulations, but is often not valid for oral drug formulations. Many factors are present in oral tablet formulations, for example, which can cause differences in the disintegration or dissolution rate characteristics of tablets, resulting in differences in the rate and extent of

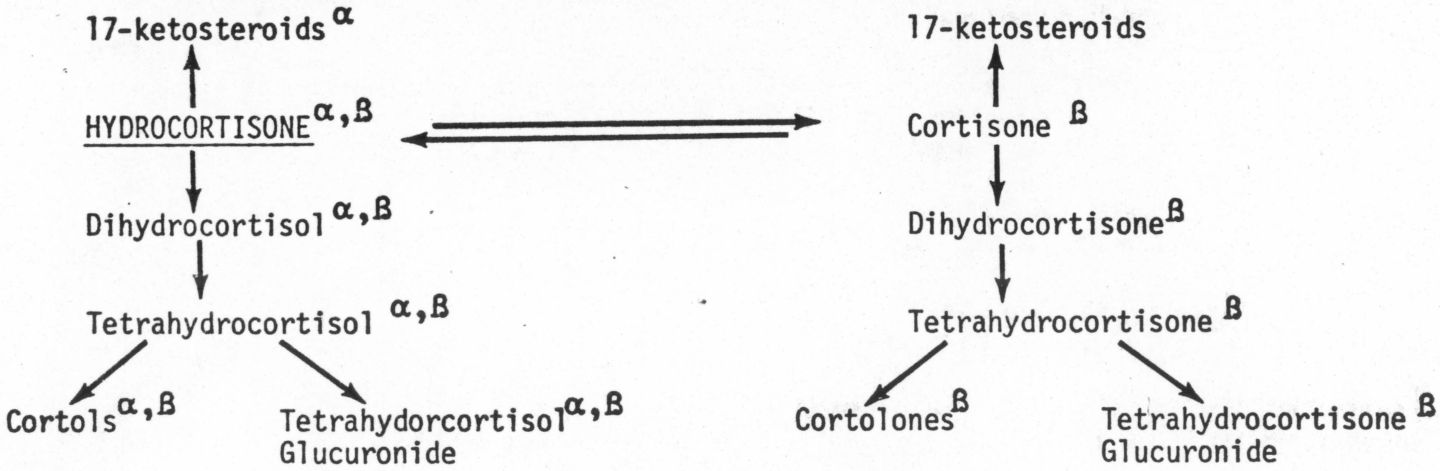
drug absorption from the gastrointestinal tract. Several documented therapeutic failures for prednisone, a synthetic glucocorticoid, have apparently been caused by poor tablet dissolution characteristics. When the time to 50% tablet dissolution (t_{50}) was measured for two samples of ineffective prednisone tablets, t_{50} values of 173 min (14) and 100 min (15) were obtained as compared to a t_{50} value of 4.3 min (15) for prednisone tablets known to be therapeutically effective. The possibility that these same tablet characteristics may be causing poor drug absorption for the more than 30 brands of hydrocortisone tablets presently on the market (16) has not yet been examined.

Regulatory efforts to prevent such therapeutic failures have been attempted by various means ever since Oser proposed the concept of physiological availability for multivitamins in 1945 (17) . In 1977, the Food and Drug Administration (FDA) issued its "Bioequivalence Requirements and In Vivo Bioavailability Procedures" (18). Included in this document are specific methods and procedures for determining drug product bioavailability, requirements for the establishment of drug product bioequivalence, what drugs are and are not covered by the regulations, as well as thorough definitions of terminology used in the regulations. Hydrocortisone tablets are listed in this document as

a drug product requiring submission of bioavailability data. Using the criteria outlined in this document, the FDA has proposed a bioequivalence requirement for hydrocortisone, pending the development of suitable *in vitro* or *in vivo* bioequivalence testing methodology. *In vitro* dissolution tests of marketed hydrocortisone tablets have been performed by the FDA using the USP Rotating Paddle method. Results for seven different tablets, shown in Figure 2, indicate extreme differences in dissolution rates, with the fastest dissolving tablet exhibiting a t_{50} of approximately 5 minutes, and the slowest tablet showing negligible dissolution even after 60 minutes. Whether these large differences in tablet dissolution will cause differences in the rate and extent of drug absorption, as represented by plasma hydrocortisone concentrations, is not yet known for several reasons. First, since oral therapeutic hydrocortisone doses from 10 to 25 mg result in plasma concentrations similar to those normally present in plasma (19, 20), close scrutiny of exogenous plasma hydrocortisone concentrations has been impeded by this significant and variable endogenous background. Second, existing assays for plasma hydrocortisone have lacked the required sensitivity and specificity. Since maximum concentrations of about 200 ng/ml have been reported for a 10 mg oral dose of hydrocortisone (20),

concentrations of 20 ng/ml or less would have to be measured to accurately establish the elimination half-life. Assay specificity is a problem due to the variety of steroids normally found in human plasma. Furthermore, hydrocortisone undergoes biotransformation to a large number of different steroidal metabolites, many of which can interfere with previously available assays that measure 17-hydroxycorticosteroid or 11-hydroxycorticosteroid activity. The major biotransformation products of hydrocortisone are shown in Scheme I (21, 22). The products with 11- or 17-hydroxy groups are indicated.

Prior to 1974, four main assay methods were used to estimate plasma hydrocortisone concentrations. One method, developed by Nelson and Samuels (23) and modified by Silber and Porter (24) and Peterson, et al (25), measured the fluorescent derivative formed by Porter and Silber's (26) reaction of phenylhydrazine with 17-hydroxycorticosteroids in concentrated sulfuric acid. The second method, introduced by Sweat in 1952 (27) and improved by DeMoor, et al (28) and Mattingly (29), made use of the native fluorescence of solutions of 11-hydroxycorticosteroids in concentrated sulfuric acid. The third method (30) measured competitive protein binding (CPB) between plasma steroids and trace amounts of radioactively-labelled steroids to transcortin, a plasma protein known to specifically bind steroids. The fourth



Scheme I: Major hepatic biotransformation products of hydrocortisone.

α : 11-hydroxycorticosteroid β : 17-hydroxycorticosteroid

method, radioimmunoassay (RIA), was introduced in 1972 (31). Background fluorescence of steroidal and non-steroidal plasma components cause the first two methods to exhibit unacceptable sensitivity limits of 20 to 30 ng of hydrocortisone per ml of plasma. In addition, these two methods require 10 to 20 ml plasma samples to achieve such sensitivity. The CPB and RIA methods exhibit the desired hydrocortisone sensitivities of about 1 to 5 ng/ml, but both show cross-reactivity with hydrocortisone metabolites as well as with other steroids normally found in plasma such as progesterone, 17-hydroxyprogesterone, cortisone, and 11-deoxycorticosterone.

Most hydrocortisone assays that have appeared since 1974 use either normal or reversed phase high pressure liquid chromatography (HPLC) with quantitation either by ultraviolet (UV) absorbance or fluorescence detection of a hydrocortisone derivative. Most of these methods use a dichloromethane extraction of hydrocortisone from plasma made basic with sodium hydroxide prior to chromatography. One UV HPLC method using an acidic plasma extraction exhibited a 20 ng/ml limit of detection for plasma hydrocortisone (32). Two methods using fluorescence detection (33, 34) had excellent sensitivities of 1 to 3 ng/ml, but sample preparation in both methods was lengthy due to the derivatization step. Three methods using normal phase UV HPLC

had acceptable sensitivity limits of about 10 ng/ml (35, 36, 37). However, one of these methods (37) was reported to have column stability problems (38). VandenBerg, et al (38) reported a method with a sensitivity limit of 1 ng of hydrocortisone but did not specify the type or size of sample. Rose, et at (39) published a method exhibiting a 5 ng/ml sensitivity from plasma using normal phase UV HPLC. Most reversed phase UV HPLC methods have sensitivities of 5 to 11 ng/ml with recoveries from plasma from 72% to 86% (40, 41, 42). Reardon, et al (43) reported a method capable of 96% recovery of hydrocortisone from plasma, with a 10 ng/ml assay sensitivity limit. All of these HPLC assays have the specificity required to quantitate hydrocortisone in plasma, and all except one method (32) appear to have the required sensitivity.

The presence of endogenous hydrocortisone has complicated the interpretation of results from many previous single dose hydrocortisone pharmacokinetic studies. As exogenous hydrocortisone can suppress endogenous secretion, pharmacokinetic results obtained after a single hydrocortisone dose should be a combination of exogenous drug disposition and depletion of endogenous concentrations. Drug kinetics obtained following hydrocortisone infusion, during multiple dose studies, or using radiolabelled drug during an infusion would be expected to represent the actual kinetics of the exogenous drug.

The development of assays capable of specifically detecting hydrocortisone has allowed studies to use this principle of steroid suppression advantageously. Endogenous hydrocortisone concentrations are decreased prior to hydrocortisone administration by giving another steroid which suppresses hydrocortisone secretion for a certain period of time. One such steroid used for this purpose is dexamethasone, a powerful synthetic glucocorticoid with approximately 25 times the activity of hydrocortisone (22). Hydrocortisone suppression by dexamethasone is used as a diagnostic test for Cushing's syndrome. As such, several different dosages and dosage regimens of dexamethasone have been examined for degree and length of hydrocortisone suppression. Nishida, et al (44) used an RIA method specific for hydrocortisone to examine suppression at 2 h and 4 h after 0.5, 1, and 2 mg of dexamethasone given at 09:00 h. He found suppression to 24%, 12%, and 17% of basal hydrocortisone concentrations by 4 h with each of the respective dosages, implying that the greatest suppression was obtained with the 1 mg dose. Measurements at 08:00 h following 0.5, 1, or 2 mg of dexamethasone at 23:00 h revealed reductions to 25%, 15%, and 23% of normal hydrocortisone concentrations at 08:00 h. Again, suppression was greatest with 1 mg, although all three dosages exhibited somewhat less suppression at 9 h after dosing than at

4 h after dosing. Mean suppressed hydrocortisone concentrations at 4 h and 9 h were 14 ng/ml and 18 ng/ml, respectively, for the 1 mg dose. Nichols, et al (9) examined the duration of suppression and the influence of time of dexamethasone administration on suppression caused by 0.5 mg doses administered for 2 days at 08:00 h, 16:00 h, or 24:00 h. In each case, suppressed levels were variable and returned to normal on the morning following the dose (32 h for the 24:00 h dose). All results were presented as graphical mean concentrations so the actual concentrations observed are not known, but many suppressed samples appeared to be below the assay sensitivity limit. Actual hydrocortisone suppression is also in doubt due to the use of a nonspecific assay for 17-hydroxycorticosteroids. Similar results were observed by Krieger, et al (7) for 0.5 mg of dexamethasone at 08:00 h or 24:00 h. Suppression from 1.0 mg of dexamethasone at 08:00 h was observed to continue until 24:00 h of the following day. Again, the use of a nonspecific 11-hydrocorticosteroid assay causes doubt as to the actual hydrocortisone suppression observed. Individual suppressed 11-hydroxycorticosteroid concentrations varied between about 20 ng/ml to 40 ng/ml. None of these studies provide long-term quantitation of suppressed hydrocortisone concentrations, and much doubt is still present concerning the relationship between

suppression and amount of administered dexamethasone. These results did suggest, however, that the maximal length of suppression would be obtained by giving dexamethasone before midnight, thereby effectively blocking the early morning secretion peak.

Besides the problems of inadequate assays and the presence of endogenous hydrocortisone, the pharmacokinetic disposition of hydrocortisone appears to be complex. Hydrocortisone distribution in the body involves significant, concentration-dependent binding to two different plasma proteins, significant red blood cell (RBC) uptake of drug, and variable sites and rates of biotransformation. Hydrocortisone binds to plasma transcortin with great affinity, but the binding becomes saturated at plasma drug concentrations greater than 200 ng/ml (45, 46). Binding of hydrocortisone to plasma albumin is characterized by low affinity and high capacity. At plasma hydrocortisone concentrations less than 200 ng/ml, 90% of the drug is bound to transcortin, about 5% is bound to albumin, and less than 5% is free. Above 300 ng/ml, the saturated transcortin binding becomes insignificant, 75% of drug is bound to albumin, and the percentage of free drug increases to 25% (47). As shown in Figure 3 (48), the resultant concentration dependence of the protein binding is biphasic. These changes in protein

binding result in a concentration-dependent drug uptake by the red blood cells. Below 200 ng/ml, approximately 5% of blood hydrocortisone is associated with red blood cells. This percentage increases curvilinearly to an asymptote of approximately 30% at blood drug concentrations above 700 ng/ml (49).

Hydrocortisone elimination is mainly by hepatic biotransformation. Free plasma hydrocortisone is filtered by the kidneys, but undergoes renal tubular reabsorption which does not appear to saturate at plasma drug concentrations below 1200 ng/ml (50, 51). Renal clearance increases from about 5 ml/min below 200 ng/ml (52) to about 50 ml/min above 200 ng/ml (51). The major biotransformation products are shown in Scheme I, page 10. Hydrocortisone is converted to its dihydro- and tetrahydro- derivatives, although as much as 50% of a dose of hydrocortisone may be converted to cortisone with subsequent conversion to dihydro- and tetrahydrocortisone (21, 53, 54). The tetrahydro- derivatives undergo glucuronide conjugation prior to renal elimination. Significant reconversion of cortisone to hydrocortisone has been reported to significantly increase the elimination half-life for hydrocortisone (54). The presence of two major sites of biotransformation, the liver (53) and the kidneys (54), suggests that mode of drug administration may cause differences in rate and extent of biotransformation beyond the usual affects due

to the first pass through the liver. Biliary secretion of hydrocortisone is negligible, and of the metabolites is normally less than 5% (55, 56).

Oral hydrocortisone is well absorbed along the first 200 to 250 cm of the small intestine (57). Drug absorption from the small intestine is proportional to intestinal drug concentrations from 0.05 to 100 μg/ml (58). The high lipophilicity of hydrocortisone plus the large intestinal absorption window result in rapid oral absorption with maximum plasma drug concentrations usually obtained at about 1 hour (19, 20, 55, 59). Enzymatic destruction of hydrocortisone in the gastrointestinal tract has been shown to be insignificant (55). Absolute bioavailability for a 200 mg oral dose has been reported to be approximately 80% (60). This concentration-dependent binding of hydrocortisone to plasma proteins may cause differences in the metabolism of oral doses during the first pass through the liver with different amounts and rates of hydrocortisone absorption. The possible dose dependence of this phenomonon has not yet been studied. One study has suggested that a difference in the intesity of the first-pass effect may exist between males and females (59).

The elimination half-life of hydrocortisone has been shown to be increased in shock (61) and liver disease (62, 63), to be decreased in obesity (63, 64), and may even follow a diurnal variation similar to hydrocortisone secretion (65, 66).

Objectives

As part of an ongoing FDA project to examine the bioequivalence of marketed drug formulations, the present study has been designed to determine the feasibility of, and methodology for, bioequivalence comparisons of marketed hydrocortisone tablets. The objectives of the study are to:

1. Develop and validate an assay for hydrocortisone in blood plasma capable of specific quantitation of hydrocortisone to a lower limit of at least 5 ng per ml of plasma.
2. Develop a method capable of suppressing plasma hydrocortisone in normal adult males to concentrations as low and as constant as possible for the twelve hour period from 08:00 h to 20:00 h.
3. Use the assay and suppression methods to determine the basic pharmacokinetics and the bioavailability of hydrocortisone in several dosage forms.

4. Study the pharmacokinetic behavior of hydrocortisone as a function of dose size and plasma concentration.
5. Examine the pharmacokinetic results, and the effects of suppressed endogenous hydrocortisone concentrations on those results, to determine the feasibility of performing a full scale bioequivalence study of hydrocortisone tablets.

The first objective will be met by combining optimal characteristics of the many HPLC assays presently available for plasma hydrocortisone in order to obtain as rapid, sensitive, and specific of an assay as possible.

To meet the second objective, several different dosages and dosage regimens of dexamethasone will be studied to determine the minimum amount of dexamethasone needed to obtain an acceptable amount and duration of hydrocortisone suppression.

The assay method and the optimal suppression regimen will then be used in a small pilot study of the pharmacokinetics of hydrocortisone tablets at three dosage levels. Results of this study will be used to test the assay method in the presence of hydrocortisone biotransformation products, determine the adequacy of the dexamethasone suppression regimen, as well as provide an initial examination of the dose-dependence of hydrocortisone pharmacokinetics. Results from this study will then be

compared to those resulting from hydrocortisone given as an intravenous bolus injection as well as an oral suspension. Both of these formulations will be given at four dose levels. Results will provide information concerning the influence of tablet dissolution and oral drug absorption on bioavailability parameters, and will allow the calculation of absolute bioavailability estimates for the two oral formulations.

The results of the assay evaluation, the suppression study, and the three hydrocortisone pharmacokinetics studies will then be used to form the basis for the examination of the bioequivalence of marketed hydrocortisone tablets.

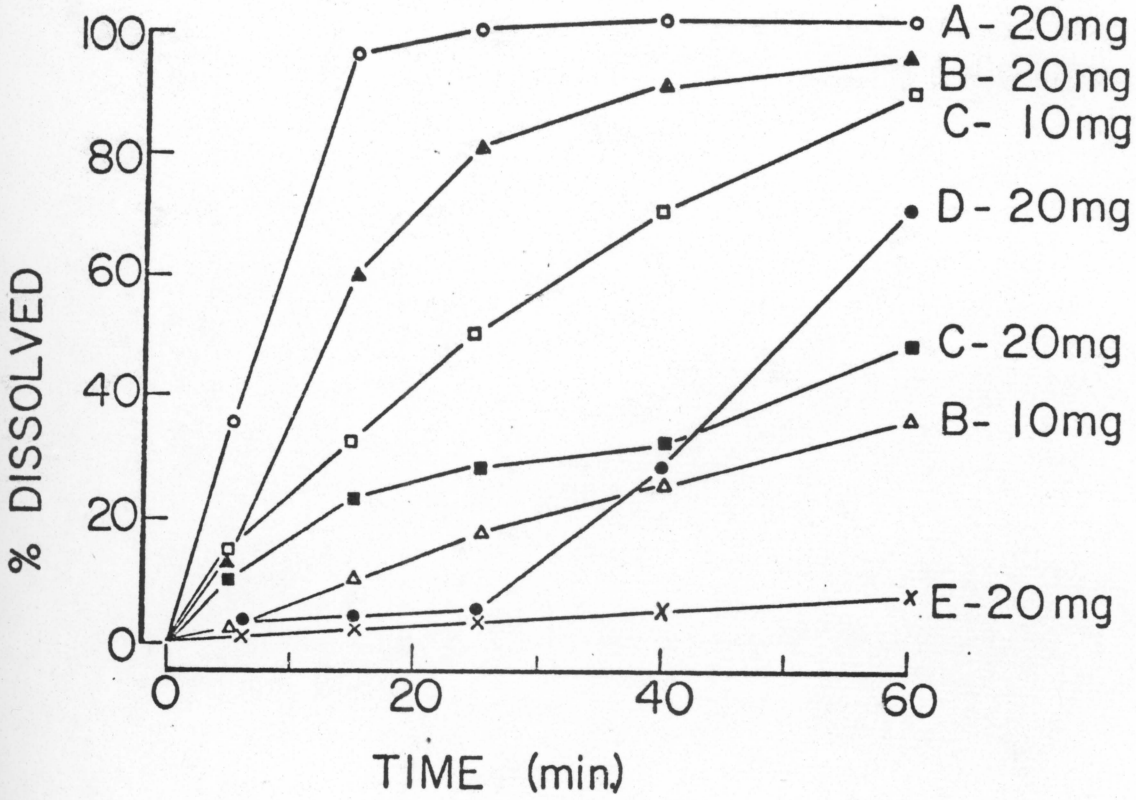


Figure 2 Tablet dissolution rate measurements obtained by USP Rotating Paddle method for 10 mg and 20 mg tablets of Manufacturers A through E. Test Conditions: distilled water (500 ml:10 mg tablets, 1000 ml:20 mg tablets), temperature: 37°C, paddle speed: 50 RPM. The test was performed by the Detroit District Laboratory of the Food and Drug Administration.

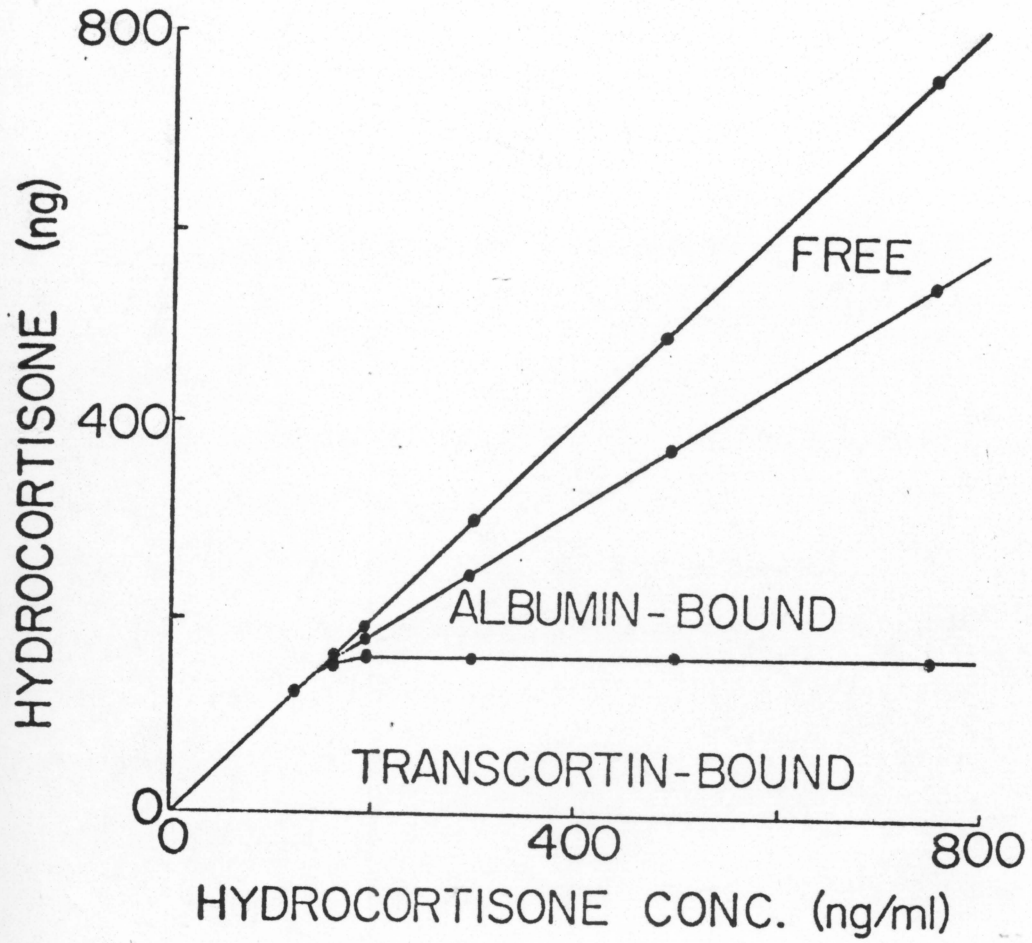


Figure 3 Concentration dependence of the amount of plasma hydrocortisone bound to transcortin, bound to albumin, and free, as determined at 4°C by Daughaday (48).

EXPERIMENTAL

Materials

Dexamethasone Assay - Dexamethasone USP reference standard (U.S. Pharmacopeial Convention, Rockville, MD 20852) was dried at 105°C to constant weight before use. Tetrazolium blue (Sigma, St. Louis, MO 63178, lot 67C-0414) was prepared as a 5 mg/ml solution in 95% ethanol. A 1/9 V/V USP tetramethylammonium hydroxide technical solution (TMAH TS) was prepared by mixing 5.00 ml of 10% TMAH in water (Eastman, Rochester, NY 14650, lot D7C) with 45.00 ml of 95% ethanol. Chloroform and 95% ethanol were analytical grade.

Hydrocortisone Assay - A series of working standard solutions from 0.025 to 15 µg/ml hydrocortisone (Sigma, lot 67C-0302) in 60% aqueous methanol was prepared from a stock solution of 75 µg/ml hydrocortisone in methanol. The internal standard, Δ^4 -Pregnen-17α, 20ß, 21-triol-3, 11-dione (Sigma, lot 20C-2180), was used as a 4 µg/ml solution in methanol. This internal standard will subsequently be referred to as IS. Dichloromethane, dichloroethane, n-butanol, and methanol (Burdick and Jackson Laboratories, Muskegon, MI 49442) were used as received. Sodium

hydroxide, sodium dichromate, and sulfuric acid were reagent grade and were used as received. Anesthetic grade diethyl ether (Baker, Phillipsburg, NJ 08865) was used as received. Distilled water was further purified using a Barnstead PCS water purification system (Boston, MA 02132). Methanolic and aqueous solutions were stored at -10°C and 5°C, respectively, and were brought to room temperature before use.

Drugs

The dexamethasone formulation used in all studies was Decadron elixir (Merck, Sharp, and Dohme, West Point, PA 19486, lot A3240) which contained 0.1 g of dexamethasone per 1 ml of 5% alcoholic solution. All subjects received TB skin tests using Aplisol (Parke-Davis, Detroit, MI 48232), a diluted tuberculin purified protein derivative. Hydrocortisone in Study 2 was administered as 10 mg tablets (Merck, Sharp, and Dohme, lot V2478, FDA sample 77-85-968). Intravenous hydrocortisone in Study 3 was A-hydrocort (Abbott, North Chicago, IL 60064, lot 11-573-AF) which was hydrocortisone sodium succinate in a 2 ml univial. Reconstitution immediately before injection resulted in a solution of 50 mg hydrocortisone activity per ml of solution. Hydorocortisone for Study 4 was Cortef (Upjohn, Kalamazoo, MI 49001, lot 027FP), a sterile aqueous intramuscular suspension of 50 mg/ml hydrocortisone. The suspension was administered in orange juice (Minute Maid,

Coca Cola, Co., Houston, TX 77001) to make the drug acceptable as an oral dosage form.

Equipment

Blood Sampling - Blood samples were removed through B-D Vacutainer 21 gauge, 1½" thin-wall sample needles directly into non-sterile B-D Vacutainer 10 ml evacuated blood collection tubes containing 143 USP units of sodium heparin (Becton-Dickinson, Rutherford, NJ 07070). Blood samples were centrifuged without transfer in a Dynac tabletop centrifuge (Clay-Adams, Parsippany, NJ 07054). Plasma samples removed after centrifugation were placed in rubber-stoppered glass tubes and stored at -20°C in a Sears Coldspot freezer (Model 1543, Sears, Roebuck, Chicago, IL 60684).

UV Absorbance - Ultraviolet wavelength absorbances were read on a Cary 16 spectrophotometer (Cary, Monrovia, CA 91016). A Cary 14 spectrophotometer (Cary, Monrovia, Ca 91016) was used for scans of absorbance versus wavelength.

Assay - Centrifugation of samples during assay was done in an IEC model 748 floor centrifuge (IEC, Boston, MA 02132). Samples were mixed using a Vortex-Genie mixer (S/P, McGaw Park, IL 60085). Evaporation steps were performed using compressed nitrogen dispensed through an N-Evap (Organomation, Shrewsburg, MA 01532).

HPLC - Mobile phase was pumped through the HPLC system by an Altex 110A constant flow pump (Altex, Berkeley, CA 94710), which was calibrated, prior to use, to deliver flow rates within 3% of the actual flow rate setting. The Rheodyne 7125 injector was fitted with a 20 µl fixed-volume sample loop. After injection, the sample passed through a 2.1 X 70 mm pre-column (Co-Pell ODS, 30-8 µm) and then through a 4.6 X 250 mm Lichrosorb C18, 10 µm analytical column (Altex). Effluent was monitored at 254 nm with a Waters 440 fixed wavelength absorbance detector (Waters, Milford, MA 01757). The resulting chromatograms were recorded on a Perkin-Elmer 023 chart recorder (Perkin-Elmer, Norwalk, CT 06856) using a 10 mV detector output and a 1 mV recorder input with record attenuation as needed.

Assay Methodology

Dexamethasone - Decadron elixir was assayed for dexamethasone content by the USP Blue Tetrazolium Assay for steroids (67).

Hydrocortisone - Plasma samples from Study 1 were assayed for hydrocortisone content at the Biopharmaceutics Division of the Food and Drug Administration in Washington, DC (subsequently referred to as the "FDA Labs") using a plasma hydrocortisone assay developed at the FDA Labs (34). This assay method employs fluorescence detection of a dansylhydrazine derivative

of hydrocortisone. The derivative is separated from other plasma components using organic extraction followed by normal-phase HPLC. This assay method will subsequently be referred to as the fluorescence method. The published version of the fluorescence method is included in this thesis as Appendix 1. All plasma samples for Study 2 were divided into two equal fractions prior to the initial freezing of the samples. One set of samples was sent to the FDA Labs for analysis by the fluorescence method. The duplicate set of plasma samples from Study 2 , as well as all plasma samples from Studies 3 and 4 were analyzed at the University of Wisconsin School of Pharmacy using a modified version of a previously described HPLC assay for hydrocortisone (41). Plasma (1.0 ml), containing 200 ng or 400 ng of IS (depending on the expected hydrocortisone concentration) and 0.1 ml of 2N aqueous sodium hydroxide, was extracted with 10 ml of dichloromethane, vortex-mixed for 1 minute, and then centrifuged for 15 minutes at 500 g in a polyethylene-stoppered, ground glass centrifuge tube. Plasma and the creamy interface were aspirated off, and the organic layer was transferred to a clean tube and evaporated to dryness under nitrogen, with the tube immersed in a room temperature water bath. Tube walls were rinsed with 1 ml of dichloromethane which was then

evaporated as before. The residue was reconstituted in 100 µl of the HPLC mobile phase (60% aqueous methanol), vortexed for 1 minute, and centrifuged for 5 minutes. A 30 µl aliquot of this solution was injected into the HPLC. Mobile phase was degassed prior to use by sonication for 10 minutes while exposed to a 380 mmHg vacuum, and was pumped through the HPLC at a flow rate of 1 ml/min resulting in a pump pressure of approximately 700 psi. Elution times for IS and hydrocortisone were 8 minutes and 10 minutes, respectively. Recorder chart speed was 20 cm/h. A second injection was performed for plasma samples with hydrocortisone:IS peak height ratios of less than 0.1 (hydrocortisone concentrations less than 25 ng/ml), and the peak height ratios from the two injections were averaged.

A separate standard curve for plasma hydrocortisone concentrations was run with each batch of samples by adding 0.2 ml of either water or the appropriate working standards to 1 ml aliquots of dexamethasone-suppressed plasma and then analyzing as described previously. Concentrations used in various standard curves were 10, 25, 50, 58, 200, 208, 500, 536, 700, 1000, 1500, and 3000 ng/ml. For each assay, four concentrations were chosen which would span the expected plasma hydrocortisone

concentrations. The same suppressed plasma sample was used for all four concentrations and as a blank, which allowed correction to be made for the endogenous suppressed hydrocortisone in that plasma sample. The hydrocortisone:IS peak height ratio for the suppressed plasma was subtracted from the peak height ratios of plasma containing added hydrocortisone. The standard curve was prepared using these corrected peak height ratios. This assay method will subsequently be referred to as the UV assay method.

UV Assay Development

When work on the assay first began, glassware was soaked and scrubbed in Alconox brand soap followed by 10 tap water rinses, 10 distilled water rinses, and drying at 75°C. Early in the assay development, the Alconox treatment was replaced by soaking glassware in a cleaning solution (92g sodium dichromate, 800 ml concentrated sulfuric acid, and 800 ml water). As this change caused a significant reduction in background noise for chromatograms of eluted samples, this new washing procedure was used for all subsequent work.

Previous assays have evaporated the extractant under air or nitrogen with the tube containing the extractant immersed in a 40 to 50°C water bath (32, 33, 39, 41). The following

experiment was performed to determine the effect of rapid extractant evaporation (high nitrogen flow rate with 40°C water bath) on variability in concentration results. Methanolic solutions of hydrocortisone (0.67 ml of 750 ng/ml) and IS (0.02 ml of 10 µg/ml) were pipetted into eight centrifuge tubes. Four tubes were placed in a 40°C water bath and evaporated at a high nitrogen flow rate. Four tubes were placed in a room temperature (approx. 20°C) water bath and evaporated with a gentle stream of nitrogen. Each tube was reconstituted in 0.1 ml of 60% aqueous methanol and a 30 µl aliquot of the reconstitution was injected into the HPLC. The variance in the resulting hydrocortisone:IS peak height ratios for the two evaporation methods was then compared.

The extent of reconstitution of the evaporated sample in the 0.1 ml of 60% aqueous methanol was examined as follows. Three standard solutions in 60% aqueous methanol were prepared. Each contained 500, 1000, or 5000 ng/ml hydrocortisone and all contained 2000 ng/ml IS. Since the reconstitution step results in a 10-fold increase in the sample concentration, direct injections of these standard solutions into the HPLC would correspond to hydrocortisone concentrations of 50, 100, and 500 ng/ml and an IS concentration of 200 ng/ml in

reconstituted samples. Methanolic solutions of hydrocortisone and IS were added to three triplicate sets of centrifuge tubes so that each set contained 50, 100, or 500 ng of hydrocortisone and all tubes contained 200 ng of IS. The methanol was evaporated and the residue was reconstituted in 0.1 ml of 60% aqueous methanol, vortex-mixed for 1 minute, and centrifuged for 15 minutes at 500 g. Aliquots of 30 μl from each tube were injected into the HPLC and the hydrocortisone:IS peak height ratios (PHR) from the three sets of 3 tubes were compared to the peak height ratios from direct injections of the standard solutions. Hydrocortisone reconstitution at each concentration was calculated by Equation 1.

$$\% \text{ reconstituted} = \frac{\text{mean PHR for tubes}}{\text{PHR for std. soln}} \times 100 \qquad (1)$$

Percent recovery of hydrocortisone and IS from plasma was determined at 50, 100, and 500 ng/ml hydrocortisone and 200 ng/ml IS. IS (200 ng) was added to 9 of 10 tubes containing 1 ml of dexamethasone-suppressed plasma. Fifty, 100, and 500 ng/ml of hydrocortisone were each added to three of the 9 tubes. The tenth tube served as a blank. All tubes were assayed by the UV assay method with a slight modification.

The volume of extractant evaporated for each tube was exactly 8.0 ml. Hydrocortisone peak heights were corrected for hydrocortisone in the blank plasma, and corrections for the 8 ml organic aliquot were made to both the hydrocortisone and IS peak heights. The obtained peak heights were compared to peak heights from direct injections of the three standard solutions. The percent recovery for IS was calculated from Equation 2.

$$\% \text{ recovery IS} = \frac{\text{mean IS peak height of extracts}}{\text{mean IS peak height of std soln}} \times 100 \quad (2)$$

Hydrocortisone peak height results for extracted tubes and for standard solutions were plotted versus hydrocortisone concentration. The percent recovery for hydrocortisone was determined by Equation 3.

$$\% \text{ recovery for hydrocortisone} = \frac{\text{slope of extracted tubes}}{\text{slope of std. solutions}} \quad (3)$$

The two common organic solutions used to extract hydrocortisone from plasma are dichloromethane and a 2:1 mixture of dichloromethane and ether. These two solutions were compared for extraction efficiency and suitability for use in the assay.

The ultraviolet absorbance spectra of four organic solvents against air, and for hydrocortisone in each solvent against pure solvent, were obtained. The results were used to select a suitable mobile phase and to determine the correct wavelength for hydrocortisone detection. Three of the four solvents which had been used as mobile phases in previous HPLC assays for hydrocortisone were 45% aqueous methanol (41), 97:3 dichloromethane:methanol (39), and 93:6.6:0.4 dichloroethane:n-butanol:water (34). The fourth solvent was pure methanol. Solutions of 50 μg/ml hydrocortisone were used for the spectra of hydrocortisone in each solvent. The absorbance scan was from 350 nm to 200 nm using a Cary 14 spectrophotometer.

Estimates of chromatographic resolution (R_s), capacity (k'), efficiency (n), selectivity (α), and the height equivalent to a theoretical plate (H) were calculated from a typical chromatogram using the following Equations,

$$R_s = \frac{2\,(d_{HC} - d_{IS})}{w_{HC} + w_{IS}} \qquad (4)$$

$$k' = \frac{d_{HC} - d_{SF}}{d_{SF}} \quad \text{or} \quad \frac{d_{IS} - d_{SF}}{d_{SF}} \qquad (5)$$

$$n = 16(d_{HC}/w_{HC})^2 \text{ or } 16(d_{IS}/w_{IS})^2 \qquad (6)$$

$$\alpha = (d_{HC} - d_{SF})/(d_{IS} - d_{SF}) = \frac{k'_{HC}}{k'_{IS}} \quad (7)$$

$$H = \frac{\text{column length}}{n} \quad (8)$$

where d_{HC}, d_{IS}, and d_{SF} are distances (mm) from injection to the center of the hydrocortisone peak, to the center of the internal standard peak, and to the start of the solvent front, respectively, and w is the peak width at the baseline (68).

The effect of the HPLC pre-column on peak resolution was examined by comparing two injections of a solution of hydrocortisone and IS, one injection with the pre-column in the system, the other with the pre-column replaced by a 7 inch piece of HPLC tubing.

UV Assay Validation

Six repetitions of a standard curve at hydrocortisone concentrations of 5, 10, 25, 50, 100, 200, 400, and 700 ng/ml were run on different days to determine between-day variability. Within-day variability was examined by performing three repetitions of a standard curve at hydrocortisone concentrations of 10, 50, 200, 500, 700, 1000, 1500, and 3000 ng/ml within one assay run.

The fluorescence and UV assay methods were each used to assay triplicate sets of dexamethasone-suppressed plasma samples containing added hydrocortisone at four different concentrations. This allowed comparison of the two assays using plasma samples which did not contain significant amounts of hydrocortisone biotransformation products. One set of triplicate samples at the four concentrations were assayed by the fluorescence method at the FDA Labs. The other set was assayed by the UV method at the University of Wisconsin School of Pharmacy. The actual sample concentrations were unknown to the persons performing the assays. Four solutions were prepared by diluting 0.02, 0.1, 0.4, or 1.0 ml of 100 µg/ml aqueous hydrocortisone to 10.0 ml with water. Aliquots of 0.5 ml of each of these solutions were mixed with 9.5 ml of dexamethasone-suppressed plasma, resulting in plasma concentrations of approximately 10, 50, 200, and 500 ng/ml. Six 1.5 ml aliquots of each solutions were placed in separate tubes and frozen. Samples were divided into two identical sets and one set was shipped in dry ice to the FDA Labs. Assay was as described previously.

All plasma samples from Study 2 were assayed by both the fluorescence and UV assay methods. Results were compared by paired t-test and by plotting the concentrations obtained by one method versus the other. As metabolites would be expected

to cause less interference with the fluorescence assay, a plot of the ratio of the concentrations obtained by the two methods versus sample time was made to see if the ratio exhibited any time-dependent trends which could be attributed to metabolite formation.

Human Subjects

Subjects for all studies were male volunteers either employed by, or attending, the University of Wisconsin. All subjects were asked to read a consent form describing, in detail, the purpose, procedures, requirements, dangers, and regulations regarding the particular study for which they had volunteered. Copies of the consent forms for Studies 1 through 4 are included as Appendix 2a through 2d. After signing the consent form, subjects were asked to fill out a "Subject Interview Eligibility Questionnaire" to determine suitability for the study. A "Subject Record" was prepared for each subject to keep track of the subject, as well as his progress through the study. Data for each subject were recorded on a "Subject Data Sheet". Copies of these three forms have been included as Appendix 3. After giving informed consent, the subjects underwent physical examinations which included blood (Sequential Multiple Analysis by 12 channel Technicon Autonalyzer System, Technicon Industrial Systems, Tarrytown NY, 10591) and urine analyses. As the administration of steroids can cause a

latent case of tuberculosis to become active, all subjects were required to show a negative reaction to a tuberculin skin test before participating in any of the studies. Prior to the start of the study, subjects were given either a sheet or a card with a summary of directions they were to follow. Copies of these summaries are included as Appendix 4. Subject details and study affiliations are given in Table 1a and 1b.

Study Protocols

Diagrams outlining the protocols for Study 1 and for Studies 2, 3, and 4 are presented in Figures 4 and 5, respectively. Food and liquid restrictions for Study 1 were identical to those for Studies 2, 3, and 4 as shown in Figure 5. Dosage designs for Studies 2, 3, and 4 are shown in Table 2. Subjects were ambulatory during blood sampling in all studies. All blood samples were centrifuged immediately. After separation from cellular components, plasma was stored at -20°C until assayed.

Study 1 - In this two-day study, nine subjects were randomly assigned to three groups of three subjects each. Plasma samples were obtained from all subjects before and after dexamethasone administration. As shown in Figure 4, each group (A through C) received one of three different dexamethasone

regimens. Basal values for plasma hydrocortisone concentrations were determined from plasma samples obtained at 08:00, 10:00, 12:00, 14:00, 16:00, 18:00, and 20:00 h on Day 1 of the study. All dexamethasone doses were given orally as 10 or 20 ml of Decadron elixir followed by 180 ml of water. Plasma samples for Day 2 were obtained at the same times as those on Day 1.

Study 2 - The three subjects who participated in this study were chosen from the subjects in Study 1. Each subject received hydrocortisone doses of 10, 30, and 50 mg at least one week apart in a a randomized design. All doses were given as multiples of 10 mg tablets. Blood samples were withdrawn from a forearm vein just prior to hydrocortisone administration, and then at 0.25, 0.5, 1.0, 1.5, 2, 3, 4, 5, 6, 7, and 8 h after dosing.

Study 3 - The six subjects in this study each received intravenous hydrocortisone sodium succinate at doses of 5, 10, 20, and 40 mg at least one week apart in a randomized block design. Drug was injected as a bolus into a forearm vein. Subjects drank 180 ml of water immediately after drug administration in order to duplicate the required water ingestion in the

two oral hydrocortisone studies. Blood samples were removed from a contralateral forearm vein just prior to hydrocortisone administration, and then at 0.17, 0.33, 0.50, 0.75, 1.0, 1.5, 2, 3, 4, 5, 6, and 8 h after dosing.

Study 4 - The eight subjects in this study were randomly assigned to four groups of two subjects each. Each subject received an oral hydrocortisone suspension in orange juice at doses of 5, 10, 20, and 40 mg of hydrocortisone at least one week apart in a randomized block design. Fifteen seconds prior to dosing, 60 ml of orange juice were added to glass beakers containing the hydrocortisone suspension (0.1 to 0.8 ml). The contents were swirled continuously for 15 seconds at which time the contents were immediately swallowed, rapidly followed by three-40 ml rinses of the beaker with additional orange juice. Beakers were rinsed with exactly 50.0 ml of methanol which was assayed for residual hydrocortisone by direct injections into the HPLC. Residual hydrocortisone concentrations were obtained from standard curves of hydrocortisone peak height versus concentration for directly-injected standard solutions. Blood samples were withdrawn from a forearm vein just prior to hydrocortisone administration, and then at 0.25, 0.5, 1.0, 1.5, 2, 3, 4, 5, 6, 7, 8, and 12 h after dosing.

Data Analysis

Study 1 - The plasma hydrocortisone concentrations of the samples were plotted against time on linear graph paper. The extent of hydrocortisone suppression by each dexamethasone regimen was examined in relation to unsuppressed concentrations observed during Day 1. Suppression was evaluated in terms of the length of suppression, the absolute concentrations of the suppressed samples, and the constancy of the suppressed concentrations. Statistical comparisons of the concentrations from the three treatments were by paired t-test (95% confidence level). Correction was made for the loss of statistical power caused by pairwise comparisons of three means. The probability of committing a Type I error when more than one pairwise comparison is made (α') is given by Equation 9,

$$\alpha' = 1 - (1 - \alpha)^c \qquad (9)$$

where α is the confidence level for the tabled t-value and c is the number of pairwise comparisons made (69). In this case, c=three comparisons (A-B, B-C,A-C) and α' was to be 0.05. Thus, the tabled t-value at $\alpha = 0.02$ was used in performing the t-test. Without the correction, a t-value

at $\alpha = 0.05$ would show significance at only the 85% confidence level. The dexamethasone regimen exhibiting the best suppression was to be used subsequently in Studies 2, 3, and 4.

Study 2, 3, and 4 - Initial estimates of pharmacokinetic parameters were obtained from plots of the logarithms of plasma hydrocortisone concentration versus time for each subject using standard curve-stripping procedures. These estimates were used to determine the appropriate pharmacokinetic models to be used to describe the data for each study.

Model-independent estimates of the area under the plasma hydrocortisone concentration versus time curves from 0 h to infinite time ($AUC_{trap}^{0\rightarrow\infty}$) were calculated using the trapezoidal method (70a) as shown in Equation 10,

$$AUC_{trap}^{0\rightarrow\infty} = \sum_{i=0}^{n-1} \frac{(C_{i+1} + C_i)(t_{i+1} - t_i)}{2} + \frac{C_n}{k} \qquad (10)$$

where C is plasma drug concentration at time t, n is the number of sample points, and k is the overall elimination rate constant. This calculation, as well as others presented later, was performed on a Casio FX502P programmable calculator. Software prepared specifically for these calculations have been included as Appendix 5. Program 541

of Appendix 5 was used for the trapezoidal AUC calculations. Concentrations for the duplicate sets of Study 2 plasma samples as obtained by the fluorescence and UV assay methods were compared by Student's paired t-test. Factor effects on pharmacokinetic parameters from all three studies were tested for significance using analysis of variance (ANOVA). Effects shown to be significant by ANOVA were further analyzed using Tukey's significant difference test (71a).

Figure 4 Diagram summarizing blood sampling periods and dexamethasone dose sizes and timing for Treatments A, B, and C of Study 1.

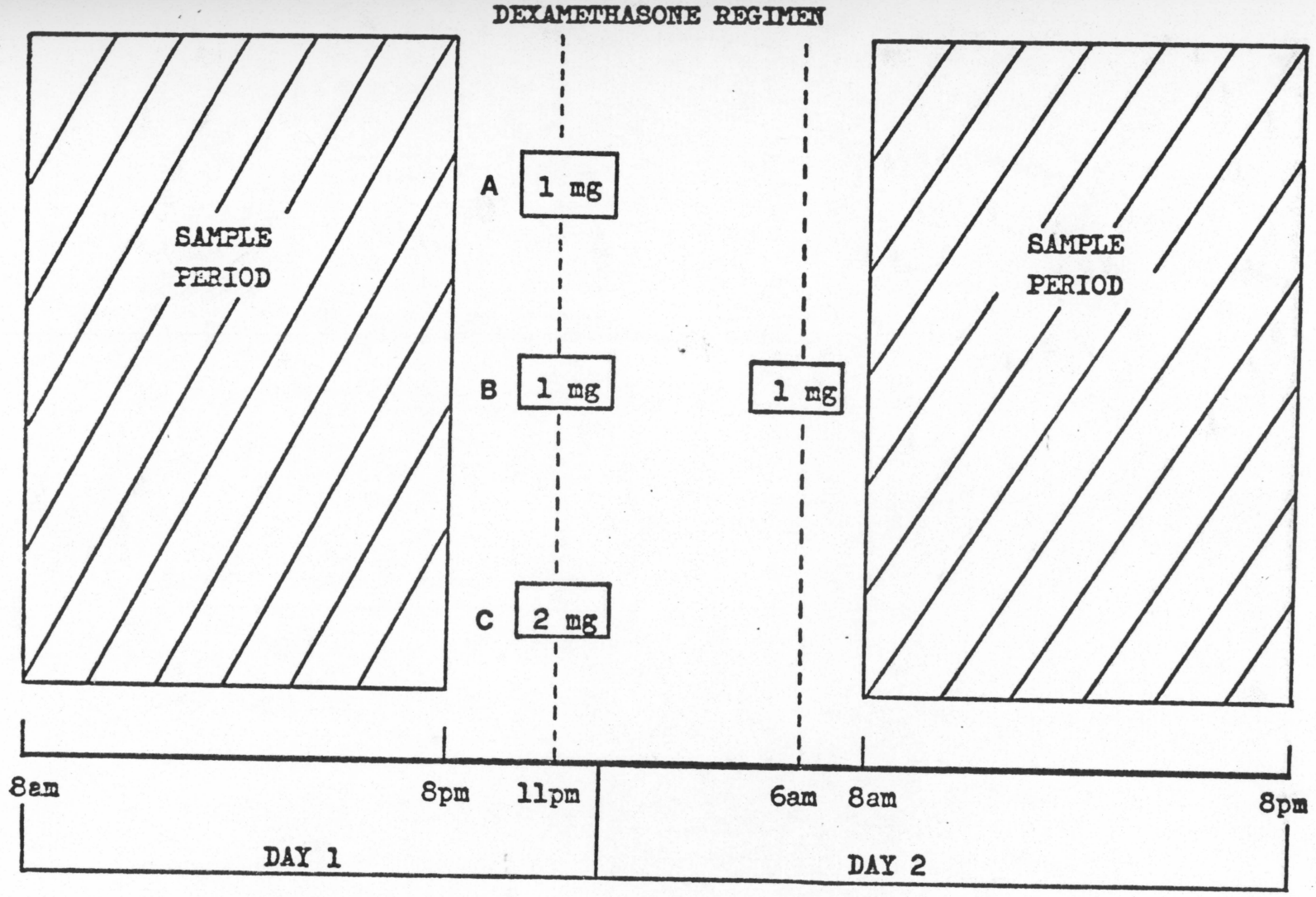

DEXAMETHASONE REGIMEN
SAMPLE PERIOD
A 1 mg
B 1 mg
C 2 mg
1 mg
SAMPLE PERIOD
8am
8pm
11pm
6am
8am
8pm
DAY 1
DAY 2

Figure 5 Diagram summarizing food and water restrictions, the blood sampling period, and times of drug administration for Studies 2, 3, and 4.

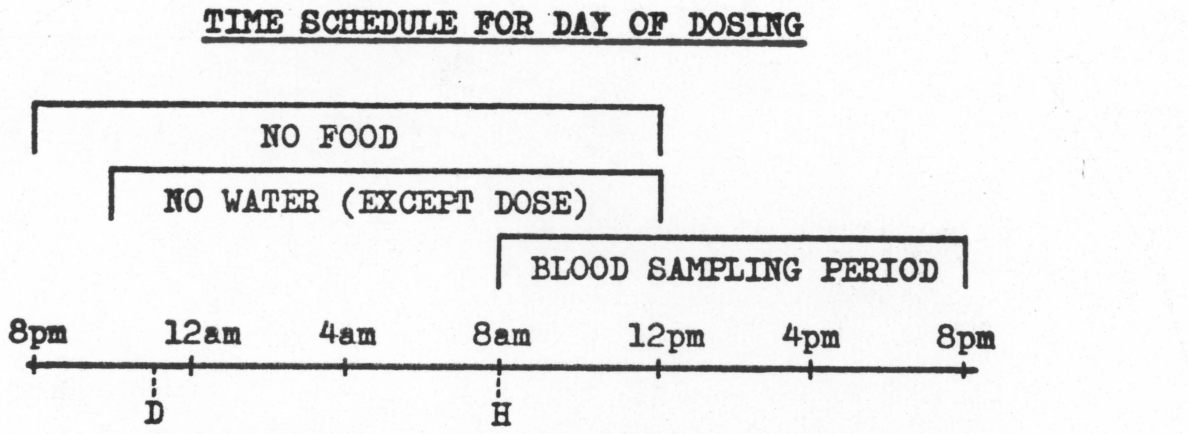


Table 1a Subject Details and Study Affiliations.

Study	Group	Subject	Subject Number	Age (yr)	Height (cm)	Weight (kg)	BSA (m^2)
1	A	a	1	28	185	73	1.96
	A	b	2	22	180	80	1.99
	A	c	3	25	170	63	1.73
	B	d	4	37	168	74	1.84
	B	e	5	31	170	77	1.88
	B	f	6	28	178	66	1.82
	C	g	7	39	185	75	1.98
	C	h	8	22	180	75	1.94
	C	i	9	45	178	64	1.80
			Mean	31	177	72	1.88
			SD	8	6	6	0.09
2	NONE	h	1	22	180	75	1.94
		f	2	28	178	66	1.82
		i	3	45	178	64	1.80
			Mean	32	179	68	1.85
			SD	12	1	6	0.08

Table 1b Subject Details and Study Affiliations.

Study	Group	Subject	Subject Number	Age (yr)	Height (cm)	Weight (kg)	BSA (m^2)
3	NONE	j	1	26	175	77	1.92
		k	2	22	196	82	2.15
		l	3	22	175	64	1.78
		m	4	23	180	82	2.02
		n	5	21	188	77	2.03
		f	6	29	178	68	1.85
			Mean	24	182	75	1.96
			SD	3	8	7	0.13
4	I	j	1	26	175	77	1.92
		o	2	23	175	68	1.82
	II	l	3	22	175	64	1.78
		p	4	35	178	73	1.90
	III	q	5	39	173	75	1.89
		r	6	35	173	67	1.80
	IV	s	7	24	183	73	1.94
		t	8	25	170	69	1.80
			Mean	29	175	71	1.86
			SD	7	4	4	0.06

Table 2 Dosage designs for Studies 2, 3, and 4. Hydrocortisone dose sizes shown are in mg.

Study	Group	Subject	WEEK 1	2	3	4
2	NONE	1	10	30	50	--
		2	30	50	10	--
		3	50	10	30	--
3	NONE	1	5	10	20	40
		2	10	40	20	5
		3	20	5	40	10
		4	40	20	10	5
		5	5	20	40	10
		6	10	40	5	20
4	I	1	10	5	20	40
		2	10	5	20	40
	II	3	5	40	10	20
		4	5	40	10	20
	III	5	20	10	40	5
		6	20	10	40	5
	IV	7	40	20	5	10
		8	40	20	5	10

RESULTS

Dexamethasone Assay

The results from the USP assay of the Decadron elixir for dexamethasone content showed the Decadron elixir to contain 100.2 μg of dexamethasone per ml of elixir. This value was within 0.2% of the 0.1 mg/ml label claim. Thus, all dosages were calculated using the label claim.

UV Assay Development

The ultraviolet absorbance scan showed hydrocortisone to exhibit a broad absorbance maximum centered at 240 to 243 nm in all four solvents. As absorbance was still near maximum at 254 nm, the detection wavelength of 254 nm chosen for use in the UV assay method resulted in only a small decrease in sensitivity. The UV cutoff was acceptable for all solvents except the 97:3 dichloromethane:methanol, which exhibited a large increase in absorbance between 310 and 200 nm. Aqueous methanol was chosen as the mobile phase for the assay.

Dichloromethane was chosen as the organic extractant for the assay. Although the 2:1 mixture of dichloromethane: diethyl ether gave a similar percent recovery of hydrocortisone

from plasma (78%), the extractant-plasma interface was more diffuse, and more extraneous peaks were seen in chromatograms of extracted plasma using the extractant mixture. The mixture ratio would also have been difficult to keep constant due to volatility.

The rate of extractant evaporation was found to have a small but significant effect on assay variability. Results are summarized in Table 3. Evaporation of the dichloromethane extract at room temperature using a gentle stream of nitrogen gas gave a smaller percent error in the peak height ratios for the four tubes than did the evaporation at 40°C using a high nitrogen flow rate. The mean peak height ratio for the slow evaporation was also found to be significantly larger (t-test, 95% confidence level) than for the fast evaporation. Thus, the slow evaporation method was chosen for the actual assay.

Results for the determination of percent recovery for hydrocortisone and IS are shown in Table 4. Recoveries for hydrocortisone and IS were 82% and 83%, respectively. The linear concentration dependence of hydrocortisone extraction over the concentration range examined can be seen in the plot of the hydrocortisone peak heights for the extracted tubes and standard solutions versus concentration as shown in Figure 6.

Results for the study of percent reconstitution in the final assay step are shown in Table 5. Reconstitution was found to be essentially complete with values of 95%, 96%, and 92% at the three hydrocortisone concentrations examined.

Estimates of chromatographic parameters obtained from the chromatogram shown in Figure 7 are presented in Table 6. Selectivities greater than 1.1 and capacity factors between 1 and 10 are normally considered optimal for analytical quantitation. The results obtained for this assay conform to these suggested values.

Removal of the HPLC pre-column caused chromatographic peak resolution to increase by 9% for the two injections examined. As resolution of hydrocortisone and IS was already acceptable, the pre-column was left in the system in order to extend the useful life of the analytical column.

UV Assay Validation

Shown in Figures 7 and 8 are representative chromatograms for the UV and fluorescence assay methods, respectively. Chromatograms from the UV method were superior to those from the fluorescence method in terms of lack of solvent front interference, baseline stability, and retention time. Furthermore, the absence of dual isomeric peaks in the UV assay greatly simplified peak quantitation.

The linearity of peak height ratio with hydrocortisone concentrations from 5 to 3000 ng/ml for the UV Assay can be seen in the standard curves shown in Figures 9 and 10. Mean concentration values, standard deviations, and percent errors for these curves are presented in Table 7a and 7b. Coefficients of variation were less than 10% at all concentrations tested and were similar for between-day and within-day runs. Experimental values were within 4% of actual concentrations at all concentrations between 25 and 3000 ng/ml. The limit of detection for a signal to noise ratio of 2.5 was approximately 4 ng/ml.

Results of the "unknown plasma sample" assay comparison are presented in Table 8. There is some doubt as to the accuracy of the actual concentrations due to a malfunctioning automatic pipettor used to prepare the samples. Agreement between the two assays was good except for the 500 ng/ml samples. The UV assay gave a lower percent error for the triplicate samples at all except the 50 ng/ml sample.

Individual and mean hydrocortisone plasma concentrations for the three subjects at the three dosage levels for Study 2 are shown in Table 9a through 9c. Concentrations obtained by the two assay methods at each sample time within each dosage level (11 sample times at each dosage level) were

compared using the paired t-test. A significant difference (95% confidence level) between the two assays was shown at only 2 of the 33 sampling times. Figure 11 shows all Study 2 concentrations obtained by the UV assay plotted against those obtained using the fluorescence method. Assay equivalence is indicated by a slope not significantly different from 1.0, and an intercept not significantly different from 0.0. The possibility of metabolite interference in the UV assay was examined by plotting the ratio of the fluorescence and ultraviolet concentrations versus sample time. The plot, shown in Figure 12, shows no significant time-dependent trend for this ratio.

Subjects

Results of the physical examinations indicated that vital signs and laboratory values for subjects in all four studies were normal. All TB skin tests were negative. No subject reported a history of hepatic or renal disease, and all subjects were free from known drug allergies. All subjects successfully completed all phases of the studies for which they had volunteered. Subject 3 in Study 3 missed week 2 of that study. Weeks 2, 3, and 4 for this subject were performed one week later than all other subjects in Study 3. Subjects 1,4, and 6 in Study 4 began one week of antibiotic therapy immediately following week three of the study due to personal

illnesses. Week 4 of Study 4 for these three subjects was performed one week later than all other subjects in Study 4 in order to avoid any possible effects of the antibiotics on the pharmacokinetics of hydrocortisone.

Study 1

Plasma hydrocortisone concentrations as obtained by fluorescence assay are presented in Table 10. Mean concentrations for each treatment are plotted as a function of sample time for Day 1 and Day 2 in Figures 13 and 14.

Concentrations from Day 1 appeared to follow the normal hydrocortisone diurnal rhythm. Initial late morning concentrations of about 160 ng/ml decreased during the day to approximately 50 ng/ml in early evening, although this decrease exhibited large fluctuations during the day. Concentrations on Day 1 were similar for all subjects within all treatment groups.

Hydrocortisone suppression was evident in all subjects on Day 2. Concentrations for all samples, except one, from 08:00 to 16:00 h were less than 20 ng/ml. Concentrations of 10 ng/ml or less were present in 43%, 86%, and 67% of all Day 2 samples from Treatments A, B, and C, respectively. All treatments caused similar suppression until 18:00 h when concentrations in Treatment A began to increase. Concentrations

and percent errors for Treatment A subject and treatment means were greater than those for Treatments B and C in all cases. Treatment B and C caused essentially identical suppression throughout the sampling period. The t-test, corrected for power loss as explained in Experimental, showed no significant differences between treatments for the Day 2 results. This is probably due to the similarity in the results between 08:00 and 16:00 h for all treatments.

All treatments lowered hydrocortisone to concentrations which would allow Studies 2, 3, and 4 to be performed. Treatment A was eliminated because the suppression was too short in duration. Treatment B required an early morning dexamethasone dose which would have interruped the sleep cycles of the subjects and might have caused interference in the assay for, or the pharmacokinetics of, hydrocortisone. Thus, Treatment C, 2 mg of dexamethasone at 23:00 h, was chosen for use in Studies 2, 3, and 4.

A copy of the published version of this study is included as Appendix 6.

Study 2

Concentration Results - Individual and mean plasma hydrocortisone concentrations from all three doses, obtained using

both assay procedures, are shown in Table 9a through 9c. Concentrations obtained prior to hydrocortisone administration have been subtracted from all concentrations obtained after dosing for each subject.

Pharmacokinetic Modeling - Mean values of the logarithm of concentration versus time for the 10, 30, and 50 mg doses are plotted in Figure 15. These plots, as well as similar graphs for each subject, exhibited linear drug elimination phases from approximately 1 to 8 h, and fairly linear drug absorption phases between 0 and 1 h, at all three doses. Thus, a one-compartment pharmacokinetic model with 1st order absorption and elimination was used to describe the individual data sets. Least squares linear regression was performed on the linear drug elimination phase for each subject using Program 445 of Appendix 5. An initial graphical estimate of the elimination rate constant, k_{el}, for each subject was obtained from the slope of the line, m, by the relation:

$$k_{el} = -2.303\ m \qquad (11)$$

A graphical estimate of the absorption rate constant, k_a, for each subject was obtained by the method of residuals (70b) using the elimination regression line and the absorption

phase data falling below that line. The third parameter needed for computer analysis of the data was FD/V, which was obtained from,

$$C_o = \frac{FD}{V}\left[\frac{k_a}{k_a - k_{el}}\right] \qquad (12)$$

where C_o is the antilogarithm of the y-intercept of the elimination phase linear regression line. Program 111 of Appendix 5 was used to calculate the k_a and C_0 values. Individual concentration versus time data sets were analyzed by nonlinear regression using the program NREG (72) on a Univac 1100 digital computer. Concentration data were analyzed without mathematical weighting and also using weighting by their reciprocal. Maximum concentrations between 0.51 h and 1.5 h were consistently underestimated by this model. Furthermore, k_a values were highly variable and exhibited unacceptable confidence intervals. A lag time of 0.5 h was added to the computer analysis to help accomodate the early maximum concentrations, and the k_{el} input was fixed at the value obtained by linear regression. As the reciprocal data weighting gave lower coefficients of determination than similar treatment of non-weighted data, and resulted in trends in plots of the least squares residual versus time, the weighting factor was excluded from all subsequent analysis of this data.

This version of the computer analysis gave satisfactory parameter confidence limits and coefficients of determination, and was free from trends in the least squares residual versus time plots. The card deck printout, program compilation, and a sample of the computer printout for this program have been included as Appendix 9. Thus, the data could be adequately represented by (73),

$$C_t = FD/V\left[k_a/(k_a-k_{el})\right]\left[e^{-k_{el}(t-t_{lag})} - e^{-k_a(t-t_{lag})}\right] \qquad (13)$$

where C_t is the plasma hydrocortisone concentration at time t after dosing, F is the fraction of oral dose D reaching the systemic circulation, V is the apparent volume of drug distribution, and t_{lag} is the lag time before drug is observed in the bloodstream. This equation, and the computer-generated mean and individual values of FD/V, k_a, k_{el}, and t_{lag}, were used to construct the curves shown in Figures 15 and 16, respectively. The proposed pharmacokinetic model adequately described the mean data at each dose obtained by both assay methods. Use of this model was also supported by the high coefficients of determination for each subject as shown in Table 11a through 11c and also by the agreement between the computer curve and the actual data as represented

by the results for Subject 3 in Figure 16. Further pharmacokinetic analysis was performed in accordance with this model. Absorption half-time, $t_{\frac{1}{2}}a$, and elimination half-life, $t_{\frac{1}{2}}el$, were obtained from the respective rate constants by $t_{\frac{1}{2}} = \ln 2/k$. The maximum drug concentration in plasma, C_{max}, and the time at which this maximum occurred, t_{max}, were calculated from Equations 14 and 15, respectively.

$$C_{max} = FD/V^{(k_a/k_{el})^{\left[k_a/(k_{el}-k_a)\right]}} \tag{14}$$

$$t_{max} = \frac{2.303}{(k_{el} - k_a)} \log (k_{el}/k_a) + t_{lag} \tag{15}$$

Elimination rate-corrected $AUC_{trap}^{0\rightarrow\infty}$ values were calculated as $AUC_{trap}^{0\rightarrow\infty}\ k_{el}$. Dose-normalized values of C_{max}, FD/V, $AUC_{trap}^{0\rightarrow\infty}$, and $AUC_{trap}^{0\rightarrow\infty}\ k_{el}$ were obtained.

Pharmacokinetic Results - Individual and mean pharmacokinetic parameter values are presented in Tables 11a through 11c and 12a through 12c. These results were statistically analyzed for significant dose, subject, and assay effects using three-way analysis of variance (ANOVA). Significant effects were further analyzed using Tukey's test. Results of the statistical analysis are given in Table 13.

Intersubject variability in all parameters was quite high, with significant subject differences in all parameters except k_a. Variation in k_a was large, which may have prevented the detection of significant factor effects. Parameters such as k_{el}, t_{max}, and t_{lag}, which were expected to be dose-independent, were significantly affected by dose size. The elimination rate constant for the 50 mg dose was significantly smaller than for the 10 and 30 mg doses. The mean t_{max} values showed significant increases corresponding to dose increases of 10 to 30 mg and 30 to 50 mg. Although t_{lag} failed to show a trend as dose was increased, values of t_{lag} at all three dose levels were significantly different. Parameters expected to increase with increasing dose (FD/V, C_{max}, AUC) were dependent on dose size with the exception that C_{max} failed to show a significant difference between the 30 and 50 mg doses. However, all three of these parameters, when dose-normalized, exhibited decreasing trends with increased dose, indicating a lack of direct proportionality to dose. This non-proportionality can also be seen in the plot of $AUC_{trap}^{0\rightarrow\infty}$ versus dose in Figure 17 where the relationship is linear but does not go through the origin.

As further support of the equivalence of the two assay methods, the assay factor of the ANOVA failed to show a significant effect on any pharmacokinetic parameter.

A manuscript describing this study has been submitted for publication. A copy of the submitted manuscript is included as Appendix 7.

Study 3

Concentration Results - Individual and mean plasma hydrocortisone concentrations for all four doses of Study 3 are shown in Table 14a through 14d. As in Study 2, all concentrations have been corrected for hydrocortisone found in plasma prior to dosing. Results from one treatment for each of two subjects were omitted from all averaged results. These two data sets are presented in Table 14e. The concentration versus time profile for the 10 mg dose in Subject 5 indicated that the subject had received the hydrocortisone dose extravascularly. Initial plasma hydrocortisone concentrations for the 20 mg dose in Subject 6 were approximately ten times greater than corresponding concentrations for the 40 mg dose. The cause of these high concentrations is unknown. Reassay of the samples gave identical results. Ill effects from this dose were not observed by either the

subject or the investigator. Missing values for results from these two data sets were replaced with "pseudo-observations" using the Yates method for missing observations (71b), and ANOVA was carried out in the normal manner using the augmented data.

Pharmacokinetic Modeling - Mean values of the logarithm of concentration versus time for the 5, 10, 20, and 40 mg doses are shown in Figure 18. These plots, as well as similar graphs for each subject, exhibited a biphasic elimination profile at all four doses with the initial rapid distribution phase lasting for less than 30 minutes after dosing. This type of profile can be described by the two compartment pharmacokinetic model with immediate drug input into the central compartment and first order elimination from the central compartment. This model was chosen to describe the concentration versus time data for Study 3. To decrease the possibility of endogenous hydrocortisone having an effect on the results, concentrations less than 10 ng/ml were omitted from all pharmacokinetic modeling. The two compartment model can be represented mathematically by (73b),

$$C_t = Ae^{-\alpha t} + Be^{-\beta t} \qquad (16)$$

where C_t is total plasma hydrocortisone concentration (bound and free) at time t after dosing, α and β are first order rate

constants governing the fast and slow phases of drug loss from the central compartment ($\alpha > \beta$), and A and B are concentration coefficients. Initial graphical estimates of these four parameters were obtained for each subject using Program 201 of Appendix 5. β was calculated from the slope, m_{slow}, of the slow elimination phase line.

$$\beta = 2.303\ m_{slow} \tag{17}$$

A value for B was obtained from the antilogarithm of the y-intercept of this line. Using the method of residuals, the slow phase line, and the fast phase data, values of α and A were obtained from the slope (m_{slow}) and y-intercept, respectively, of the residual line

$$\alpha = 2.303\ m_{fast} \tag{18}$$

These initial parameters were used to calculate the parameters required for computer analysis,

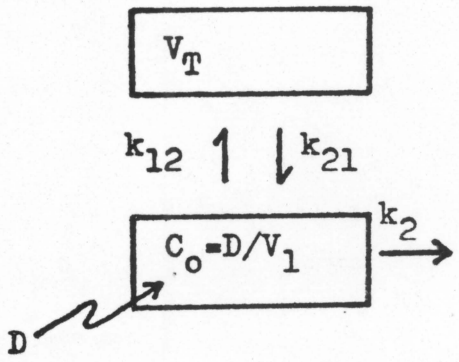
$$k_{21} = \frac{A\beta + B\alpha}{A+B} \tag{19}$$

$$k_2 = \frac{\alpha\beta}{k_{21}} \tag{20}$$

$$k_{12} = \alpha + \beta - k_{21} - k_{12} \tag{21}$$

$$\frac{D}{V_1} = A + B \tag{22}$$

where D is the administered dose, V_1 is the apparent volume of distribution of the central compartment, and k_{12}, k_{21}, and k_2 are the rate constants for drug movement from the central to the peripheral compartment, from the peripheral to the central compartment, and for elimination from the central compartment, respectively. A schematic drawing of the model appears below,



where C_0 is the extrapolated plasma drug concentration at the time of dosing and V_T is the apparent peripheral compartment distribution volume.

Improved estimates of k_{12}, k_{21}, k_2, D/V, α , and β were obtained by nonlinear regression of each concentration versus time data set using the program NREG on a Univac 1100 digial computer. As a further precaution against interference from endogenous hydrocortisone, the regression was performed using non-weighted data to give stronger emphasis to high concentrations. Coefficients of determination for the regression of

each data set using this model are given in Table 15a through 15d and show the excellent agreement between the model and the data. Parameter confidence limits, as computed by NREG, were acceptable and plots of the least squares residuals versus time did not reveal any noticeable trends. Mean and individual values of A, B, α , and β were used in Equation 16 to construct the curves shown in Figures 18 and 19, respectively, using Program 204 of Appendix 5. Excellent agreement was seen between such curves and both the mean and individual concentration versus time data.

Further pharmacokinetic analysis was performed in accordance with the two-compartment model. Calculations were performed using Program 202 of Appendix 5. Half-lives for the fast elimination phase, for the slow elimination phase, and for elimination from the central compartment were obtained from the respective rate constants by $t_{\frac{1}{2}} = \ln 2/k$. The apparent peripheral compartment distribution volume, V_T, and the apparent volume of distribution at steady state, Vd_{ss}, were obtained from Equations 23 and 24.

$$V_T = V_1 \frac{k_{12}}{k_{21}} \tag{23}$$

$$Vd_{ss} = V_1 \left[1 + \frac{k_{12}}{k_{21}}\right] \tag{24}$$

Plasma clearance was calculated as $V_1 k_2$. Values of dose-normalized trapezoidal area under the curve values, AUC/D, and trapezoidal area under the curve corrected for elimination rate differences, AUCβ, were also obtained.

Pharmacokinetic Results - Individual and mean pharmacokinetic parameter values are presented in Tables 15a through 15d and 16a through 16d. These results were statistically analyzed for significant dose and subject effects using two-way analysis of variance (ANOVA). Significant effects were further analyzed using Tukey's test. Results of the statistical analysis are given in Table 17.

Kinetic rate constants for the distribution and elimination of hydrocortisone are shown in Table 15a through 15d. The rate constants for drug loss from the central compartment, k_{12} and k_2, were not affected by dose size. The distribution phase rate constant, α, was also independent of dose size. However, increases in dose significantly decreased the rate constant for return of drug to the central from the peripheral compartment, k_{21}, and the overall elimination rate constant, β. This dependence suggests that a shift in equilibrium towards more drug in the peripheral compartment is occurring as dose is increased.

The dependence of the apparent volumes of distribution on hydrocortisone dose size is illustrated in Figure 20. While the apparent central compartment volume, V_1, was constant over the 5 to 40 mg dose range, the apparent peripheral compartment volume, V_T, increased linearly with increasing dose, resulting in a linear increase in Vd_{ss} as dose was increased. These increases in V_T and Vd_{ss} were found to be significant for the 40 mg dose only.

The overall elimination half-life, $t_{\frac{1}{2}}\beta$, exhibited dose-dependent behavior similar to that seen with Vd_{ss}. Figure 21 shows the high correlation between these two parameters ($r=0.771$).

Although the mean values of V_1 and k_2 were essentially constant with respect to dose, intersubject variability in these parameters caused the mean value of plasma clearance, V_1k_2, to increase with dose, with the value at 40 mg showing a significant difference. However, this trend was not consistently observed in the V_1k_2 versus dose results for the individual subjects.

All doses exhibited significantly different AUC values. Both mean and individual AUC values were linearly related to dose with y-intercepts not significantly different from zero,

as shown in Figure 22. Doubling the dose at 5, 10, and 20 mg resulted in respective AUC increases of 1.9, 1.9, and 1.6. The elimination rate-corrected area values, AUC_{β}, exhibited a negative deviation from linearity as dose was increased.

The subject blocking variable did not have a significant effect ($p<0.05$) on any of the pharmacokinetic parameters.

A manuscript describing Study 3 is in preparation, a copy of which has been included as Appendix 8.

Study 4

Dose Verification - The hydrocortisone dose in Study 4 was administered as a suspension in orange juice. Some of the viscous liquid remained in the dosing containers after drug administration. Residue in the dosing containers was analyzed for hydrocortisone content as described in the Experimental Section. The results, shown in Table 18, indicate that residual hydrocortisone did not exceed 1% of the administered dose in any container. As amounts of 1% or less were considered negligible, dose correction was not necessary.

Concentration Results - Individual and mean plasma hydrocortisone concentrations for all four doses of Study 4 are shown in Table 19a through 19d. As in Studies 2 and 3, all concentrations have been corrected for hydrocortisone found in plasma prior to dosing.

Pharmacokinetic Modeling - Plots of the mean values of the logarithm of concentration versus time for the 5, 10, 20, and 40 mg doses are shown in Figure 23. Individual and mean data sets were characterized by fast drug absorption phases followed by log-linear elimination phases. Drug absorption in this study was more rapid than in Study 2. Although sample times in both studies were the same, only 44% of the Study 2 data sets had one or two data points before maximum concentrations were reached, whereas 84% of Study 4 data sets had only one or two points. The shortage of absorption phase data in Study 2 resulted in large uncertainty in the calculated absorption parameters. This shortage in the majority of the Study 4 data sets prevented the calculation of absorption parameters for this study. Consequently, computer analysis of the Study 4 data was not attempted, and absorption rate estimates were not obtained.

The elimination rate constant, k_{el}, was obtained from the slope of the linear elimination phase by Equation 11. The slope was obtained from a least squares linear regression of the elimination phase data using Program 445 of Appendix 5. As in Study 3, all concentrations less than 10 ng/ml were omitted from the data analysis. The C_{max} and t_{max} values

were the actual observed maximum concentrations and times of maximum concentration. Trapezoidal AUC results were used to calculate $AUCk_{el}$. Dose-normalized values of AUC, $AUCk_{el}$, and C_{max} were also calculated. Estimates of k_a, t_{lag}, and FD/V could not be obtained.

Pharmacokinetic Results - Figure 24 shows individual data for Subject 3 at all four doses. The elimination phase line in this figure was obtained by linear regression. The elimination phase line in Figure 23 was obtained from the mean k_{el} value at each dose. Individual and mean values for the pharmacokinetic parameters are presented in Table 20a through 20d. Results of the statistical analysis of these values are shown in Table 21.

Mean values of k_{el} decreased linearly as dose was increased, with the values at 5 and 10 mg significantly different from those at 20 and 40 mg. This linear decrease caused $t_{½}el$ to increase asymptotically. No such trends could be observed for the individual k_{el} and $t_{½}el$ values for each subject. Mean C_{max} and AUC values increased linearly with dose, as shown in Figure 25, and both exhibited large, positive y-intercepts similar to that observed in

Figure 17 of Study 2. Doubling the dose at 5, 10, and 20 mg resulted in AUC increases of 1.5, 1.9, and 1.6, respectively. This lack of direct proportionality to dose was also evident in the observed decreases in $AUCk_{el}/D$ and AUC/D as dose was increased. The mean t_{max} values tended to increase at higher doses, although much scatter was present due, in part, to the t_{max} values being actual observations rather than graphical estimates.

Subject-effect was significant on all parameters. However, differences were mostly just between the high and low values and the rank order of the subject differences appeared to be random. There was a significant group effect on $t_{\frac{1}{2}}el$, t_{max}, and all AUC parameters. There was a pronounced repetition in rank order for group differences in these parameters.

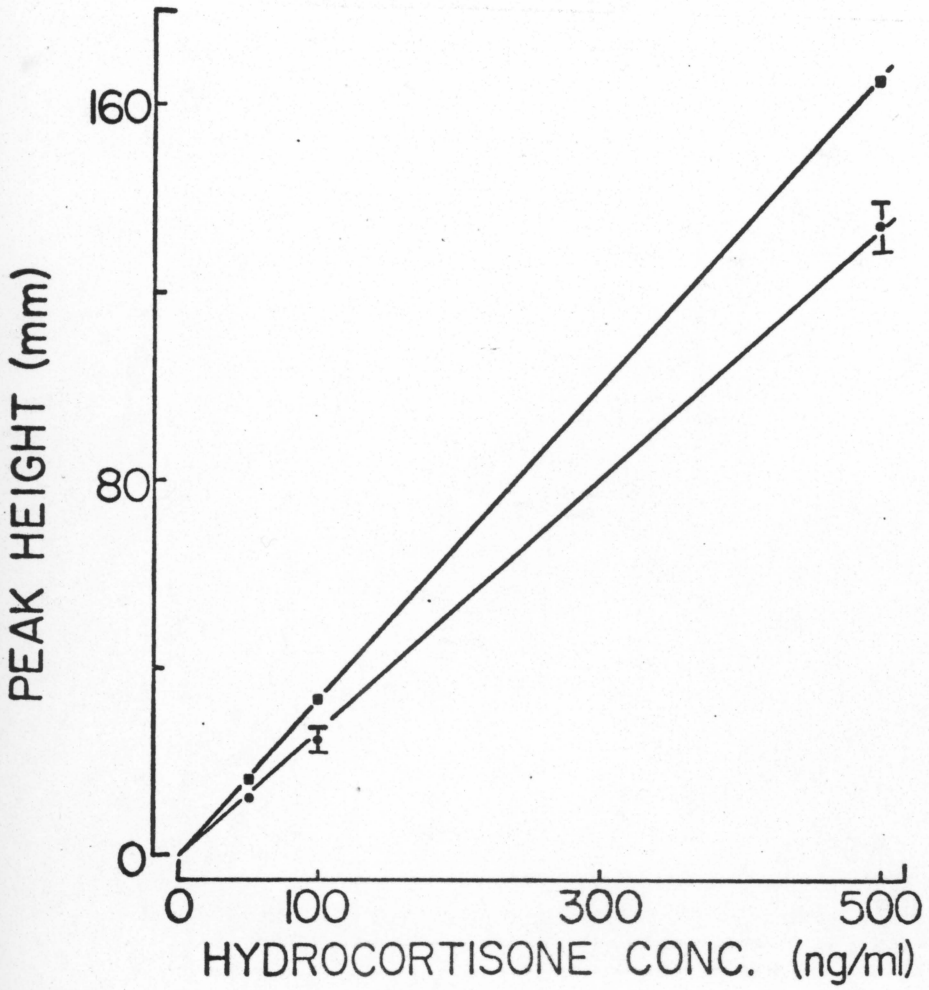


Figure 6 Peak heights for direct HPLC injections of hydrocortisone standard solutions (■) and for hydrocortisone recovered from plasma (●) (mean ±1 SD, n=3) at hydrocortisone concentrations of 50, 100, and 500 ng/ml. Data are presented in Table 4. Standard solutions:

$Y = 0.3300\ (\pm 0.0004)X - 0.01\ (\pm 0.12)$ $\quad r^2 = 1.000$

plasma extracts:

$Y = 0.270\ (\pm 0.005)X - 1.5\ (\pm 1.6)$ $\quad r^2 = 0.9974$

$\%\ \text{recovery} = \frac{0.270}{0.330} = 81.8\%$

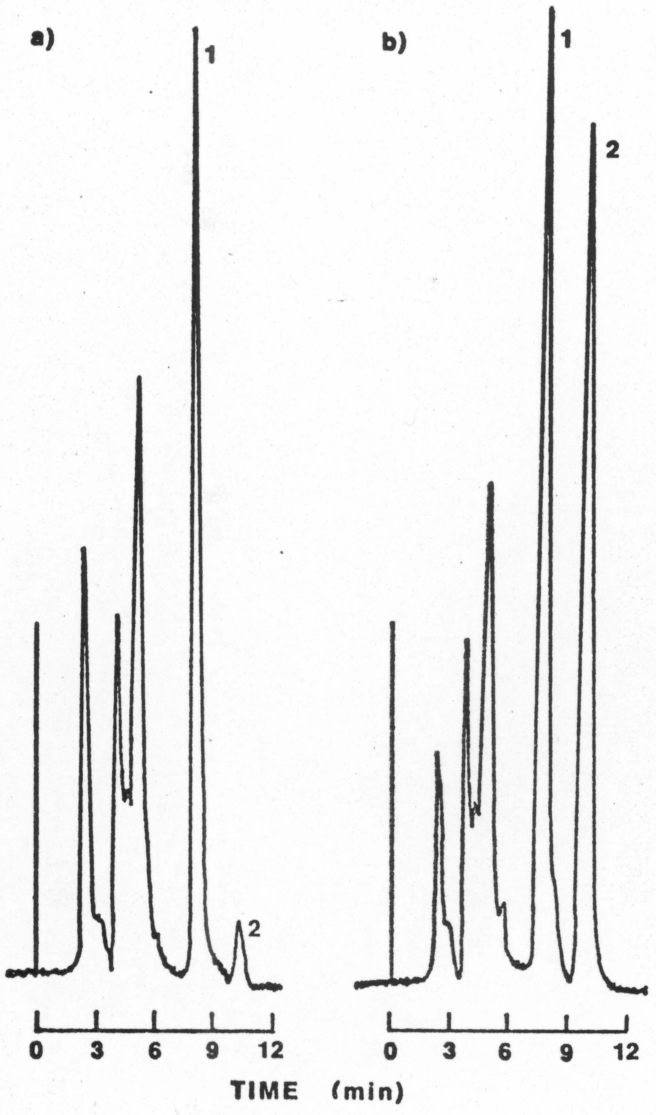


Figure 7 Representative chromatograms for UV assay method for plasma samples containing 200 ng/ml internal standard (1) and, a) 11 ng/ml endogenous hydrocortisone (2), or b) 149 ng/ml dosed and endogenous hydrocortisone (2).

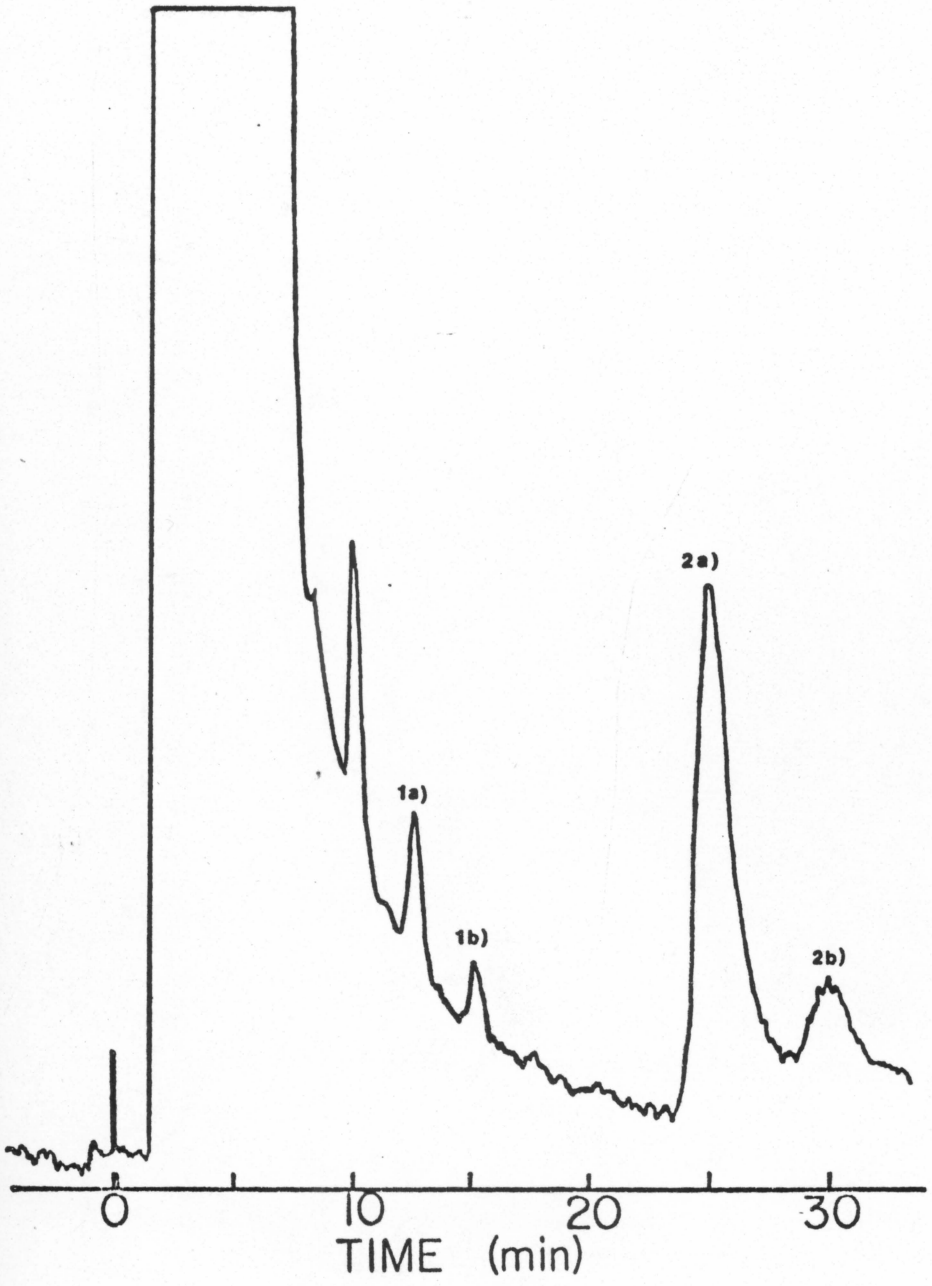


Figure 8 Representative chromatogram from fluorescence assay method for plasma sample containing 100 ng/ml hydrocortisone (1a and 1b) and 100 ng/ml internal standard (2a and 2b).

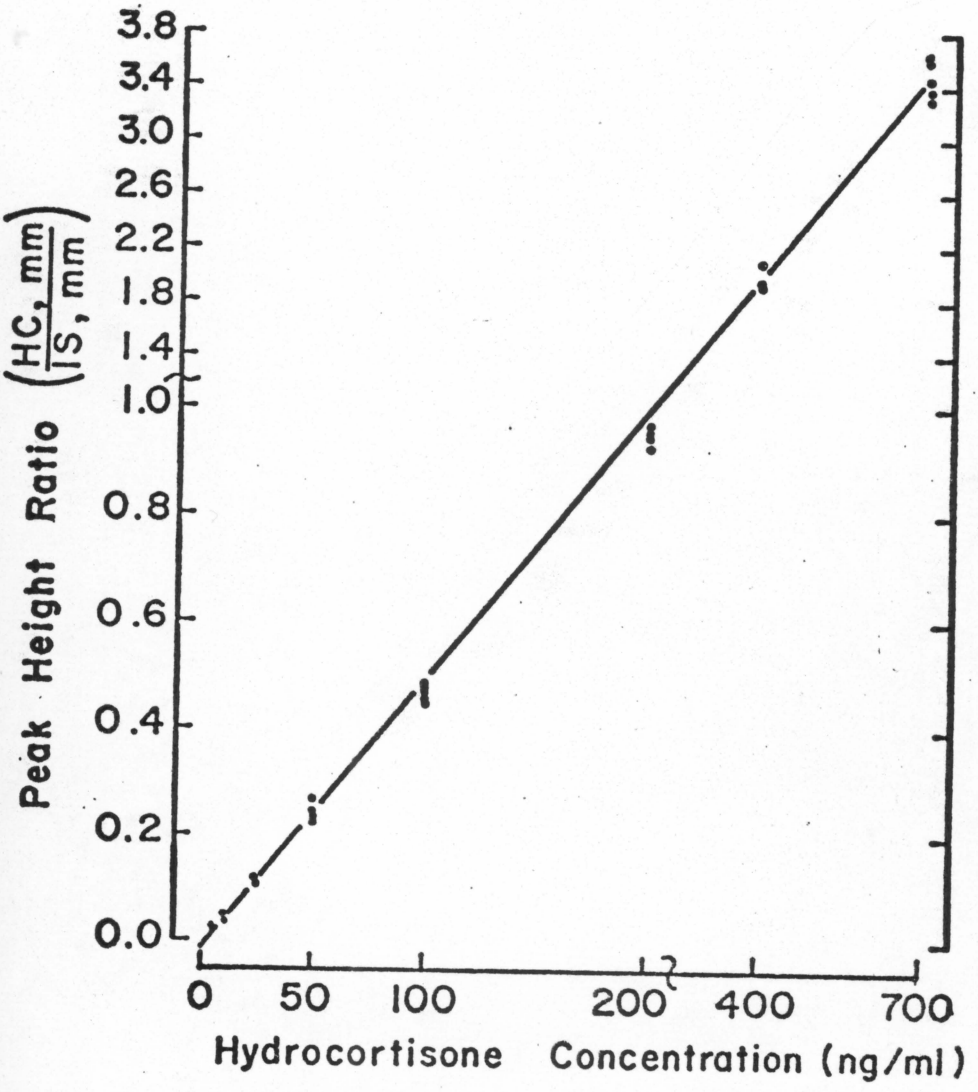


Figure 9 Standard curve for UV assay method obtained from six repetitive analyses at each concentration performed on different days. Data are presented in Table 7a. (HC: hydrocortisone, IS: internal standard)

$Y = 0.00497\ (\pm 0.00003)X - 0.014\ (\pm 0.010)$

$r^2 = 0.9978$ $n = 6$

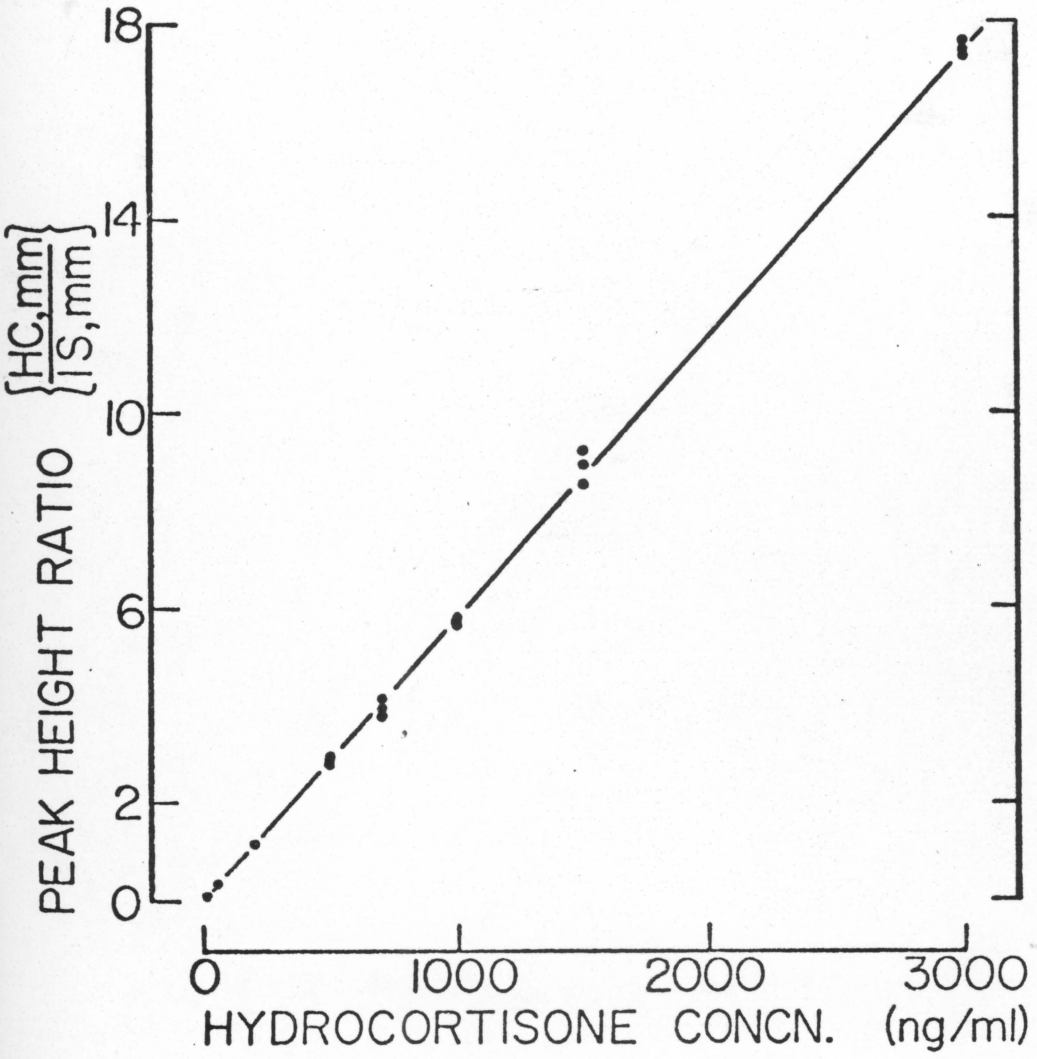


Figure 10 Standard curve for UV assay method obtained from analysis of triplicate samples at each concentration during one assay run. Data are presented in Table 7b. (HC: hydrocortisone, IS: internal standard)

$Y = 0.00582\ (\pm 0.00003)X - 0.02\ (\pm 0.04)$

$r^2 = 0.9993$ $n = 3$

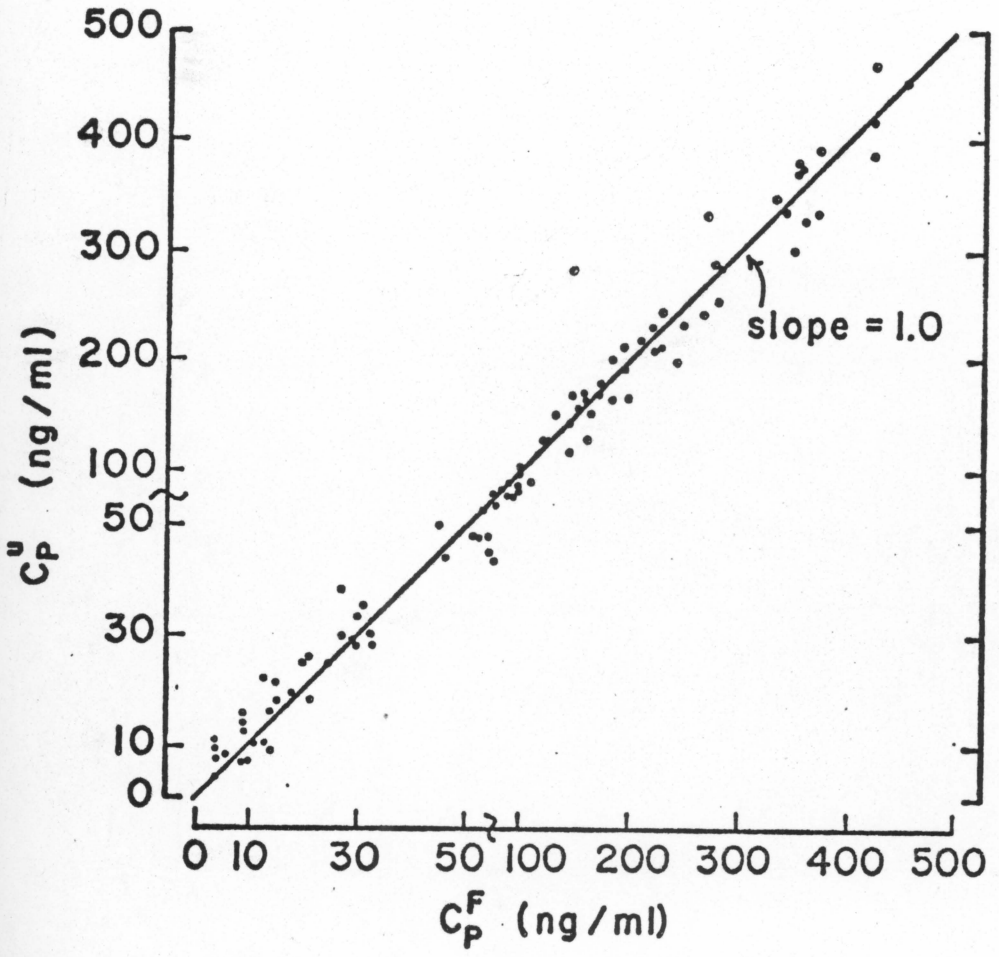


Figure 11 Plasma hydrocortisone concentrations from Study 2 as obtained by the UV assay method (C_p^u) versus those obtained by the fluorescence assay method (C_p^F).

$Y = 0.992\ (\pm 0.018)X - 2\ (\pm 3)$

$r = 0.986$ $n = 94$

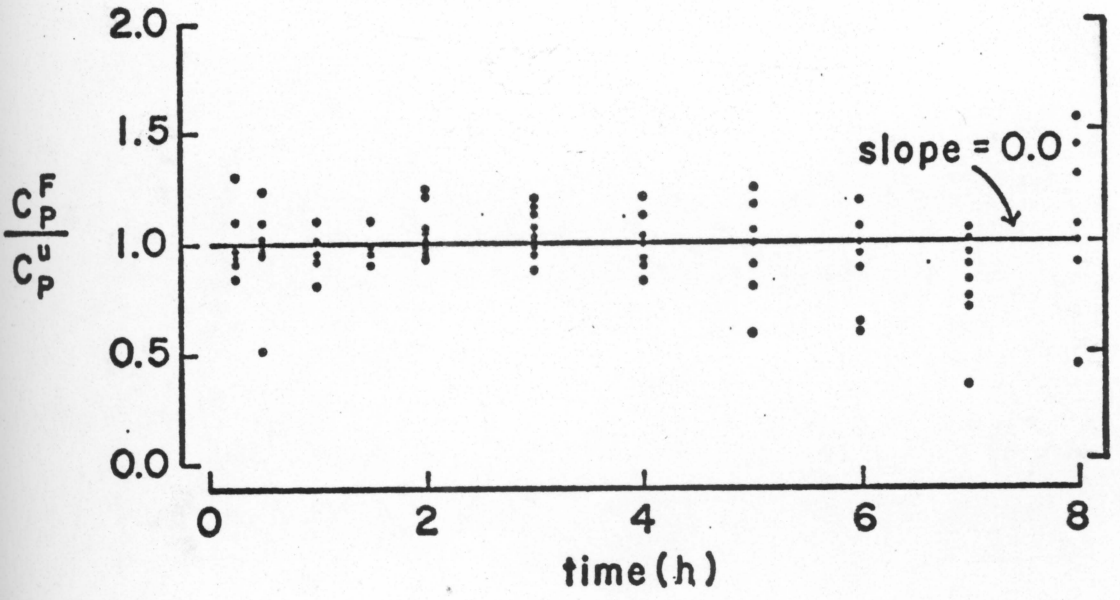


Figure 12 Ratio of plasma hydrocortisone concentrations obtained by the fluorescence (C_p^F) and UV (C_p^u) assay methods versus blood sampling time.

$Y = -0.009\ (\pm 0.007)X + 1.02\ (\pm 0.03)$

$n = 94$

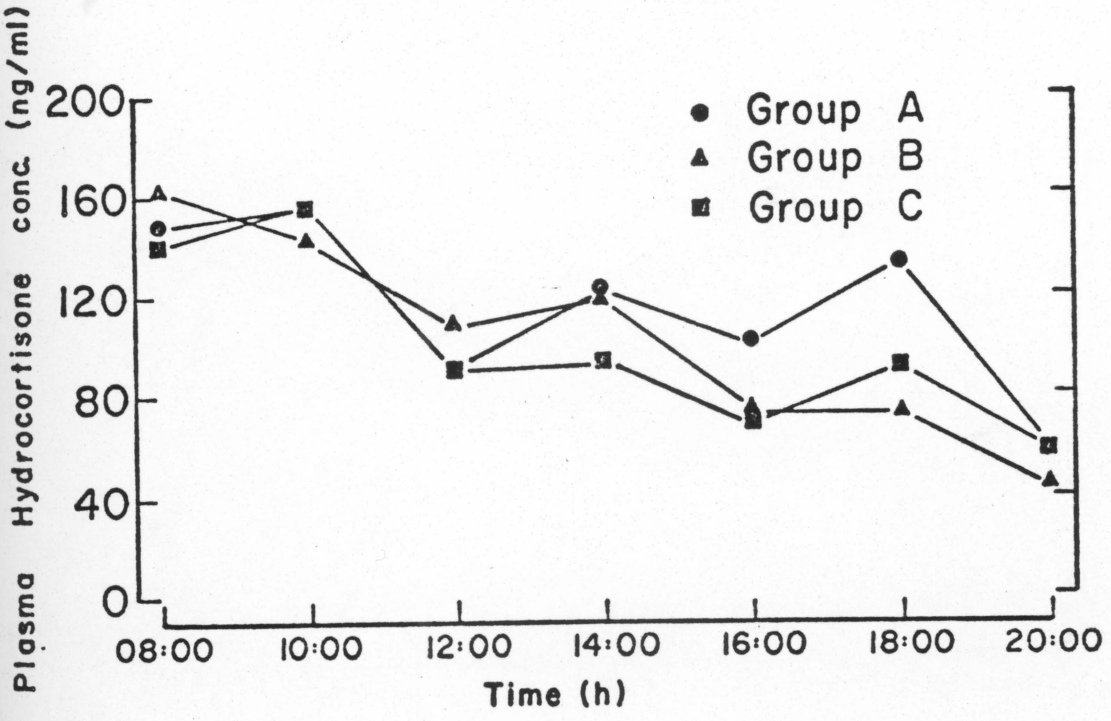


Figure 13 Mean plasma hydrocortisone concentrations versus time for Groups A (●), B (▲), and C (■) during Day 1 of Study 1.

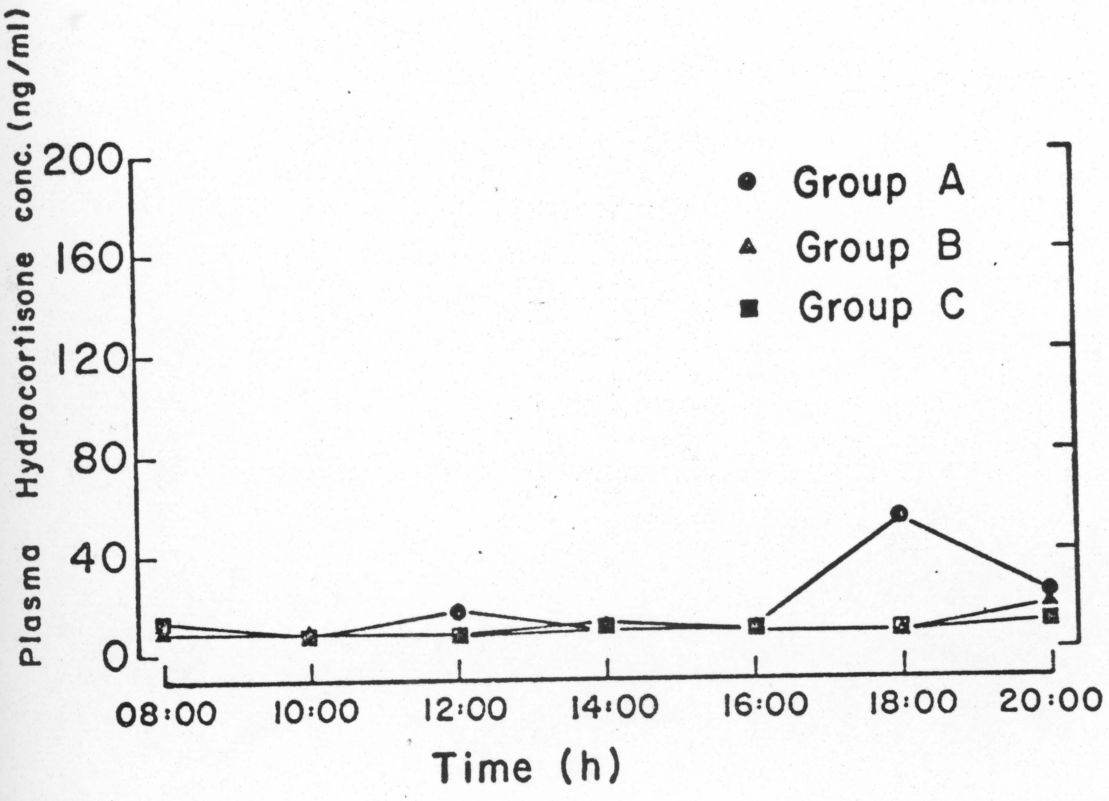


Figure 14 Mean plasma hydrocortisone concentrations versus time for Groups A (●), B (▲), and C (■) during Day 2 of Study 1. Groups received treatments as indicated in Figure 4.

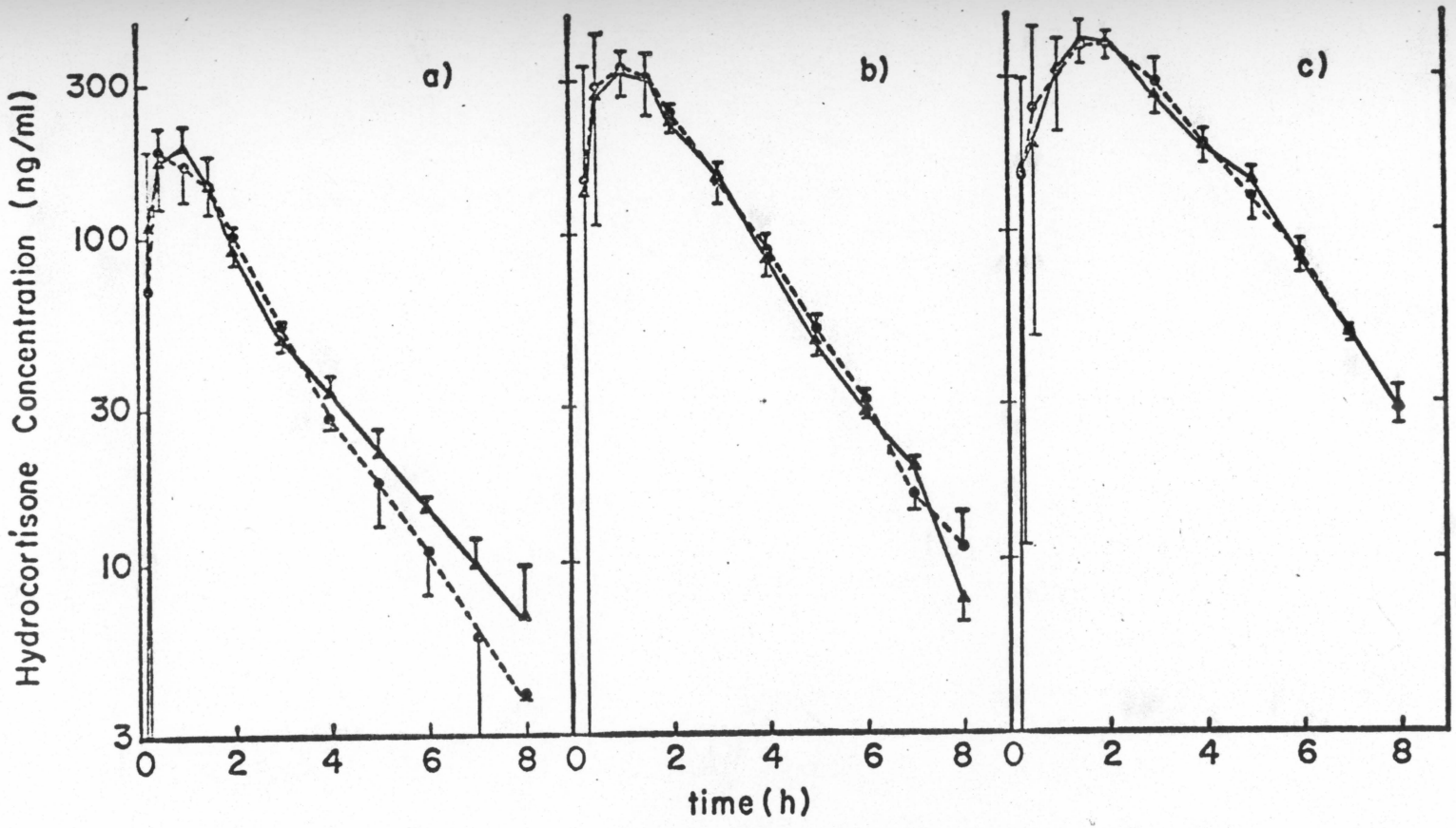


Figure 15 Mean plasma hydrocortisone concentrations (±1 SD) versus time for the 3 subjects of Study 2 at oral hydrocortisone tablet doses of a) 10 mg b) 30 mg, and c) 50 mg.

▲——▲ UV assay method

●- - - - - -● Fluorescence assay method

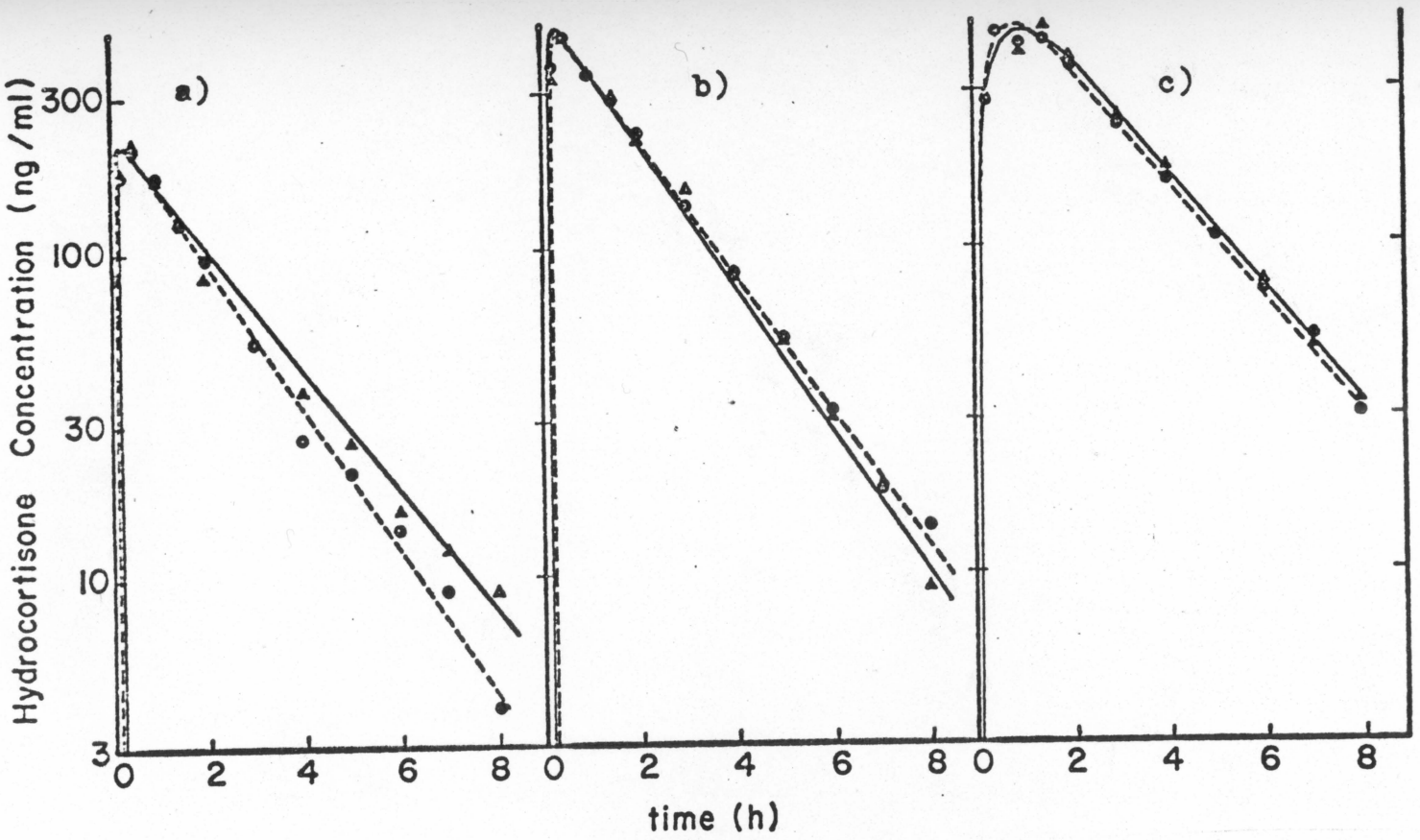


Figure 16 Plasma hydrocortisone concentrations versus time with computer-generated curves, for subject 3 of Study 2 at oral hydrocortisone tablet doses of a) 10 mg, b) 30 mg, and c) 50 mg.

▲——▲ UV assay ●- - - - - - - ● Fluorescence assay

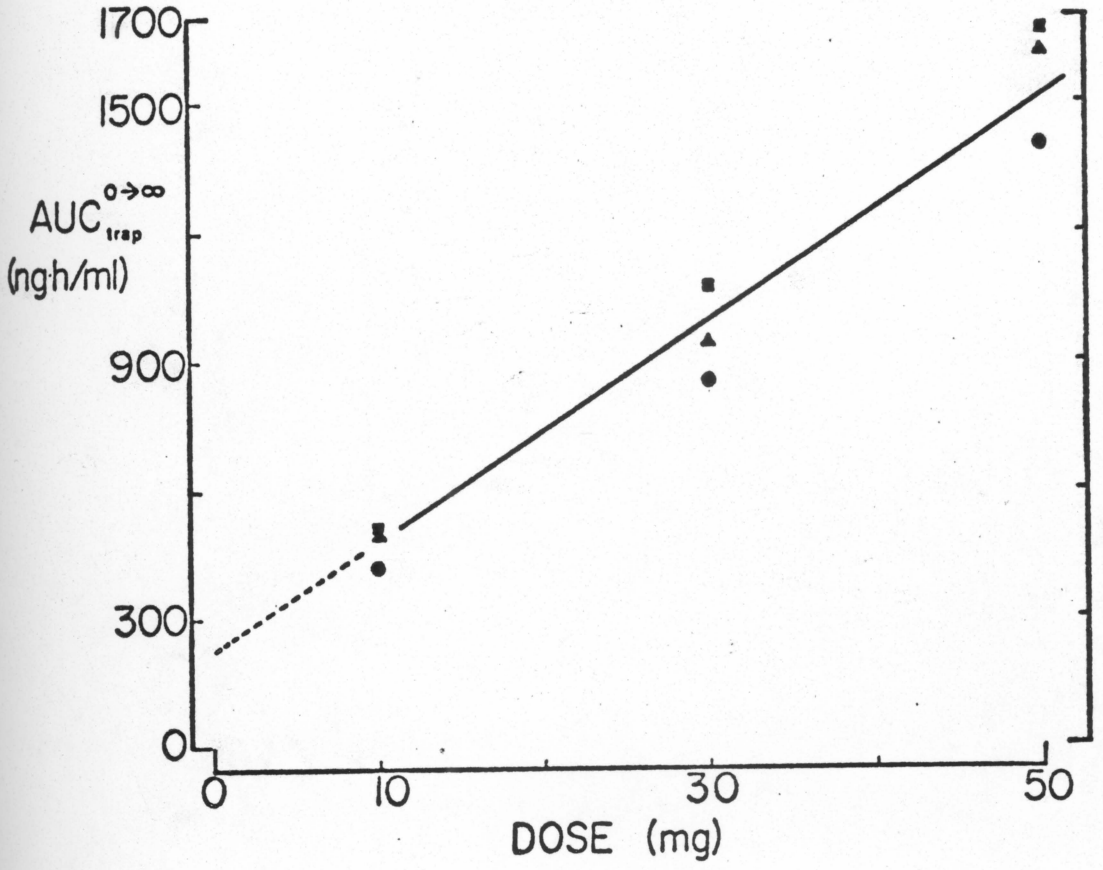


Figure 17 Trapezoidal area under the curve values from time 0 to ∞ ($AUC^{0\rightarrow\infty}_{trap}$) for Subjects 1 (●), 2 (▲), and 3 (■) of Study 2.

$Y = 26.2\ (\pm 1.9)X + 198\ (\pm 64)$

$r^2 = 0.9657$

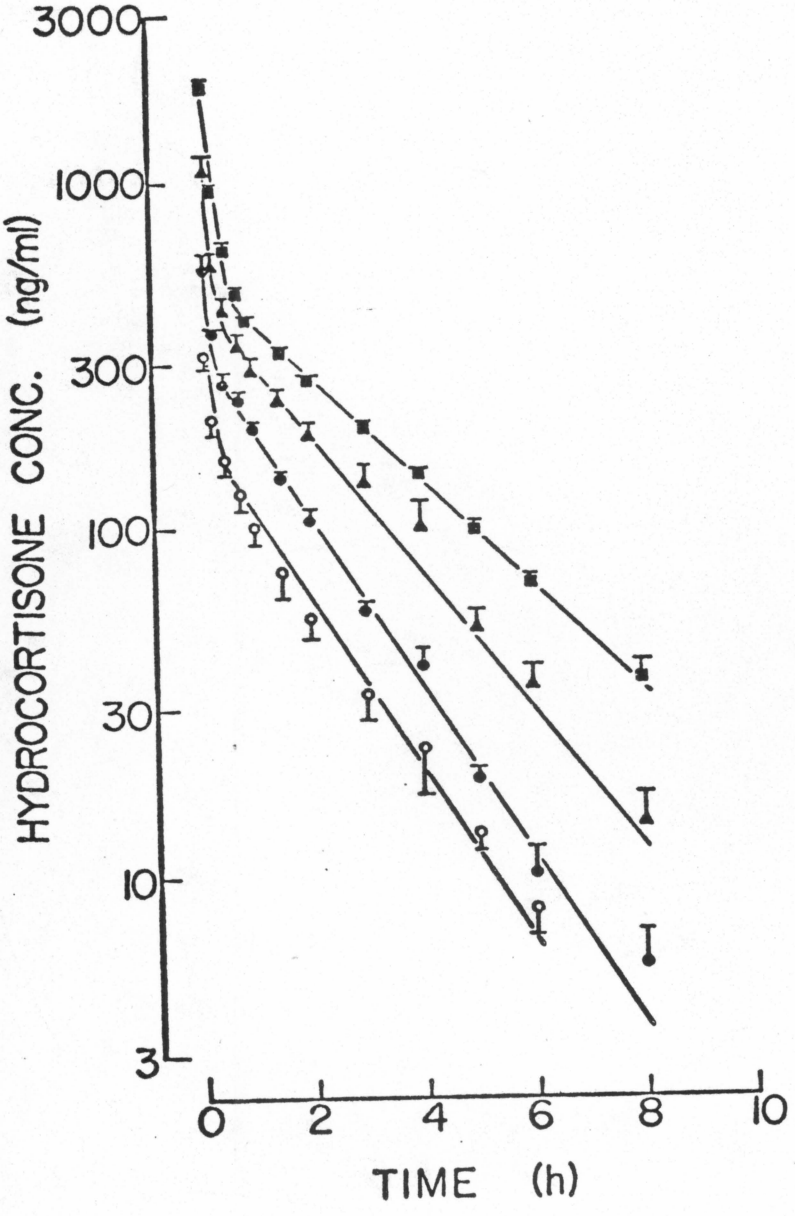


Figure 18 Mean plasma hydrocortisone concentrations (±1 SE) versus time for the 6 subjects of Study 3 at intravenous hydrocortisone sodium succinate doses of 5 mg (○), 10 mg (●), 20 mg (▲), and 40 mg (■).

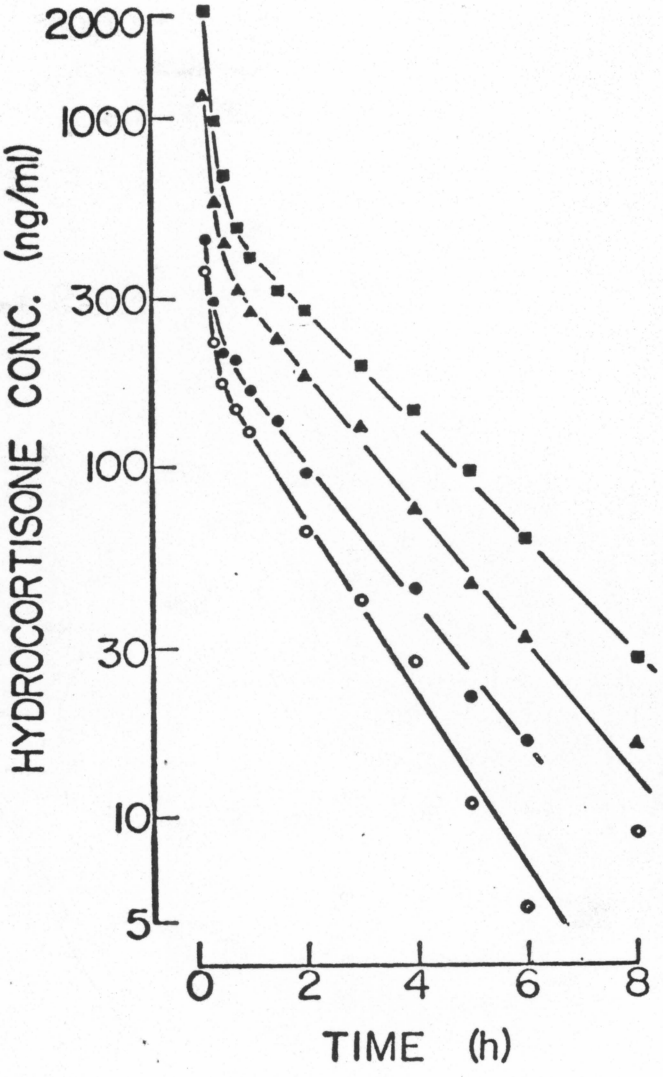


Figure 19 Plasma hydrocortisone concentrations versus time, with computer-generated curves, for Subject 1 of Study 3 at intravenous hydrocortisone doses of 5 mg (○), 10 mg (●), 20 mg (▲), and 40 mg (■).

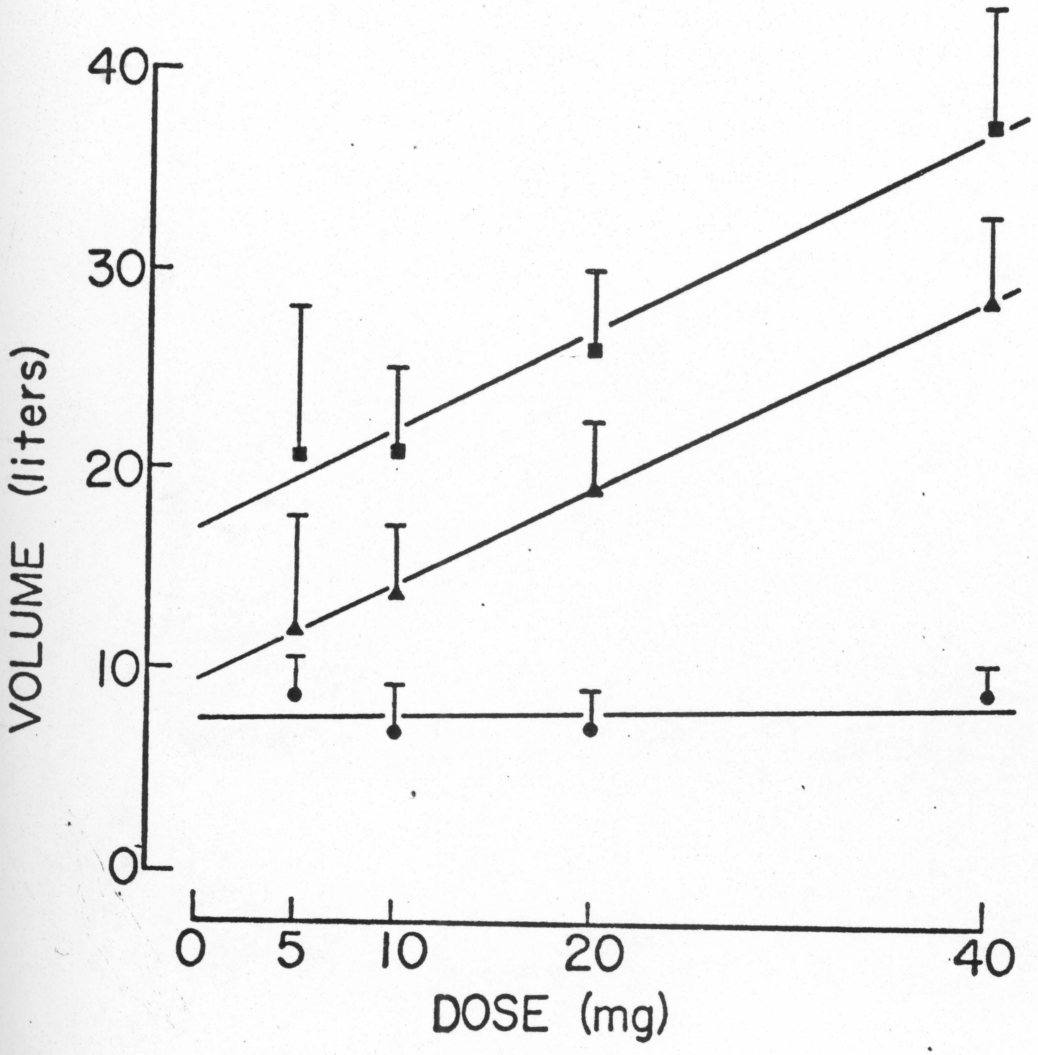


Figure 20 Apparent hydrocortisone volumes of distribution (±1 SD) for the central compartment, V_1 (●), the peripheral compartment, V_T (▲), and at steady state, Vd_{ss} (■) versus intravenous hydrocortisone dose (D) for Study 3.

$V_1 = 0.02\ (\pm 0.03)D + 7.4\ (\pm 0.7)$ $\quad r^2 = 0.0219$

$V_T = 0.48\ (\pm 0.06)D + 9.2(\pm 1.4)$ $\quad r^2 = 0.7310$

$Vd_{ss} = 0.50\ (\pm 0.08)D + 17.0\ (\pm 1.9)$ $\quad r^2 = 0.6320$

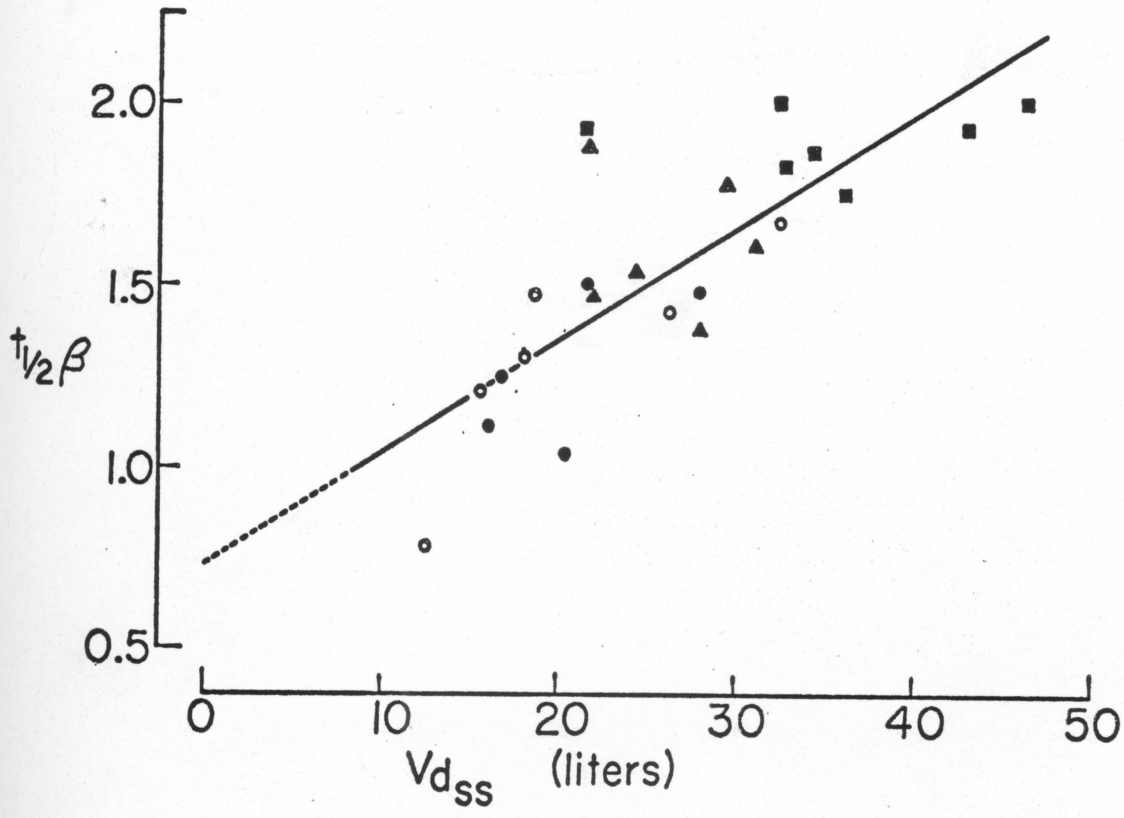


Figure 21 Relationship between the overall hydrocortisone elimination half-life, $t_{\frac{1}{2}}\beta$, and the apparent volume of distribution at steady state for intravenous hydrocortisone sodium succinate doses of 5 mg (○), 10 mg (●), 20 mg (▲), and 40 mg (■) in Study 3.

$t_{\frac{1}{2}}\beta = 0.029\ (\pm 0.005)Vd_{ss} + 0.79\ (\pm 0.14)$

$r = 0.771$

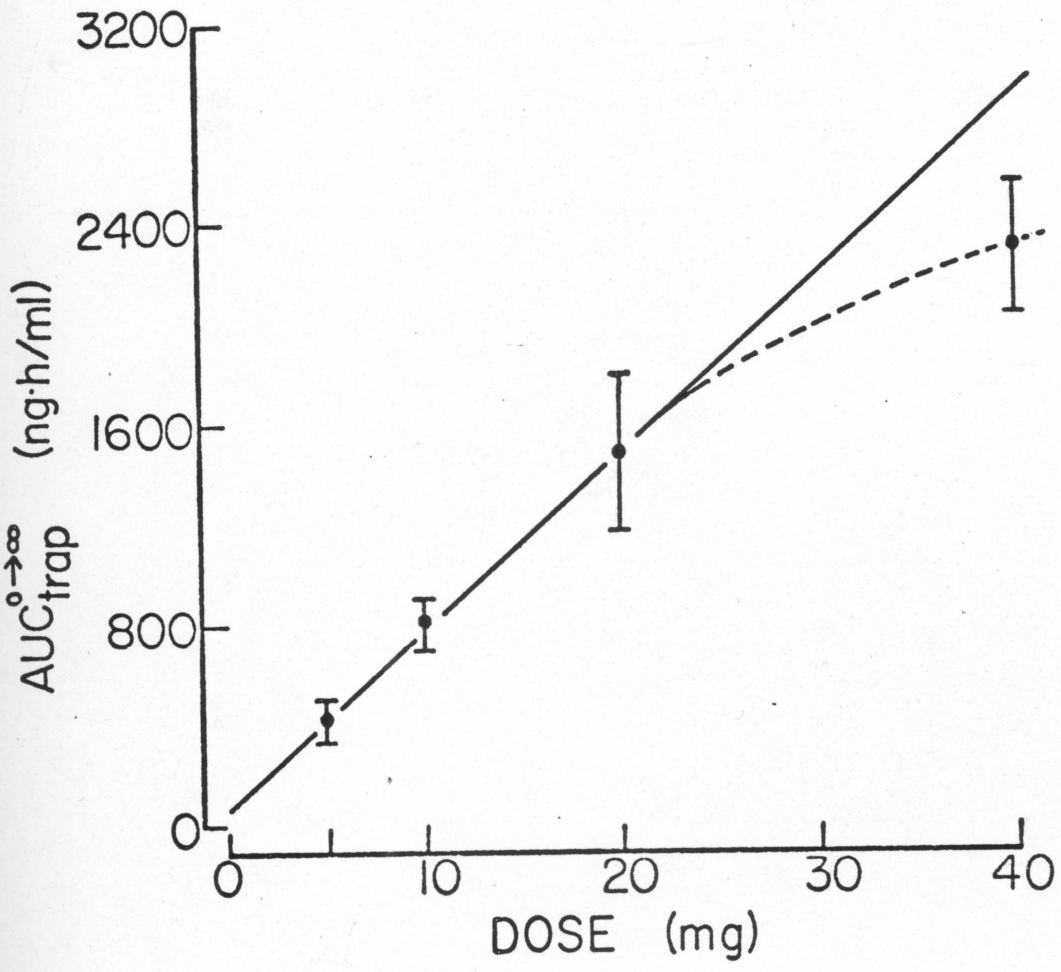


Figure 22 Mean trapezoidal area under the curve values from time 0 to ∞ ($AUC^{0\rightarrow\infty}_{trap}$) versus dose for the 6 subjects of Study 3 ($\pm$1 SD).

Linear regression of data for 5 mg, 10 mg, and 20 mg doses gives:

$$Y = 69(\pm 8)X + 72(\pm 98) \qquad r = 0.9242$$

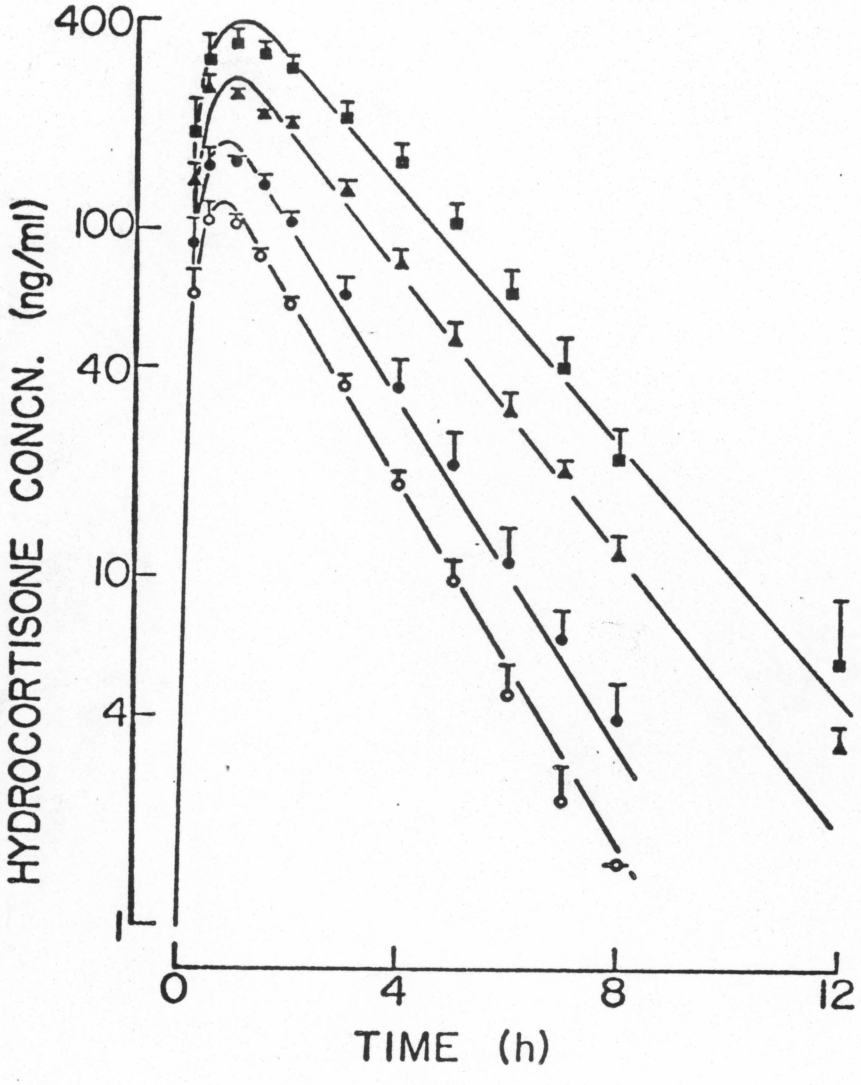


Figure 23 Mean plasma hydrocortisone concentrations (±1 SE) versus time for the 8 subjects of Study 4 at oral hydrocortisone suspension doses of 5 mg (○), 10 mg (●), 20 mg (▲), and 40 mg (■).

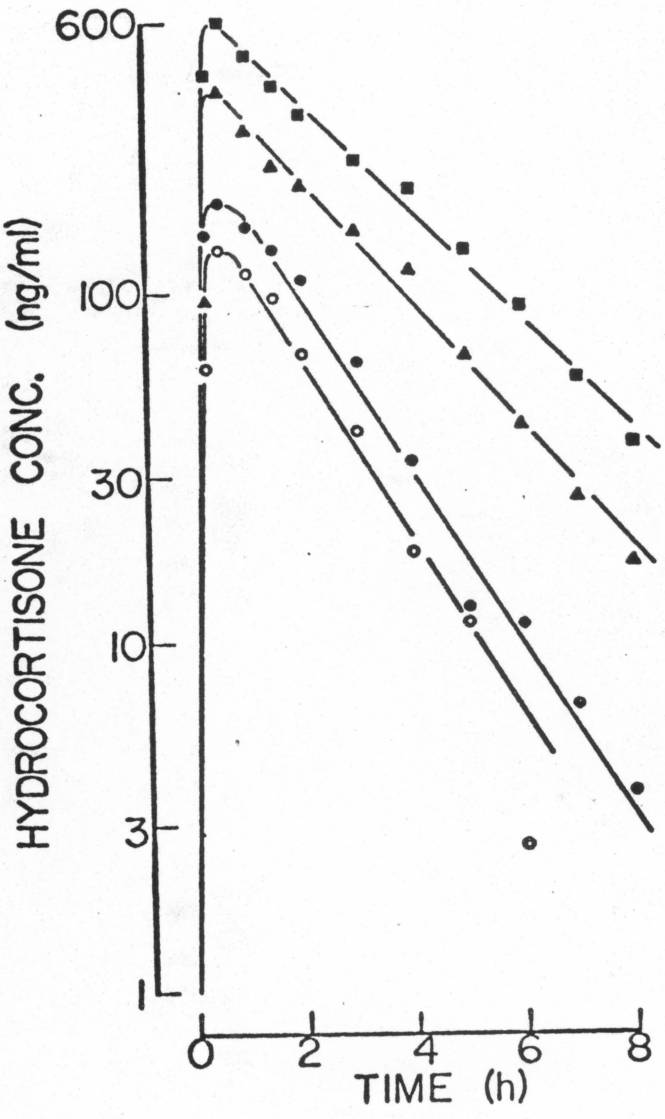


Figure 24 Plasma hydrocortisone concentrations versus time for Subject 3 of Study 4 at oral hydrocortisone suspension doses of 5 mg (○), 10 mg (●), 20 mg (▲), and 40 mg (■).

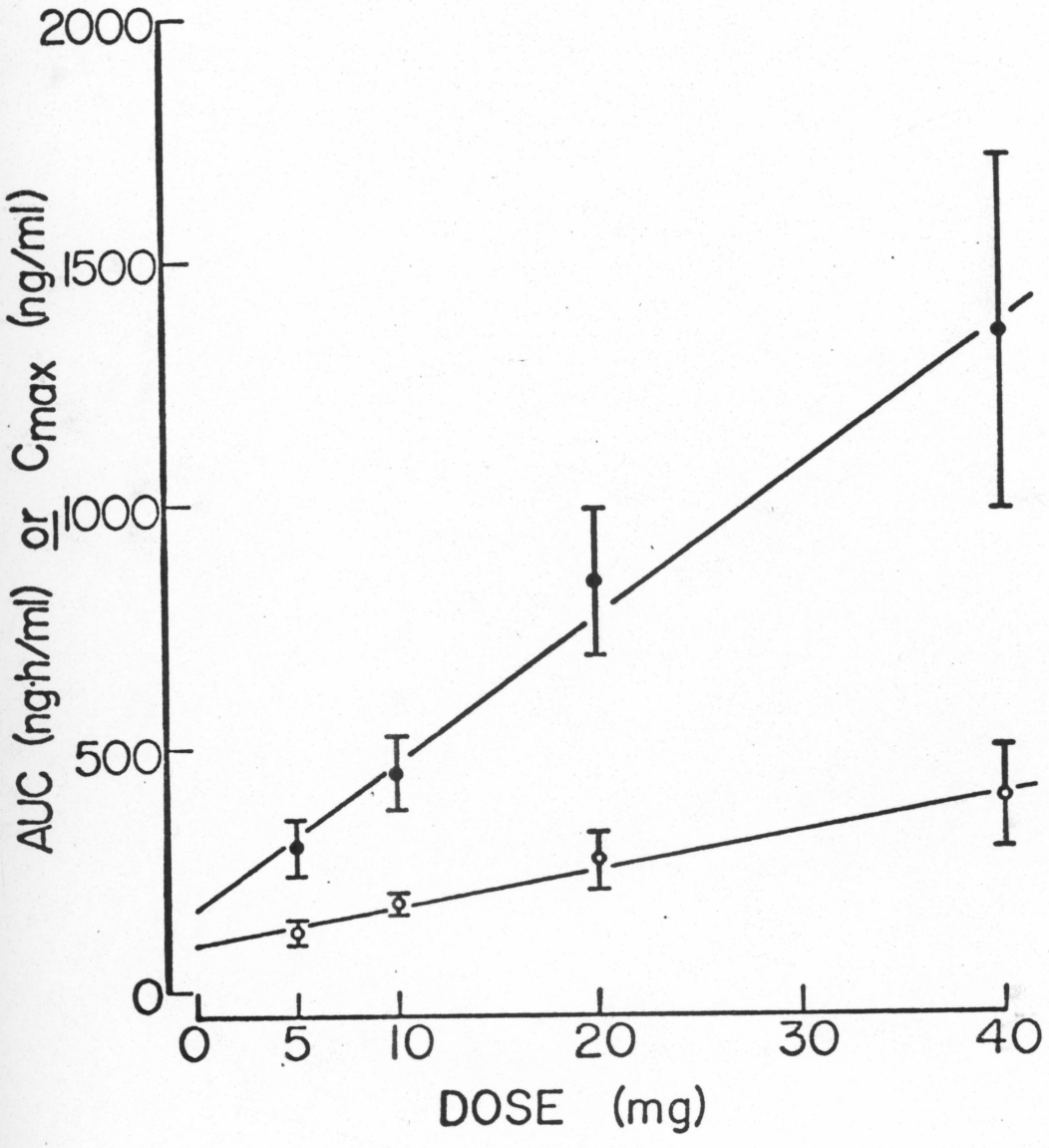


Figure 25 Mean values of trapezoidal area under the curve from time 0 to ∞, AUC (●), and maximum observed plasma hydrocortisone concentration, C_{max} (o) versus dose for the 8 subjects of Study 4.

AUC = 30(±3)D + 165(±60) r^2 = 0.8167

C_{max} = 7.5(±0.8)D + 95(+18) r^2 = 0.7550

Table 3 Peak height ratios of hydrocortisone:IS for study of the effect of fast and slow evaporation on variability. SD is standard deviation.

	Fast Evaporation	Slow Evaporation
Tube 1	2.136	2.264
Tube 2	1.992	2.608
Tube 3	2.000	2.463
Tube 4	2.359	2.418
Mean	2.12	2.44
SD	0.17	0.14
% Error	8.1%	5.8%

Table 4 Individual and mean peak heights (mm) of hydrocortisone (HC) and internal standard (IS) extracted from plasma and of direct injections of standard solutions for the determination of % recovery of HC and IS from plasma. SD is standard deviation.

h^{HC} [a,b]		HC Concentration (ng/ml) 50	100	500	
Tube Set	A	11.9	26.2	131.6	
	B	13.1	26.1	139.8	
	C	13.0	22.3	129.8	
Mean		12.7	24.9	133.6	
SD		0.7	2.3	2.7	
h^{IS} [b]					
Tube Set	A	56.8	54.3	55.3	
	B	57.5	56.3	55.5	
	C	54.9	51.9	54.3	
Mean					55.2
SD					1.7
h for standard solutions					
	h^{HC}	16.4	33.1	165.0	
	h^{IS}	66.9	67.0	66.6	

mean h^{IS} std. soln = 66.8 SD = 0.2

a: Corrected for blank
b: Corrected for extractant volume

Table 5 Individual and mean hydrocortisone:IS peak height ratios for reconstitution study. SD is standard deviation.

	Hydrocortisone Concentration (ng/ml)		
	50	100	500
Tube Set A	0.233	0.449	2.285
Tube Set B	0.225	0.445	2.294
Tube Set C	0.225	0.476	2.351
Mean	0.228	0.457	2.310
SD	0.005	0.017	0.036
% error	2.1%	3.7%	1.6%
Standard solutions	0.240	0.457	2.310
% reconstitution	95%	96%	92%

Table 6 Chromatographic parameter values calculated from chromatogram shown in Figure 7b for the hydrocortisone (HC) and internal standard (IS) peaks.

Parameter	Symbol	HC Peak	IS Peak	Mean	Value[a]
Resolution	R_s	---[b]	---	---	1.9
Capacity	k'	3.7	2.7	3.2	---
Efficiency	n	1326	784	1055	---
Selectivity	α	---	---	---	1.4
Height equivalent to a theoretical plate	H	0.19 mm	0.32 mm	0.26 mm	---

a: Computed using both peaks
b: Not applicable

Table 7a

Accuracy and precision of UV assay method for between-day runs. SD is standard deviation.

Theoretical Conc.(ng/ml)	Experimental Conc. (ng/ml) Mean	SD	n	Coefficient of Variation (%)
5	7.2	0.5	6	7.6
10	11.7	0.9	6	8.0
25	25.5	0.5	6	1.9
50	51	3	6	5.7
100	96	3	6	2.9
200	193	3	6	1.6
400	402	17	5	4.1
700	695	27	6	3.9

Table 7b Accuracy and precision of UV assay method for a within-day run (3 samples per concentration). SD is standard deviation.

Theoretical Conc. (ng/ml)	Experimental Conc. (ng/ml) Mean	SD	Coefficient of Variation (%)
10	14.5	0.2	1.2
50	58	0.7	1.3
200	199	2	0.8
500	498	14	2.9
700	685	40	5.8
1000	988	9	0.9
1500	1530	56	3.7
3000	3004	30	1.0

Table 8 Concentration results (ng/ml) for Fluorescence (F) and UV assay of "unknown" plasma samples.

Actual Conc.	F Conc.	% Error	UV Conc.	% Error
10	14.3	8.3	14.4	5.2
50	59	8.5	54	9.4
200	185	6.4	186	5.1
500	411	10.3	456	2.0

Table 9a

Individual and mean corrected plasma hydrocortisone concentrations as determined by the fluorescence (F) and UV assay methods for the 10 mg oral tablet dose in Study 2. SD is standard deviation.

Assay	Subject	Time (h) .25	.50	1.0	1.5	2	3	4	5	6	7	8	C_{pre}
F	1	20	144	126	183	109	54	27	13	9	4	--[a]	6
	2	119	195	197	133	100	54	31	21	9	6	4	3
	3	172	210	172	123	96	53	27	21	14	9	4	3
	Mean	70	183	165	146	102	53.7	28	18	11	6	4	4
	SD	88	35	36	32	7	0.6	2	5	3	3	0	2
UV	1	25	117	—[b]	165	90	48	30	22	15	11	8	6
	2	127	191	213	—	102	45	35	18	14	8	4	5
	3	178	218	169	127	84	53	38	26	16	12	9	3
	Mean	110	175	191	146	92	49	34	22	15.0	10	7	5
	SD	78	52	31	27	9	4	4	4	1.0	2	3	2

a: Not detectable b: Insufficient sample

Table 9b

Individual and mean corrected plasma hydrocortisone concentrations as determined by the fluorescence (F) and UV assay methods for the 30 mg oral tablet dose in Study 2. SD is standard deviation.

Assay	Subject	Time (h) .25	.50	1.0	1.5	2	3	4	5	6	7	8	C_{pre}
F	1	13	242	371	266	227	147	86	46	29	15	9	7
	2	75	153	279	375	249	160	100	55	33	15	10	5
	3	360	453	341	282	224	132	80	53	32	18	14	3
	Mean	149	283	330	308	233	146	89	51	31	16.0	11	5
	SD	185	154	47	59	14	14	10	5	2	1.7	3	2
UV	1	10	197	336	241	212	143	77	44	29	18	7	7
	2	68	156	253	392	238	163	97	44	28	21	7	5
	3	328	452	337	287	210	150	81	53	30	19	9	5
	Mean	135	268	309	307	220	153	85	47	29.0	19.3	7.7	5.7
	SD	169	160	48	77	16	10	11	5	1.0	1.5	1.2	1.2

Table 9c

Individual and mean corrected plasma hydrocortisone concentrations as determined by the fluorescence (F) and UV assay methods for the 50 mg oral tablet dose in Study 2. SD is standard deviation.

Assay	Subject	Time (h) .25	.50	1.0	1.5	2	3	4	5	6	7	8	C_{pre}
F	1	11	96	220	356	353	250	184	135	89	51	30	6
	2	147	146	271	335	425	351	199	138	91	45	25	5
	3	278	461	425	425	353	230	157	105	72	52	30	3
	Mean	145	234	305	372	377	277	180	126	84	49	28	4.7
	SD	134	198	107	47	42	65	21	18	10	4	3	1.5
UV	1	10	88	230	375	371	234	200	129	89	48	28	8
	2	169	282	330	349	418	302	165	151	77	50	25	5
	3	288	--[c]	389	468	380	244	170	--[d]	77	48	33	3
	Mean	156	185	316	397	390	260	178	140	81	48.7	29	5
	SD	139	137	80	63	25	37	19	16	7	1.2	4	3

c: Sample spilled d: Sample broke in centrifuge

Table 10 Plasma hydrocortisone concentrations (ng/ml) for Day 1 and Day 2 of Study 1. SD is standard deviation.

Group		A			B			C		
Subject		1	2	3	4	5	6	7	8	9
Day	Time									
1	08:00	116	182	150	114	162	212	208	138	78
	10:00	148	218	102	126	120	182	210	164	98
	12:00	56	108	108	100	94	130	92	92	86
	14:00	76	164	128	76	144	138	74	122	86
	16:00	94	120	90	62	60	100	78	88	42
	18:00	102	148	148	28	94	96	88	126	58
	20:00	44	102	24	14	46	68	42	104	28
2	08:00	7	8	12	10	10	7	16	8	16
	10:00	12	7	4	7	9	7	9	6	8
	12:00	24	8	18	6	9	10	7	8	11
	14:00	10	8	12	10	11	10	11	10	14
	16:00	13	7	9	11	8	8	7	7	12
	18:00	40	82	36	10	7	9	8	9	7
	20:00	14	44	14	8	36	9	8	19	9
Day 2 Mean		17	23	15	8.9	13	8.6	9	10	11
Day 2 SD		11	29	10	1.9	10	1.3	3	4	3
Day 2 % error		65%	126%	67%	21%	77%	15%	33%	40%	27%
Treatment Mean			18			10			10	
Treatment SD			18			6			4	
Treatment % error			100%			60%			40%	

Table 11a Pharmacokinetic rate parameters and coefficients of determination (r^2) for the 10 mg oral tablet dose in Study 2 for the fluorescence (F) and UV assays. SD is standard deviation.

Assay	Subject	k_a (h^{-1})	k_{el} (h^{-1})	$t_{½}a$ (h)	$t_{½}el$ (h)	t_{max} (h)	t_{lag} (h)	r^2 ---
F	1	2.3	0.67	0.31	1.03	0.97	0.20	0.957
	2	5.0	0.56	0.14	1.24	0.62	0.13	0.997
	3	116.0	0.51	0.04	1.36	0.39	0.17	0.999
	Mean	7.7	0.58	0.16	1.21	0.66	0.17	0.984
	SD	7.3	0.08	0.13	0.17	0.29	0.04	0.024
UV	1	3.6	0.44	0.20	1.58	0.89	0.22	0.960
	2	4.4	0.53	0.16	1.31	0.65	0.10	0.988
	3	59.8	0.43	0.012	1.61	0.30	0.22	0.992
	Mean	23	0.47	0.12	1.50	0.61	0.18	0.980
	SD	32	0.06	0.10	0.17	0.30	0.07	0.017

Table 11b Pharmacokinetic rate parameters and coefficients of determination (r^2) for the 30 mg oral tablet dose in Study 2 for the fluorescence (F) and UV assays. SD is standard deviation.

Assay	Subject	k_a (h^{-1})	k_{el} (h^{-1})	$t_{½}a$ (h)	$t_{½}el$ (h)	t_{max} (h)	t_{lag} (h)	r^2 ---
F	1	21	0.53	0.03	1.31	0.64	0.46	0.997
	2	1.4	0.56	0.50	1.24	1.28	0.18	0.977
	3	57	0.48	0.012	1.44	0.31	0.23	1.000
	Mean	26	0.52	0.18	1.33	0.74	0.29	0.991
	SD	28	0.04	0.28	0.10	0.49	0.15	0.012
UV	1	7.7	0.52	0.09	1.33	0.79	0.42	0.996
	2	1.3	0.58	0.52	1.19	1.29	0.18	0.964
	3	14.0	0.51	0.05	1.36	0.25	0.18	0.998
	Mean	7.7	0.54	0.22	1.29	0.78	0.26	0.986
	SD	6.3	0.04	0.26	0.09	0.52	0.14	0.019

Table 11c Pharmacokinetic rate parameters and coefficients of determination (r^2) for the 50 mg oral tablet dose in Study 2 for the fluorescence (F) and UV assays. SD is standard deviation.

Assay	Subject	k_a (h^{-1})	k_{el} (h^{-1})	$t_{½}a$ (h)	$t_{½}el$ (h)	t_{max} (h)	t_{lag} (h)	r^2 ---
F	1	1.2	0.40	0.58	1.73	1.75	0.37	0.990
	2	0.7	0.48	0.96	1.44	1.76	0.07	0.972
	3	3.1	0.40	0.22	1.73	0.81	0.05	0.996
	Mean	1.7	0.43	0.59	1.63	1.44	0.16	0.986
	SD	1.3	0.05	0.37	0.17	0.55	0.18	0.013
UV	1	1.2	0.42	0.57	1.65	1.72	0.39	0.987
	2	1.0	0.46	0.71	1.51	1.46	0.00	0.988
	3	2.0	0.41	0.36	1.69	1.01	0.00	0.989
	Mean	1.4	0.43	0.55	1.62	1.40	0.13	0.988
	SD	0.5	0.03	0.18	0.09	0.36	0.23	0.001

Table 12a Pharmacokinetic concentration parameters for the 10 mg oral tablet dose in Study 2 for the fluorescence (F) and UV assays. SD is standard deviation.

Assay	Subject	$\frac{FD}{V}$ (ng/ml)	C_{max} (ng/ml)	F/V (10^{-6}/ml)	C_{max}/D (10^{-6}/ml)	$AUC_{eqn}^{0\rightarrow\infty}$ (ngh/ml)	$AUC_{trap}^{0\rightarrow 8h}$ (ngh/ml)	$AUC_{trap}^{0\rightarrow\infty}$ (ngh/ml)	$AUC_{trap}^{0\rightarrow\infty}/D$ (10^{-6}h/ml)
F	1	280	168	28.0	16.8	418	510[a]	516	51.6
	2	269	204	26.9	20.4	480	466	473	47.3
	3	242	216	24.2	21.6	475	467	475	47.5
	Mean	264	196	26.4	19.6	458	467[b]	488	48.8
	SD	20	25	2.0	2.5	34	0.7	24	2.4
UV	1	205	153	20.5	15.3	466	401	419	41.9
	2	275	206	27.5	20.6	519	487	495	49.5
	3	225	217	22.5	21.7	523	487	508	50.8
	Mean	235	192	23.5	19.2	503	458	474	47.4
	SD	36	34	3.6	3.4	32	50	48	4.8

a: $AUC_{trap}^{0\rightarrow 7h}$, C_{8h} for Subject 1 was not detectable. b: n=2

Table 12b

Pharmacokinetic concentration parameters for the 30 mg oral tablet dose in Study 2 for the fluorescence (F) and UV assays. SD is standard deviation.

Assay	Subject	$\frac{FD}{V}$ (ng/ml)	C_{max} (ng/ml)	F/V (10^{-6}/ml)	C_{max}/D (10^{-6}/ml)	$AUC^{0\rightarrow\infty}_{eqn}$ (ngh/ml)	$AUC^{0\rightarrow 8h}_{trap}$ (ngh/ml)	$AUC^{0\rightarrow\infty}_{trap}$ (ngh/ml)	$AUC^{0\rightarrow\infty}_{trap}/D$ (10^{-6}h/ml)
F	1	480	437	16.0	14.6	906	910	927	30.9
	2	573	309	19.1	10.3	1476	958	976	32.5
	3	506	486	16.9	16.2	1521	1063	1092	36.4
	Mean	520	411	17.3	13.7	1301	977	998	33.3
	SD	48	91	1.6	3.0	343	78	85	2.8
UV	1	430	354	14.3	11.8	1193	838	852	28.4
	2	580	305	19.3	10.2	1000	933	945	31.4
	3	520	459	17.3	15.3	1020	1059	1076	35.9
	Mean	510	373	17.0	12.4	1071	943	958	31.9
	SD	75	79	2.5	2.6	106	111	113	3.8

Table 12c

Pharmacokinetic concentration parameters for the 50 mg oral tablet dose in Study 2 for the fluorescence (F) and UV assays. SD is standard deviation.

Assay	Subject	$\frac{FD}{V}$ (ng/ml)	C_{max} (ng/ml)	F/V (10^{-6}/ml)	C_{max}/D (10^{-6}/ml)	$AUC_{eqn}^{0\rightarrow\infty}$ (ngh/ml)	$AUC_{trap}^{0\rightarrow 8h}$ (ngh/ml)	$AUC_{trap}^{0\rightarrow\infty}$ (ngh/ml)	$AUC_{trap}^{0\rightarrow\infty}/D$ (10^{-6}h/ml)
F	1	578	333	11.6	6.7	1445	1316	1391	27.8
	2	814	362	16.3	7.2	1696	1550	1602	32.0
	3	648	478	13.0	9.6	1620	1563	1638	32.8
	Mean	680	391	13.6	7.8	1587	1476	1544	30.9
	SD	121	77	2.4	1.5	129	139	133	2.7
UV	1	607	347	12.1	6.9	1445	1330	1397	27.9
	2	757	386	15.1	7.7	1646	1559	1613	32.3
	3	690	456	13.8	9.1	1683	1585	1666	31.2
	Mean	785	396	13.7	7.9	1591	1491	1559	30.5
	SD	75	55	1.5	1.1	128	140	142	2.3

Table 13 Statistical analysis of pharmacokinetic parameters for Study 2. Factor-effect significance level as determined by ANOVA is shown as P-value. Actual differences are as determined by Tukey's test.

	EFFECT			
	DOSE		SUBJECT	
	P	Difference	P	Difference
k_a	> 0.05	ns[a]	>0.05	ns
k_{el}	< 0.01	50 < 10 30	<0.05	3U 1U 3F < 2U 2F 1F[b]
FD/V	< 0.01	10 < 30 < 50	<0.01	1U < 1F < 3F < 3U < 2U 2F
C_{max}	< 0.01	10 < 30 50	<0.01	1U 2F 2U 1F < 3U 3F
t_{max}	< 0.01	10 < 30 < 50	<0.01	3F 3U < 1F 1U 2U 2F
AUC	< 0.01	10 < 30 < 50	<0.01	1U < 1F < 2F 2U < 3F 3U
t_{lag}	< 0.05	50 < 10 < 30	<0.01	2U < 2F 3U < 3F < 1U 1F

a: No significant difference at $p \leq 0.05$

b: F = fluorescence assay, U = UV assay

Table 14 a

Individual and mean corrected plasma hydrocortisone concentrations for the 5 mg intravenous dose in Study 3. SD is standard deviation.

Subject	Time (h) .17	.33	.50	.75	1.0	1.5	2	3	4	5	6	8	C_{pre}
1	363	226	173	149	125	—[a]	66	42	28	11.1	5.5	9.0	8.6
2	238	141	106	73	64	46	37	24	17.3	13.5	11.9	8.9	4.2
3	289	210	193	142	129	106	84	42	25	16.1	5.3	--[b]	8.7
4	255	159	124	101	76	57	46	27	19.5	15.8	11.5	10.7	6.7
5	347	248	176	166	110	99	61	48	44	10.6	6.6	--	12.0
6	379	245	169	129	102	64	38	13.7	2.8	--	--	--	8.6
Mean	312	205	157	127	101	74	55	33	23	13	8	9.5	8
SD	59	45	34	34	26	27	18	13	14	3	3	1.0	3

a: Sample broke during assay
b: Not detectable

Table 14 b

Individual and mean corrected plasma hydrocortisone concentrations for the 10 mg intravenous dose in Study 3. SD is standard deviation.

Subject	Time (h) .17	.33	.50	.75	1.0	1.5	2	3	4	5	6	8	C_{pre}
1	445	300	213	201	166	137	97	—[c]	45	22	16.5	--[b]	13.2
2	653	402	297	279	232	155	134	63	32	15.7	10.0	--	6.7
3	587	309	237	186	157	128	108	61	57	18.8	7.5	--	7.5
4	446	295	225	227	190	128	83	47	27	16.2	7.0	7.0	17.0
6	732	512	329	264	204	151	114	59	39	21	11.1	4.1	4.5
Mean	573	364	260	231	190	140	107	58	40	19	10	6	10
SD	127	94	50	40	30	13	19	17	12	3	4	2	5

c: Sample spilled
b: Not detectable

Table 14c Individual and mean corrected plasma hydrocortisone concentrations for the 20 mg intravenous dose in Study 3. SD is standard deviation.

Subject	Time (h) .17	.33	.50	.75	1.0	1.5	2	3	4	5	6	8	C_{pre}
1	1162	574	440	320	277	234	181	131	75	46	32	16.1	6.4
2	910	418	306	242	197	181	134	93	67	33	22	5.4	9.2
3	1457	728	547	402	387	286	238	203	162	75	44	19.4	9.3
4	937	565	410	347	257	215	172	98	61	41	26	6.4	14.9
5	1007	592	437	362	289	254	206	139	95	59	50	19.4	11.1
Mean	1095	575	428	335	281	234	186	133	101	51	35	14	10
SD	225	110	86	60	69	40	39	44	43	16	12	7	3

Table 14 d

Individual and mean corrected plasma hydrocortisone concentrations for for 40 mg intravenous dose in Study 3. SD is standard deviation.

Subject	Time (h) .17	.33	.50	.75	1.0	1.5	2	3	4	5	6	8	C_{pre}
1	2060	979	679	483	398	318	280	196	145	97	62	28	6.8
2	1584	780	554	428	350	256	245	173	123	90	68	50	3.5
3	2022	977	677	487	381	293	250	203	138	103	68	29	8.4
4	1514	869	508	399	361	303	232	185	130	72	48	29	7.2
5	1695	911	693	534	445	375	259	199	158	106	86	30	16.5
6	2246	1129	725	538	423	351	298	214	157	113	77	52	4.9
Mean	1854	941	639	478	393	316	261	195	142	97	68	36	8
SD	296	118	87	56	36	42	24	14	11	14	13	11	5

Table 14 e

Corrected plasma hydrocortisone concentrations for two subjects in Study 3 which were excluded from all mean calculations.

Subject	Dose	Time (h) .17	.33	.50	.75	1.0	1.5	2	3	4	5	6	8	C_{pre}
5	10	212	266	273	1170	1438	321	135	117	50	37	17.0	16.7	10.5
6	20	23006	5927	3626	3298	2140	964	523	224	148	64	45	18.1	8.1

Table 15 a

Pharmacokinetic rate parameters, equation parameters, and coefficients of determination (r^2) for the 5 mg intravenous dose in Study 3. SD is standard deviation.

Subject	α (h^{-1})	β (h^{-1})	k_2 (h^{-1})	k_{12} (h^{-1})	k_{21} (h^{-1})	A (ng/ml)	B (ng/ml)	r^2 ---
1	8.44	0.570	1.91	4.58	2.52	676	223	0.9997
2	5.19	0.415	1.59	2.66	1.35	369	90	0.9991
3	7.49	0.534	1.21	3.51	3.30	344	227	0.9978
4	5.55	0.474	1.47	2.76	1.79	356	125	0.9981
5	4.59	0.470	1.16	2.04	1.86	374	190	0.9952
6	6.50	0.909	2.18	2.52	2.71	514	244	0.9997
Mean	6.3	0.56	1.6	3.0	2.3	439	183	0.9983
SD	1.5	0.18	0.4	0.9	0.7	132	62	0.0017

Table 15 b

Pharmacokinetic rate parameters, equation parameters, and coefficients of determination (r^2) for the 10 mg intravenous dose in Study 3. SD is standard deviation.

Subject	α (h^{-1})	β (h^{-1})	k_2 (h^{-1})	k_{12} (h^{-1})	k_{21} (h^{-1})	A (ng/ml)	B (ng/ml)	r^2 ---
1	7.13	0.468	1.42	3.83	2.35	674	266	0.9987
2	10.2	0.621	2.36	5.77	2.68	1542	422	0.9992
3	9.08	0.459	2.65	5.31	1.57	1769	262	0.9991
4	12.7	0.646	2.72	7.59	3.00	1399	339	0.9974
5[a]	8.31	0.540	1.79	4.69	2.36	865	402	---
6	4.74	0.559	1.62	2.04	1.63	997	344	0.9990
Mean	8.7	0.55	2.1	4.9	2.3	1210	340	0.9987
SD	2.7	0.08	0.6	1.9	0.6	430	70	0.0007

a: Yates "pseudo-observations"

Table 15 c

Pharmacokinetic rate parameters, equation parameters, and coefficients of determination (r^2) for the 20 mg intravenous dose in Study 3. SD is standard deviation.

Subject	α (h^{-1})	β (h^{-1})	k_2 (h^{-1})	k_{12} (h^{-1})	k_{21} (h^{-1})	A (ng/ml)	B (ng/ml)	r^2 ---
1	8.29	0.445	2.52	4.75	1.47	3028	455	0.9993
2	9.21	0.430	2.99	5.33	1.33	2890	330	0.9995
3	8.02	0.368	2.19	4.85	1.35	3694	544	0.9986
4	6.62	0.502	1.78	3.48	1.86	1596	455	0.9994
5	6.73	0.391	1.61	3.88	1.64	1837	451	0.9997
6[a]	6.69	0.598	2.40	3.34	1.56	2940	515	---
Mean	7.6	0.46	2.3	4.3	1.5	2660	460	0.9992
SD	1.1	0.08	0.5	0.8	0.2	790	70	0.0004

a: Yates "pseudo-observations"

Table 15 d

Pharmacokinetic rate parameters, equation parameters, and coefficients of determination (r^2) for the 40 mg intravenous dose in Study 3. SD is standard deviation.

Subject	α (h^{-1})	β (h^{-1})	k_2 (h^{-1})	k_{12} (h^{-1})	k_{21} (h^{-1})	A (ng/ml)	B (ng/ml)	r^2 ---
1	7.16	0.378	2.46	3.98	1.10	5042	601	0.9994
2	7.18	0.359	2.20	4.17	1.17	3744	505	0.9990
3	6.72	0.371	2.34	3.68	1.06	4645	565	0.9990
4	6.01	0.344	1.82	3.40	1.14	2960	484	0.9989
5	7.07	0.397	1.91	4.08	1.47	3520	675	0.9989
6	6.42	0.346	2.20	3.55	1.01	4966	609	0.9998
Mean	6.8	0.37	2.2	3.8	1.16	4146	573	0.9992
SD	0.5	0.02	0.2	0.3	0.16	858	71	0.0004

Table 16 a

Pharmacokinetic disposition and concentration parameters for the 5 mg intravenous dose in Study 3. SD is standard deviation.

Subject	$t_{½}\alpha$ (h)	$t_{½}\beta$ (h)	$t_{½}el$ (h)	V_1 (l)	V_T (l)	Vd_{ss} (l)	$V_1 k_2^a$	$AUC_{trap}^{0\rightarrow\infty}$ (ngh/ml)	AUCβ (ng/ml)	AUC/D (10^{-6}h/ml)
1	0.082	1.22	0.363	5.56	10.1	15.7	160	486	277	97.2
2	0.134	1.67	0.436	10.9	21.5	32.4	232	306	127	61.2
3	0.093	1.30	0.573	8.76	9.29	18.1	172	476	254	95.2
4	0.125	1.46	0.471	10.4	16.0	26.4	218	365	173	73.0
5	0.151	1.47	0.597	8.87	9.76	18.6	146	481	226	96.2
6	0.107	0.76	0.318	6.60	6.14	12.7	224	356	324	71.2
Mean	0.12	1.3	0.46	8.5	12	21	190	410	230	82
SD	0.03	0.3	0.11	2.1	6	7	40	80	70	16

a: Plasma clearance normalized for body surface area (ml/min m^2)

Table 16 b

Pharmacokinetic disposition and concentration parameters for the 10 mg intravenous dose in Study 3. SD is standard deviation.

Subject	$t_{½\alpha}$ (h)	$t_{½\beta}$ (h)	$t_{½el}$ (h)	V_1 (l)	V_T (l)	Vd_{ss} (l)	$V_1 k_2$ [a]	$AUC_{trap}^{0\rightarrow\infty}$ (ngh/ml)	AUC_β (ng/ml)	AUC/D (10^{-6}h/ml)
1	0.097	1.48	0.488	10.6	17.3	28.0	227	683	320	68.3
2	0.068	1.12	0.294	5.09	10.9	16.1	161	870	540	87.0
3	0.076	1.51	0.262	4.92	16.6	21.6	211	789	362	78.9
4	0.055	1.07	0.255	5.75	14.5	20.3	223	679	439	67.9
5 [b]	0.112	1.45	0.478	8.37	13.6	22.0	186	912	483	91.2
6	0.146	1.24	0.428	7.46	9.33	16.8	188	836	467	83.6
Mean	0.09	1.3	0.37	7.0	14	21	200	800	440	80
SD	0.03	0.2	0.11	2.2	3	4	30	100	80	10

a: Plasma clearance normalized for body surface area (ml/min m^2)
b: Yates "pseudo-observations"

Table 16 c

Pharmacokinetic disposition and concentration parameters for the 20 mg intravenous dose in Study 3. SD is standard deviation.

Subject	$t_{½}\alpha$ (h)	$t_{½}\beta$ (h)	$t_{½}el$ (h)	V_1 (l)	V_T (l)	Vd_{ss} (l)	V_1k_2 [a]	$AUC_{trap}^{0\rightarrow\infty}$ (ngh/ml)	AUCβ (ng/ml)	AUC/D $\left(\frac{10^{-6}h}{ml}\right)$
1	0.084	1.56	0.275	5.74	18.5	24.3	217	1464	651	73.2
2	0.075	1.61	0.232	6.21	24.9	31.1	249	1138	489	56.9
3	0.086	1.88	0.316	4.72	16.9	21.7	167	1981	729	99.1
4	0.105	1.38	0.389	9.75	18.2	28.0	248	1193	599	59.7
5	0.103	1.77	0.430	8.74	20.7	29.4	200	1467	574	73.4
6 [b]	0.118	1.92	0.324	6.36	15.2	21.6	226	1659	726	83.0
Mean	0.10	1.7	0.33	6.9	19	26	220	1480	630	74
SD	0.02	0.2	0.07	1.9	3	4	30	310	90	16

a: Plasma clearance normalized for body surface area (ml/min m^2)
b: Yates "pseudo-observations"

Table 16 d

Pharmacokinetic disposition and concentration parameters for the 40 mg intravenous dose in Study 3. SD is standard deviation.

Subject	$t_{½}\alpha$ (h)	$t_{½}\beta$ (h)	$t_{½}el$ (h)	V_1 (l)	V_T (l)	Vd_{ss} (l)	$V_1 k_2{}^a$	$AUC_{trap}^{0\rightarrow\infty}$ (ngh/ml)	AUCβ (ng/ml)	AUC/D (10^{-6}h/ml)
1	0.097	1.83	0.282	7.09	25.7	32.7	262	2396	906	59.9
2	0.097	1.93	0.315	9.41	33.5	43.0	278	2095	752	52.4
3	0.103	1.87	0.296	7.68	26.6	34.3	291	2332	865	58.3
4	0.115	2.01	0.381	11.6	34.6	46.3	302	1926	663	48.2
5	0.098	1.75	0.363	9.53	26.5	36.0	259	2312	918	57.8
6	0.108	2.00	0.315	7.17	25.2	32.4	246	2677	926	66.9
Mean	0.103	1.90	0.33	8.7	29	37	270	2290	840	57
SD	0.007	0.10	0.04	1.8	4	6	20	260	110	6

a: Plasma clearance normalized for body surface area (ml/min m^2)

Table 17 Statistical analysis of pharmacokinetic parameters for Study 3. Factor-effect significance level as determined by ANOVA is shown as P-value. Actual differences are as determined by Tukey's test.

The subject factor did not have a significant effect on any parameter.

	Dose effect P-value	Differences between dose levels
α	>0.05	ns[a]
β	<0.05	$\overline{5\ \ 10} > \underline{20\ \ 40}$
k_2	>0.05	ns
k_{12}	>0.05	ns
k_{21}	<0.025	$\overline{5\ \ 10} > \underline{20} > 40$
A	<0.001	$40 > 20 > \overline{10\ \ 5}$
B	<0.001	$\overline{40\ \ 20} > 10 > 5$
$t_{1/2}\alpha$	>0.05	ns
$t_{1/2}\beta$	<0.005	$\overline{40} > \underline{20} > \overline{10\ \ 5}$
$t_{1/2}el$	>0.05	ns
V_1	>0.05	ns
V_T	<0.001	$40 > \overline{20\ \ 10\ \ 5}$
Vd_{ss}	<0.001	$40 > \overline{20\ \ 10\ \ 5}$
V_1k_2	<0.001	$40 > \overline{20\ \ 10\ \ 5}$
$AUC^{0\rightarrow\infty}_{trap}$	<0.001	$40 > 20 > 10 > 5$
$AUC\beta$	<0.001	$\overline{40} > \underline{20} > \overline{10} > 5$
AUC/D	<0.025	$\overline{5\ \ 10} > \underline{20} > 40$

a: No significant difference at $p \leq 0.05$

Table 18 Percent of hydrocortisone dose in Study 4 which remained in dosing container after drug administration.

Subject	WEEK 1	2	3	4
1	0.45	0.93	0.87	0.38
2	0.26	0.08	0.33	0.41
3	0.46	0.14	0.74	0.41
4	0.21	0.24	0.63	0.54
5	0.15	0.94	0.26	0.60
6	0.39	---[a]	0.49	1.00
7	0.10	0.09	0.26	0.45
8	0.11	0.21	0.76	0.49

a: Sample spilled

Table 19 a Individual and mean corrected plasma hydrocortisone concentrations for the 5 mg oral suspension dose in Study 4. SD is standard deviation.

Subject	Time (h) .25	.50	1.0	1.5	2	3	4	5	6	7	8	12	C_{pre}
1	110	128	103	77	55	31	18.7	10.0	7.5	3.0	---[a]	---	10.3
2	83	120	107	73	48	33	17.6	11.1	5.6	3.6	---	---	6.7
3	61	134	114	97	67	40	18.0	11.4	2.6	0.3	---	---	8.4
4	21	41	69	71	69	56	30	16.3	6.1	1.7	---	---	6.3
5	11.2	32	126	87	64	29	15.4	5.2	2.6	---	---	---	8.2
6	90	147	115	86	63	29	16.1	11.9	4.5	3.1	1.5	---	4.6
7	69	120	102	70	48	34	12.5	2.6	---	---	---	---	7.3
8	69	106	88	104	66	30	18.4	10.1	3.0	---	---	---	6.6
Mean	64	104	103	83	60	35	18	10	4.6	2.3	1.5	---	7.3
SD	33	43	18	13	8	9	5	4	1.9	1.3	---	---	1.7

a: Not detectable

Table 19 b

Individual and mean corrected plasma hydrocortisone concentrations for the 10 mg oral suspension dose in Study 4. SD is standard deviation.

Subject	.25	.50	1.0	1.5	2	3	4	5	6	7	8	12	C_{pre}
1	136	189	152	131	101	50	23	14.8	7.1	4.4	2.2	---[a]	5.7
2	101	181	150	113	71	37	21	14.9	7.8	4.0	1.1	---	5.4
3	147	183	158	135	110	64	33	12.2	11.1	6.6	3.7	---	4.3
4	29	66	121	141	118	95	73	46	24	13.2	8.2	---	5.1
5	98	156	154	99	89	39	14.5	6.0	1.3	---	---	---	11.3
6	6.2	47	179	147	117	95	47	27	14.4	5.3	2.5	---	7.3
7	100	203	157	124	89	47	23	14.8	6.1	2.2	---	---	6.1
8	105	168	154	171	131	85	46	32	19.3	10.7	5.4	---	5.7
Mean	90	149	153	133	103	64	35	21	11	7	4	---	6
SD	49	59	16	22	20	25	19	13	7	4	3	---	2

a: Not detectable

Table 19 c

Individual and mean corrected plasma hydrocortisone concentrations for the 20 mg oral suspension dose in Study 4. SD is standard deviation.

Subject	Time (h)												C_{pre}
	.25	.50	1.0	1.5	2	3	4	5	6	7	8	12	
1	125	240	220	193	169	112	68	38	27	18.5	10.7	---[a]	5.5
2	51	182	224	180	152	83	47	26	18.0	10.0	5.1	---	4.5
3	96	378	283	232	204	150	116	66	42	26	16.8	---	5.1
4	130	174	181	188	198	142	82	49	25	14.5	12.2	---	5.9
5	173	225	247	199	224	115	67	42	21	17.7	11.1	---	12.8
6	106	263	265	219	196	129	84	46	29	19.6	12.5	4.9	6.3
7	202	297	237	213	180	116	65	40	25	17.8	7.0	1.9	5.5
8	178	234	252	250	249	165	103	78	53	32	18.0	3.1	5.5
Mean	133	249	239	209	197	127	79	48	30	20	12	3.3	6
SD	50	66	31	24	31	26	22	17	12	7	4	1.5	3

a: Not detectable

Table 19d

Individual and mean corrected plasma hydrocortisone concentrations for the 40 mg oral suspension dose in Study 4. SD is standard deviation.

Subject	Time (h)												C_{pre}
	.25	.50	1.0	1.5	2	3	4	5	6	7	8	12	
1	306	382	292	258	224	135	115	76	44	22	12	---[a]	4.4
2	120	284	293	234	217	146	93	59	30	12.1	6.5	---	3.9
3	423	599	479	391	323	240	199	133	91	57	37	2.6	6[b]
4	64	77	126	263	255	223	179	143	89	59	28	---	5.4
5	174	373	363	289	269	180	108	68	36	18.8	8.7	---	6.7
6	42	119	347	342	309	249	169	117	65	44	24	---	2.7
7	256	379	417	345	263	176	118	67	42	24	11.6	---	6.2
8	111	191	368	410	434	317	252	165	123	86	50	8.8	9.1
Mean	187	301	336	317	287	208	154	104	65	40	22	6	6
SD	132	170	105	65	70	61	55	41	33	26	15	4	2

a: Not detectable

b: C_{pre} (40 mg) = Average of C_{pre} for 5, 10, and 20 mg for this subject. Actual sample was lost during assay.

Table 20 a

Pharmacokinetic results for the 5 mg oral dose in Study 4. SD is standard deviation.

Subject	k_{el} (h^{-1})	$t_{½el}$ (h)	t_{max} (h)	C_{max} (ng/ml)	$AUC_{trap}^{0\rightarrow\infty}$ (ngh/ml)	$AUCk_{el}$ (ng/ml)	AUC/D (10^{-6}h/ml)	$AUCk_{el}$/D (10^{-6}/ml)	C_{max}/D (10^{-6}/ml)
1	0.570	1.22	0.5	128	279	149	55.8	29.8	25.6
2	0.546	1.27	0.5	120	268	146	53.6	29.2	24.0
3	0.577	1.20	0.5	134	395	228	79.0	45.6	26.8
4	0.495	1.40	1.5	71	359	178	71.8	35.6	14.2
5	0.707	0.980	1.0	126	228	161	45.6	32.2	25.2
6	0.596	1.16	0.5	147	296	176	59.2	35.2	29.4
7	0.630	1.10	0.5	120	244	154	48.8	30.8	24.0
8	0.654	1.06	0.5	106	271	177	54.2	35.4	21.2
Mean	0.60	1.17	0.69	119	293	171	59	34	24
SD	0.07	0.13	0.37	23	57	26	11	5	5

Table 20b

Pharmacokinetic results for the 10 mg oral dose in Study 4.
SD is standard deviation.

Subject	k_{el} (h^{-1})	$t_{½el}$ (h)	t_{max} (h)	C_{max} (ng/ml)	$AUC_{trap}^{0\rightarrow\infty}$ (ngh/ml)	$AUCk_{el}$ (ng/ml)	AUC/D (10^{-6}h/ml)	$AUCk_{el}/D$ (10^{-6}/ml)	C_{max}/D (10^{-6}/ml)
1	0.598	1.16	0.5	189	427	255	42.7	25.5	18.9
2	0.593	1.17	0.5	181	368	218	36.8	21.8	18.1
3	0.564	1.23	0.5	183	469	265	46.9	26.5	18.3
4	0.419	1.66	1.5	141	528	221	52.8	22.1	14.1
5	0.769	0.901	0.5	156	341	262	34.1	26.2	15.6
6	0.500	1.39	1.0	179	475	238	47.5	23.8	17.9
7	0.605	1.14	0.5	203	410	248	41.0	24.8	20.3
8	0.495	1.40	1.5	171	559	277	55.9	27.7	17.1
Mean	0.57	1.26	0.81	175	447	248	45	25	18
SD	0.10	0.23	0.46	19	75	21	8	2	2

Table 20c Pharmacokinetic results for the 20 mg oral dose in Study 4. SD is standard deviation.

Subject	k_{el} (h^{-1})	$t_{½el}$ (h)	t_{max} (h)	C_{max} (ng/ml)	$AUC_{trap}^{0\rightarrow\infty}$ (ngh/ml)	$AUCk_{el}$ (ng/ml)	AUC/D (10^{-6}h/ml)	$AUCk_{el}$/D (10^{-6}/ml)	C_{max}/D (10^{-6}/ml)
1	0.428	1.62	0.5	240	748	320	37.4	16.0	12.0
2	0.527	1.32	1.0	224	595	314	29.8	15.7	11.2
3	0.403	1.72	0.5	378	1026	413	51.3	20.7	18.9
4	0.504	1.37	2.0	198	774	390	38.7	19.5	9.90
5	0.469	1.48	1.0	247	811	380	40.6	19.0	12.4
6	0.448	1.55	1.0	265	856	383	42.8	19.2	13.3
7	0.451	1.54	0.5	297	816	368	40.8	18.4	14.9
8	0.422	1.64	1.0	252	1053	444	52.7	22.2	12.6
Mean	0.46	1.53	0.94	263	835	377	42	19	13
SD	0.04	0.14	0.50	55	148	44	7	2	3

Table 20 d

Pharmacokinetic results for the 40 mg oral dose in Study 4.
SD is standard deviation.

Subject	k_{el} (h^{-1})	$t_{½el}$ (h)	t_{max} (h)	C_{max} (ngh/ml)	$AUC^{0\rightarrow\infty}_{trap}$ (ngh/ml)	$AUCk_{el}$ (ng/ml)	AUC/D (10^{-6}h/ml)	$AUCk_{el}/D$ (10^{-6}/ml)	C_{max}/D (10^{-6}/ml)
1	0.437	1.58	0.5	382	1088	475	27.2	11.9	9.55
2	0.502	1.38	1.0	293	921	462	23.0	11.6	7.33
3	0.353	1.96	0.5	599	1851	653	46.3	16.3	15.0
4	0.357	1.94	1.5	263	1216	434	30.4	10.9	6.58
5	0.485	1.43	0.5	373	1151	558	28.8	14.0	9.33
6	0.409	1.69	1.0	347	1346	551	33.7	13.8	8.68
7	0.497	1.40	1.0	417	1241	617	31.0	15.4	10.4
8	0.350	1.98	2.0	434	1925	674	48.1	16.9	10.9
Mean	0.42	1.67	1.00	389	1340	553	34	14	10
SD	0.07	0.26	0.53	103	360	90	9	2	3

Table 21

Statistical analysis of pharmacokinetic parameters for Study 4. Factor-effect significance level as determined by ANOVA is shown as P-value. Actual differences are as determined by Tukey's test.

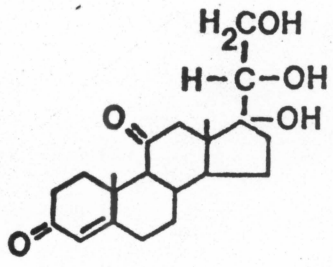
	EFFECT					
	DOSE		GROUP		SUBJECT	
	P	Difference	P	Difference	P	Difference
k_{el}	< 0.001	$\overline{5\ \ 10} > \overline{20\ \ 40}$	> 0.05	ns[a]	<0.05	$\overline{5\ 7\ 2\ 1\ 6\ 8\ 3\ 4}$
$t_{½el}$	< 0.001	$\overline{40\ \ 20} > \overline{10\ \ 5}$	< 0.05	$\overline{II_{>}\ IV\ \ I}\ III$	<0.05	$\overline{4\ 3\ 8\ 6\ 1\ 7\ 2\ 5}$
t_{max}	> 0.05	ns	< 0.05	$\overline{II_{>}\ IV\ \ III}\ I$	<0.001	$\overline{4\ 8\ 6\ 5\ 2\ 7\ 3\ 1}$
C_{max}	< 0.001	$40 > 20 > \overline{10\ \ 5}$	> 0.05	ns	<0.025	$\overline{3\ 7\ 8\ 1\ 6\ 5\ 2\ 4}$
$AUC_{trap}^{0\to\infty}$	< 0.001	$40 > 20 > \overline{10\ \ 5}$	< 0.025	$\overline{II\ \ IV_{>}\ III}\ I$	<0.01	$\overline{8\ 3\ 6\ 4\ 7\ 1\ 5\ 2}$
$AUCk_{el}$	< 0.001	$40 > 20 > 10 > 5$	< 0.005	$\overline{IV\ \ II_{>}\ III}\ I$	<0.005	$\overline{8\ 3\ 7\ 5\ 6\ 4\ 1\ 2}$
AUC/D	< 0.001	$5 > \overline{10\ \ 20} > 40$	< 0.001	$\overline{II_{>}\ IV}\ III_{>}\ I$	<0.005	$\overline{3\ 8\ 4\ 6\ 1\ 7\ 5\ 2}$

a: No significant difference at $p \leq 0.05$

DISCUSSION

Assay Development

The internal standard, Δ^4 - Pregnen-17α, 20β, 21-triol-3, 11-dione, shown below (II), was selected on the basis of its similarity to hydrocortisone. Chemical formulae, and the number



II

and types of functional groups are identical for the two compounds. Transposition of the C-11 hydroxy and the C-20 carbonyl groups of hydrocortisone apparently increases the polarity of the internal standard, as it is eluted before hydrocortisone in the reversed-phase HPLC of present assay, and is eluted after hydrocortisone in the normal phase HPLC of the fluorescence assay. The similar recoveries from plasma of 82% and 83% for hydrocortisone and IS, respectively, suggest that this polarity difference is small. Elution of IS after hydrocortisone would have been preferred, as hydrocortisone metabolites would be expected to elute before hydrocortisone due to greater polarity. However, as no peaks were observed

to interfere with the IS peak, this was not a major drawback. In fact, chromatograms from many samples showed a small peak eluting shortly after hydrocortisone, which might have interfered with a different internal standard.

Although standard curves run on different days were linear and axis intercepts were approximately zero, slopes were observed to differ. Consequently, all sample concentrations were computed using standard curves analyzed at the same time as the sample assay. The slope variance may be a result of inter-day differences in elution times. An increased elution time would cause the hydrocortisone:IS peak height ratio to decrease slightly. Such an effect could be lessened by computing the ratio of peak height areas or the products of peak height and retention time for the two compounds. While these calculations might have decreased inter-day slope variability, they might have also resulted in less reliable sample concentration estimates. Grant (74) has compared seven methods of chromatogram quantitation for gas chromatography. Of the methods tested, peak height measurements were found to be the most affected by elution time changes. However, for chromatograms with constant elution times, peak height measurements gave much lower error variance for carefully reproduced chromatograms

than did any other method tested, with the exception of electronic integration. Peak height measurements were preferred to electronic integration in situations with extraneous peak interference or baseline instability. Due to the high baseline noise level and the possibility of unpredicted peak interference in the UV assay, peak height ratio was chosen as the quantitation method, and separate standard curves were obtained for each assay execution.

The 60% aqueous methanol mobile phase used in the UV assay was relatively inexpensive, easy to prepare, and showed only minor UV absorbance at 254 nm. Retention times were not greatly affected by small (±3%) composition changes. Baseline noise was significantly decreased by the sonication of the mobile phase under vacuum. Any composition changes that this treatment may have caused were apparently minor. The significant UV absorbance of 97:3 dichloromethane: methanol at wavelengths less than 310 nm, as observed in this study, is odd since this solvent has been used previously as an HPLC mobile phase with UV detection at 254 and 280 nm (39). Rose(39) presented an absorbance spectrum for hydrocortisone, but it had been performed in 1:1 acetonitrile:ethanol, rather than the mobile phase. The sensitivity limit for plasma hydrocortisone of 5 ng/ml, as reported by Rose, might have been even lower if a different mobile phase had been used.

The rate of extractant evaporation apparently had a small, but significantly adverse, effect on assay variability. Sample preparation time for the UV assay could have been decreased by approximately 50% by the use of a rapid nitrogen gas flow rate and an elevated water bath temperature. While this modification might be permissible with a different evaporation apparatus or with larger tubes, the slow evaporation was chosen in order to maximize assay sensitivity and minimize variation using available equipment.

Percent recovery for hydrocortisone was acceptable and well within the range of previously reported values for similar extraction methods (40, 41, 42). A theoretical increase to 100% recovery would have lowered the sensitivity limit by only 18%, assuming extraction of interfering plasma components would not have been similarly increased.

Hydrocortisone stability in plasma was not examined, as hydrocortisone has previously been shown to be stable in plasma for 72 hours at 25°C (60), and for several months when deep frozen (75).

Assay Validation

The UV assay proved to be rapid, sensitive, and specific. As many as 30 plasma samples could be assayed per day. Coefficients of variation below 10% for concentrations from 5 to 3000 ng/ml demonstrated the excellent repeatability of the

assay. Accuracy at concentrations below 25 ng/ml was not great, even with the use of double sample injections as explained in Experimental. This appeared to be related to uncertainty in the blank concentration correction values, and could be improved by analyzing triplicate blank samples. However, this would decrease sample throughput, and would require larger, or more, suppressed blood samples to be obtained prior to hydrocortisone administration. Assay sensitivity was better than most of the other UV HPLC assays presently available (see Introduction), and was almost as good as the two fluorescence assays for hydrocortisone (33, 34). The similar sensitivities for the fluorescence and UV assays are probably due to the significant baseline increase caused by the large amount of excess fluorescent reagent required to obtain a high product yield in the fluorescence methods.

Concentrations for the Study 2 plasma samples as obtained by the fluorescence and UV assay methods were virtually identical. The identical results and the lack of a time-dependent trend in Figure 12, supported the idea of a lack of metabolite interference in either assay. The alternative would be that metabolite interference was identical for both assays. This is highly unlikely because of the derivatization, the different extraction conditions, and the second extraction employed by the fluorescence method.

The UV assay was basically a modification of the method of Scott (41). Table 22 lists differences between the two methods. The amount of sodium hydroxide, added to decrease plasma component interference (76), was increased to an amount similar to that used by Rose (39), in view of the 5 ng/ml sensitivity limit obtained with this method even with the significant UV absorbance of the mobile phase. Organic extractant volume, length of vortex-mixing, and evaporated extractant volume were changed to optimize percent recovery from plasma. The change in centrifugation was required as the ground glass tubes broke at speeds greater than 500 g. Nitrogen was used as the evaporation gas to minimize the possibility of oxidation during evaporation. Injector loop volume was decreased to allow multiple injections of each sample if desired. These changes resulted in a 40% decrease in elution time, similar percent recoveries, and an approximate 2-fold improvement in the limit of detection.

Plasma concentrations of several of the main hydrocortisone metabolites could possibly be quantitated using the UV assay with minor modifications. This would require the availability of the pure metabolite compounds, similar partitioning behavior, and similar UV absorbance characteristics. The second requirement should be possible since

the major hydrocortisone metabolites, the tetrahydro-derivatives, lack the C_4 double bond and the C_3 carbonyl group, which should result in even greater solubility in dichloromethane. However, the lack of these two groups may seriously decrease UV absorbance.

Study 1

This study provided the first examination of long-term suppression of actual plasma hydrocortisone concentrations by dexamethasone. In contrast to the variable concentrations between 20 and 40 ng/ml observed by Nichols (9) and Krieger (7) using non-specific assays, actual hydrocortisone concentrations were found to be fairly constant between 6 to 14 ng/ml during maximal suppression.

Maximum constancy and length of suppression were obtained by both Treatments B and C using 2 g of dexamethasone. In contrast with results by Nishida (44), the 1 g dose was shown to be as effective as 2 g in causing suppression at 08:00 h. However, suppression using 1 g of dexamethasone decreased at around 18:00 h and was, thus, unsuitable for use in the subsequent studies.

Dexamethasone has been shown to undergo biphasic elimination with a terminal elimination half-life of approximately 150 minutes (77). Two hours after an 8 g intravenous dose,

the plasma dexamethasone concentration was reported to be approximately 60 ng/ml. At 9 h after administration, the concentration had decreased to 10 ng/ml. Assuming direct dose proportionality and bioavailability similar to hydrocortisone, plasma dexamethasone concentrations at 08:00 h should be about 7 ng/ml and 2 ng/ml for dexamethasone Treatments B and C, respectively, of Study 1. As dexamethasone has a 12 minute retention time for the chromatographic conditions of the UV assay, assay interference from dexamethasone is unlikely for Studies 2, 3, and 4. Dexamethasone does not interfere with the fluorescence assay method (34). Lack of interference is also supported by the constant hydrocortisone concentrations seen for Day 2 of Study 1. The small amounts of dexamethasone present during hydrocortisone administration should not significantly affect protein binding of hydrocortisone. Any possible effects were minimized by the use of Treatment C rather than Treatment B.

Suppressed Concentration Correction

Reported concentrations for all samples from Studies 2, 3, and 4 have been corrected for endogenous hydrocortisone supposedly present during blood sampling. Correction

was made by subtracting the concentration observed for the pre-dose blood sample of a data set, from concentrations for all other samples of that data set. The validity of this correction depends on the assumption that endogenous hydrocortisone remained at the pre-dose concentration throughout the sampling period. This assumption was based on the constancy observed in the hydrocortisone concentrations for Day 2 of Study 1 and was supported, to some extent, by the apparent independence of suppression seen for the three different dexamethasone dosage regimens, and by the approach to a horizontal asymptote at late sample times which was observed for semi-logarithmic plots of uncorrected concentration versus time data for individual subjects in Studies 2, 3, and 4. However, hydrocortisone administration in Studies 2, 3, and 4 may have caused some type of change in endogenous concentrations during the sampling period. To try to determine whether such a change had occurred, data simulations for the effects of five different endogenous concentration profiles on an ideal set of concentration versus time data were constructed. An ideal (I) plasma hydrocortisone concentration data set was generated with a C_{max} of 240 ng/ml, a t_{max} of 1.0 h, and a 1.5 h elimination half-life. Concentrations were calculated at times corresponding to the sample times of Study 4. Five endogenous

concentration profiles at these same times were then obtained:

A. Linear decrease from C_{0h} = 5 ng/ml to C_{12h} = 1 ng/ml

B. Linear increase from C_{0h} = 5 ng/ml to C_{12h} =10 ng/ml

C. Radial change with C_{0h} = 5 ng/ml, C_{6h} = 1 ng/ml, C_{12h} = 5 ng/ml

D. Radial change with C_{0h} = 5 ng/ml, C_{6h} = 10 ng/ml, C_{12h} = 5 ng/ml

E. 1st order decrease from C_{0h} = 5 ng/ml to C_{12h} = 0.03 ng/ml with $t_{\frac{1}{2}}$ = 1.5 h

Of these simulations, B and D would be the most unlikely to actually occur, A would simulate a decrease caused by a zero-order process, C would approximate a 1st order decrease followed by a 1st order increase, and E would simulate cessation of endogenous secretion with elimination of the endogenous hydrocortisone by the same processes affecting exogenous hydrocortisone. Five "uncorrected" data sets were obtained by adding concentrations for profiles A through E to the I concentrations. These five data sets were then "corrected" by subtracting 5 ng/ml, the pre-dose concentration for each profile, from each concentration. The resulting profiles A through E are plotted along with I in Figure 26.

Deviations from ideal are large at concentrations below 10 ng/ml. As a result, all corrected concentrations less than 10 ng/ml in Studies 3 and 4 were omitted from all calculations. Elimination half-lives calculated by least squares linear regression of elimination phase data greater than 10 ng/ml for A through E were 1.43, 1.64, 1.37, 1.70, and 1.34 h, with percent errors from ideal of 4.7%, 9.3%, 8.7%, 13.3%, and 10.7%, respectively. These errors are significant even with the omission of the concentrations below 10 ng/ml, but are still well within half-life errors due to subject variability. This problem could be studied further by administering radio-labelled hydrocortisone using the same experimental protocol, and analyzing for both total and radio-labelled hydrocortisone. The lack of an animal model exhibiting similar distribution, binding, and metabolic characteristics to man would require this study be done in humans. The use of a radioactive drug would pose problems in executing such a study.

This simulation suggests that the data should exhibit greater error variance at low concentrations. Thus, non-weighted least squares was used for all linear regressions, and also for the nonlinear regressions in Studies 2 and 3, in order to give greater emphasis to high concentrations.

None of the data sets from Studies 2, 3, or 4 exhibited deviations at low concentrations as great as those seen in the simulations. Deviations similar to B, but only about 50% as severe, occurred in two of the 24 data sets from Study 3. Eight of the 32 data sets of Study 4 exhibited a milder version of deviation A. Nonlinearities in six of the nine data sets from Study 2 occurred at high concentrations and were, thus, probably not attributable to the concentration correction.

Results from Study 1 and previous studies of dexamethasone suppression (7, 9, 44) suggest that a greater degree of suppression prior to hydrocortisone administration cannot be obtained. Error in the pharmacokinetics of hydrocortisone would be much greater if suppression was omitted. Thus, samples for future bioavailability studies should be obtained during suppression and concentration data, corrected for suppressed concentrations, greater than 10 ng/ml should be analyzed by non-weighted, nonlinear least squares regression.

Study 2

The large subject variability in the pharmacokinetic parameters for this study was largely due to the small subject size (n=3). As this study was planned as a pilot study to test the assay method, the suppression scheme, the experimental protocol, and to obtain an initial idea of the dose-

dependence of hydrocortisone pharmacokinetics, the small subject number was adequate. However, caution must be used in obtaining pharmacokinetic generalizations from this study.

The lack of samples between 0 h and 1 h post-dosing caused uncertainty in the values of the first order absorption rate constants. In fact, doubt remains concerning the actual order of the absorption rate process. This prompted the addition of earlier sampling times to Study 3 and the suggestion of earlier times for Study 4. This suggestion for Study 4 was vetoed by one of the various human experimentation committees involved in protocol approval. Thus, the same uncertainty in the absorption rate process was also seen in Study 4 as discussed later.

An additional cause of Study 2 parameter variability was the inclusion of all measurable sample concentrations in the calculations. Data for this study were analysed before the full significance of the possible effects of residual endogenous concentrations was realized. Data were corrected for endogenous concentrations and non-weighted least squares regressions were used as in Studies 3 and 4, but concentrations below 10 ng/ml were not omitted from the regressions.

The inclusion of an absorption lag time was necessary to allow successful predictions of maximum concentrations using the proposed model. Inclusion of such a parameter is logical, considering transit time to the small intestine absorption site, and time required for tablet disintegration, particle dissolution, and drug absorption processes.

Elimination phases for mean concentration values for the 10, 30, and 50 mg doses were concave, linear, and convex with respect to the x-axis as shown in Figure 15. This could simply be due to subject variability as this pattern was not evident in all three subjects. However, the significant decrease in k_{el} at 50 mg suggests the occurrence of a decreased biotransformation rate either due to enzyme saturation at higher concentrations or decreased presentation of drug to the biotransformation site attributable to an increased distribution volume. The apparent convexity for the 50 mg dose agrees with possible enzyme saturation. The lack of a significant difference between C_{max} values at 30 mg and 50 mg would suggest an increased distribution volume at higher doses, but may also be an absorption rate effect associated with the number of tablets given. Results for Study 2 can not be used to decide which of these, or other, factors are actually responsible for the apparent pharmacokinetic dose dependence.

Results from Study 2 showed the UV assay to be essentially equivalent to the fluorescence assay in terms of sensitivity, specificity, and repeatibility. The UV assay was faster, simpler, and provided chromatograms of better quality as compared to the fluorescence assay. Plasma hydrocortisone concentrations obtained after a 10 mg oral hydrocortisone dose were high enough to establish elimination half-lives with only minor interference from suppressed, endogenous hydrocortisone. Rapid drug absorption requires samples to be drawn at sample times no less frequent than 10, 20, 30, 45, and 60 minutes post-dosing, in order to accurately establish the absorption kinetics. Pharmacokinetic results suggested the existence of dose-dependent absorption, distribution, or elimination for hydrocortisone.

Study 3

The lack of a significant subject effect on any pharmacokinetic parameter for Study 3 can be attributed to the larger subject size (n=6) and to administration of the drug intravenously rather than orally. Drug disposition for all doses in all subjects was definitely biphasic, with a well-defined, first order distribution phase followed by a slower first-order elimination phase.

Linearity of the elimination phases was excellent for concentrations above 10 ng/ml. Adoption of a more complicated pharmacokinetic model could not be justified in view of the predictive success seen with the two-compartment model. As previously mentioned, only two out of the 24 data sets exhibited non-linearities which might be attributable to endogenous interference. The omission of concentrations less than 10 ng/ml and the use of non-weighting in the regressions helped to guard against any such effects.

Rate and distribution parameters were similar for the 5, 10, and 20 mg doses. The decreased mean rate constant for the return of drug from the peripheral compartment, and the increased mean peripheral compartment and mean steady state distribution volumes at 40 mg, suggest that drug distribution at higher plasma drug concentrations is occurring into different body regions than was allowed at lower plasma concentrations. This occurrence would be in agreement with the known plasma protein binding characteristics of hydrocortisone as presented in Introduction. The increased free fraction of drug in plasma at concentrations above 200 ng/ml should allow increased access of drug to tissues such as muscle and fat. Midgeon, et at (56) has reported that plasma protein binding apparently prohibits access of

hydrocortisone into muscle and fat regions at physiological concentrations, since amounts of drug found in these areas were not much greater than that attributable to the tissue blood supply. Elimination phase plasma drug concentrations in Study 3 were below 200 ng/ml with the 5 and 10 mg doses, and were above 200 ng/ml for only 1.5 hours with the 20 mg dose, but were above this concentration for 3 to 4 hours with the 40 mg dose. The high correlation between $t_{\frac{1}{2}}\beta$ and Vd_{SS} suggested that this increased distribution is responsible for the increased half-life at 40 mg, possibly a result of a smaller fraction of total drug being presented to the hepatic biotransformation sites. This decrease in elimination rate, though, is partially compensated by increased renal clearance (51) and decreased competition between transcortin and hepatic biotransformation enzymes (78) due to the increased free fraction and predominance of binding of hydrocortisone to plasma albumin at drug concentrations above 200 ng/ml.

The execution of Weeks 2, 3, and 4 of Study 3 one week later for Subject 3 than for other subjects did not have a noticeable effect on any of the results. The extremely high initial plasma drug concentrations for the 20 mg dose in Subject 6 (see Table 14e) are difficult to explain.

The apparent volume of distribution, as computed by D/C_0 where C_0 was obtained by NREG, was 65 ml. Use of the 10 minute concentration for C_0 gave a distribution volume of 870 ml. Obviously, this is physiologically impossible. Even using the 10 minute concentration, a 70 mg dose would have to have been given in order to obtain a 3 l distribution volume. This would have required 1.4 ml of drug solution which would have been difficult to fit into the 1 ml syringe used. If the C_0 value is assumed to be correct, 19 ml of drug would have to have been given. The only explanation presently available is that reconstitution of the solid drug was incomplete at the time the syringes were filled, and a piece of undissolved drug was aspirated into the syringe. Reassay of the plasma sample gave identical results, so the concentrations appear to be real. Assuming the concentrations are correct, the pharmacokinetic results should be of interest. Although uncertainty concerning the administered dose precludes calculation of distribution volumes, the obtained $t_{\frac{1}{2}}el$ and $t_{\frac{1}{2}}\beta$ values of 0.05 h and 0.58 h suggest an extremely large increase in elimination rate at the high concentrations. This is supported by the observation that, although the concentration at 10 minutes was approximately 20 times greater for Subject 6 than the mean value for the 20 mg dose at that time, concentrations for Subject 6 at 4, 5, 6, and 8 h were all within 1

standard deviation of mean values for the 20 mg dose at the same times. If real, this effect is probably due to saturation of both plasma protein binding and renal tubular reabsorption. This result emphasizes the fact that the present study is examining only a very small portion of the normal hydrocortisone dosage range of from 5 mg to 500 mg.

Although subject variability was still large, the use of a greater number of subjects (n=6) resulted in increased confidence in the pharmacokinetic results. The use of non-weighted regression of corrected concentrations greater than 10 ng/ml minimizes possible effects from residual endogenous hydrocortisone. Drug distribution and elimination characteristics are independent of dose size for 5, 10, and 20 mg intravenous doses, but changes in these parameters occur with 40 mg doses as a result of changes of drug distribution in the body.

Study 4

As mentioned previously, a proposed increase in the frequency of early blood sampling times was rejected by one of the human experimentation committees. Consequently, sampling times were identical to Study 2. The increased absorption rate seen with the suspension for Study 4 as compared to the tablets of Study 2, resulted in a shortage of absorption phase

data points. The increased rate was most likely due to the absence of tablet disintegration and granule dissolution processes with the suspension. The uncertainty in the observed C_{max} and t_{max} values, and the inability to characterize the absorption rate process, are valid reasons that the protocol for all future oral hydrocortisone bioavailability studies must have a higher frequency of sampling times. The originally proposed times of 10, 20, 30, 45, and 60 minutes should provide sufficient data. This author does not consider this number or frequency of human sampling times to be excessive or dangerous.

Mean elimination phases for the four dose levels of Studies 3 and 4 (Figures 18 and 23) are essentially superimposable. Thus, dose-effects on elimination and steady state distribution should be similar for hydrocortisone in intravenous or suspension form. As C_{max} values for all 4 oral doses are below the initial concentrations observed for the 10 mg intravenous dose, and dose effects for the intravenous dose were mainly present at doses above 20 mg, dose dependence resulting from distribution or elimination effects should be absent from all oral suspension doses. However, almost identical decreases in k_2, with increasing dose, were observed as with β of Study 3, with k_2 values at 5 and 10 mg significantly greater than those at 20 and 40 mg.

The 20 and 40 mg suspension doses gave concentrations above 200 ng/ml for 2 and 3 h, respectively, which may have been responsible for the observed decrease in k_2. If k_a could have been obtained, drug distribution volumes could have been calculated and examined for dose dependence. The similarity of the results for the elimination rate constants of Studies 3 and 4 suggests that distribution volume in Study 4 increases with increasing dose as in Study 3.

The lack of difference in C_{max} and AUC values at 5 and 10 mg may be related to a drug absorption or a hepatic first-pass effect since AUC values in Study 3 were significantly different for all four doses. Dose-dependence in the AUC values for Studies 2, 3, and 4 will be further examined in a later section of this Discussion.

The execution of Week 4 of this study one week later for Subjects 1, 4, and 6 than for the other subjects did not have an observable effect on the results. In contrast to Study 3, all parameters exhibited small, but significant subject-effect differences. As the differences appeared random, they were attributed to normal subject variability in hydrocortisone dispostion and suggest that a subject size greater than 8 is required for the study of hydrocortisone bioavailability. The cause of the significant group effect

is unknown. The repetition in the group order of the differences suggests the influence of an uncontrolled factor. This factor is unknown since assignment of subject to groups was done randomly.

The inclusion of a 12 h sample in this study revealed that hydrocortisone concentrations at this time (C_{12}) were below the pre-dosing concentrations in all but 5 subjects. This implies that the exogenous hydrocortisone did cause additional suppression of endogenous hydrocortisone. Most of the C_{12} values were within 4 ng/ml of the C_{pre} value. A profile similar to A in Figure 26, but somewhat less pronounced, was seen in 8 of the 32 subjects in Study 4. However, elimination phases for concentrations greater than 10 ng/ml were linear for all subjects. If additional suppression did occur, it probably had an insignificant effect on the results. This re-emphasizes the importance of omitting corrected concentrations less than 10 ng/ml from the pharmacokinetic analysis.

Results from Study 4 stress the need for a high frequency of early blood sampling times. Oral hydrocortisone doses from 5 to 40 ng/ml result in almost identical elimination phase concentrations as those obtained from the same doses given intravenously. Drug distribution volume may show

an increase with dose with the oral suspension, similar to that observed for intravenous hydrocortisone. Administration of hydrocortisone may cause a slight increase in endogenous hydrocortisone suppression, but this should not have a significant effect on corrected endogenous concentrations greater than 10 ng/ml.

Statistics

As most subject-effect differences in Study 4 were significant at just above the 95% confidence level, an increase in subject size from 8 to 12 should be sufficient to decrease, or possibly eliminate, significant subject effects. Subject variability will still be present, but the increased subject size will allow better determination of the difference between subject variability and actual subject differences.

The correct subject size can be obtained directly by the use of the Power Approach by specifying the maximum difference (71 c). A non-centrality parameter, ϕ', which describes the inequality of means, can be obtained from:

$$\phi' = \frac{(X_i^{max} - \bar{X})}{SD}\sqrt{\frac{1}{2k}} \qquad (25)$$

where X_i^{max} is the parameter value most different from the parameter mean $\overline{X}$, SD is the parameter standard deviation, and k is the number of means. Using Equation 25, non-centrality parameters of 0.57, 0.71, and 0.57 were calculated for k_{el}, C_{max}, and AUC of Study 4. The power of the ANOVA in Study 4 was then obtained from published tables (79). For k = 4 doses, n = 8 subjects, α (Type I error probability) = 0.05, and $\Phi' = 0.6$, the power of the test is approximately 0.8, with a β value (probability of Type II error) of (1-Power) = 0.2. As this is normally the maximum power used in bioavailability tests (73c), the subject size of 8 appears statistically valid. Similarly, increasing the subject size to 12 would increase the power of the test to approximately 0.95.

One possible reason why more significant effects were observed in Study 4 than in Study 3 may have been due to the ANOVA examination of 4 factor effects in Study 4 as compared to 2 in Study 3. This had the effect of decreasing the error sums of squares, resulting in higher levels of significance for the differences.

It was predicted, due to the parametric nature of ANOVA and the non-parametric nature of Tukey's test, that ANOVA would detect significant factor effects at times that Tukey's

test would fail to find a significant difference at the same confidence level. This was not observed to occur in any of the 83 ANOVA/Tukey comparisons of Studies 2, 3, or 4. The excellent agreement between the tests supports the validity of the observed parameter differences.

"Pseudo-observations" for missing parameters as obtained by the Yates method in Study 3 appeared to agree well with observed trends in actual parameter values. This method of data replacement is suitable when fewer than three observations are missing from an ANOVA table. Another method that could have been used would have been to perform ANOVA by regression analysis without replacing the missing values. However, regular ANOVA techniques are normally preferred over the regression approach (71d).

Bioavailability Study Suggestions

With the design and execution of four human subject studies and participation in at least six other studies, this author has had the opportunity to develop several practical suggestions which may help other workers confronted with similar arduous tasks.

Protocol approval for human subject studies can often require several months to obtain. Time can be saved if the experimenter plans the originally submitted protocol to cover

all possible future experiments and protocol changes. Submission of the "worst possible protocol" at the start may save as much as a year by avoiding subsequent protocol changes and additions.

Volunteers for the studies must understand the importance of the study and the reasons why experimental treatments cannot simply be missed and made up later. They must also understand the importance of the precise sample timing in order to avoid statements such as, "Oh, I'm 5 minutes late. That won't matter, will it?". It is most desirable to confine the subjects or keep them near the sampling station, but this is often not possible. Physical examinations are extremely important, especially for drugs eliminated by biotransformation. Physicals should be as thorough as possible with acceptable laboratory values set by the doctor and the investigator prior to the physical. When possible, direct measurements of liver and kidney function should be included. Consistency in food and liquid prohibition, sampling procedures, sample isolation procedures, and in all aspects of the experimental protocol is imperative. When sample timing and drug protein binding characteristics permit, the use of a heparin well or a non-heparinized indwelling catheter is recommended. This decreases subject discomfort and insures

that the blood sample will be obtained at exactly the right time. The effects of heparin, or other anti-coagulants, on drug displacement from plasma proteins should be determined prior to use.

AUC Dose Dependence

AUC was proportional to dose in Study 3 at 5, 10, and 20 mg as shown in Figure 22. The slight non-linearity at 40 mg was probably due to the increase in the apparent steady state drug distribution volume for the 40 mg intravenous dose. The mean and 5 of the 6 individual plots of AUC versus dose exhibited zero intercepts when the 40 mg data points were excluded. In contrast, mean and individual AUC versus dose results for the oral doses in Studies 2 and 4 were essentially linear from 5 to 50 mg but had large, positive y-intercepts as shown in Figures 17 and 25. Colburn, et al (80) measured plasma hydrocortisone concentrations in 23 subjects after receiving various doses of oral cortisone acetate. A similar positive y-intercept was observed in a plot of AUC versus dose per kg body weight in that study. Thus, the intercept phenomenon is reproducible in larger subject groups than those used in the present studies, and appears to be related to the oral administration of the drug. Possible causes could be dose-dependence in drug absorption,

in the hepatic first pass effect, or in the drug distribution volume. The third of these choices can be eliminated since distribution volume changes were absent with the IV dose at plasma drug concentrations covering the entire oral dosage range. The other two choices will be explored further.

Let ω represent overall systemic drug bioavailability as given by the product of the fraction of dose absorbed from the gastrointestinal tract, F, and the fraction of absorbed drug surviving the hepatic first-pass effect, as shown in Equation 26.

$$\omega = fF \tag{26}$$

Thus, ω represents the fraction of dose which reaches the systemic circulation as unchanged drug. Established pharmacokinetic theory gives the following relationships between AUC, dose, ω, steady state distribution volumes, and overall elimination rate constants for one-compartment oral and two-compartment IV pharmacokinetic models.

a) One-Compartment Oral

$$AUC_0 = \frac{\omega D_0}{Vdk_{el}}$$

$$\frac{AUC_0}{D_0} = \frac{\omega}{Vdk_{el}}$$

$$\frac{AUC_0 k_{el}}{D_0} = \frac{\omega}{Vd}$$

b) Two-Compartment IV

$$AUC_I = \frac{D_I}{Vd_\beta \beta} \qquad (27 \text{ a \& b})$$

$$\frac{AUC_I}{D_I} = \frac{1}{Vd_\beta \beta} \qquad (28 \text{ a \& b})$$

$$\frac{AUC_I \beta}{D_I} = \frac{1}{Vd_\beta} \qquad (29 \text{ a \& b})$$

There is no ω term for IV administration as the entire dose is assumed to reach the systemic circulation. Concordance between Vd and Vd_{β}, and between k_{el} and β, is not direct due to inherent differences in the two models, but the parameters are comparable in terms of overall drug distribution volumes and elimination rate constants. Equations for AUC_I in terms of Vd_{ss} and β are complex and could not be easily comparable to the one-compartment equations. Thus, AUC was described in terms of Vd_{β} which is related to Vd_{ss} by Equation 30, using the usual two-compartment model terminology.

$$Vd_{\beta} = Vd_{ss} + \frac{(k_2 - \beta) V_1}{k_{21}} \qquad (30)$$

This substitution of volume terms is justified by the close, linear relationship between Vd_{β} and Vd_{ss} for Study 3 as shown in Figure 27.

Graphical comparisons between Study 3 and Studies 2 and 4 of the relationships between dose size and the terms in Equation 28 and 29 should provide information concerning the relative effect of ω, distribution volume, and elimination rate constant changes on the dose-dependency of AUC. Such comparisons are shown in Figures 28 and 29. The increasing negative slope of the line for Study 3 in Figure 28 reflects

the increasing distribution volume and the compensating decrease in β as dose was increased. A large decrease in Figure 28 for Studies 2 and 4 is observed at doses of 5 to 10 mg where the AUC/D values for Study 3 are essentially constant. The differences between the two modes of administration are more obvious in Figure 29. The similarity of the lines for Studies 2 and 4 in Figure 28 and 29 implies that the non-proportionality is not related to the elimination rate constant, but is due to a decrease in ω, an increase in Vd, or both. The constancy of the Study 4 line in Figure 29 supports the absence of a distribution volume effect. Thus, the oral dose non-proportionality appears to be related to overall systemic bioavailability.

Dose effects on ω can be further analysed in terms of changes in f and F. For a given dose, ω can be experimentally determined from oral and IV AUC values using Equation 31.

$$\omega = \frac{AUC_O}{AUC_I} \tag{31}$$

Gibaldi has presented theory which allows the calculation of f from oral or IV AUC data assuming F = 1. The equations are

$$f_O = \frac{Q_L}{Q_L + \frac{FD_O}{AUC_O}} \tag{32}$$

and

$$f_I = 1 - \frac{D_I}{Q_L AUC_I} \qquad (33)$$

where Q_L is hepatic blood or plasma flow rate (81). These equations are based on the assumptions that the rates of drug transport from blood to liver and from liver to blood are equal, and that this rate is blood or plasma flow rate limited. As hydrocortisone equilibration between red blood cells and plasma is rapid (49), the value of Q_L used should be that for hepatic blood flow (81). The results from Equations 32 or 33 can be used with the experimental ω value from Equation 31 to obtain two estimates of F from Equation 26. Alternatively, Equation 32 and 33 can be used to obtain similar equations for ω_0 and ω_I, which can be combined and solved for F in terms of AUC_0, AUC_I, and Q_L. When this is done (See Appendix 10 for derivation), the following equation for F is obtained.

$$F = \frac{AUC_0 Q_L D_I}{D_0(A_I Q_L - D_I)} \qquad (34)$$

The values of F and ω from Equations 34 and 31 can be used in Equation 26 to obtain an estimate of f. This method was applied to the mean AUC results from Studies 3 and 4, and also

to individual AUC values for Subjects 1 and 3 of Studies 3 and 4 as these two subjects participated in both studies. A value of 1.7 l/min was used for hepatic blood flow, Q_L (81). AUC versus dose plots from Studies 3 and 4 (IV and oral suspension) for these three data sets are shown in Figure 30a through 30c. The resulting values of ω, F, and f are shown in Figure 31a through 31c. Figure 31 shows that, while the first-pass effect is essentially constant, the fraction absorbed shows extreme variations with dose, which results in large variations in systemic bioavailability of between 50% and 90%. These findings, however, must be evaluated with caution due to the large number of assumptions involved in the equation derivations. Several possibilities of assumption violations exist:

1) Blood-liver drug exchange may not be hepatic blood flow rate limited, or the characteristics of the exchange may change as a function of changes in the plasma protein binding of drug.
2) Red blood cell uptake is concentration dependent.
3) AUC calculations are based on total drug concentrations, not unbound drug, so changes in binding affinity are ignored.

4) Calculations of F assume a constant Q_L value of 1.7 l/min which may differ between subjects and may not be constant during each experiment.

5) Gibaldi's three-compartment model (81) has not been shown to adequately represent data obtained in these studies.

Regardless of the relative effects of F and f, the oral dose-nonproportionality appears to be related to systemic bioavailability, ω, rather than to changes in drug distribution volumes or elimination rate constants.

An intuitive explanation for the non-zero AUC versus dose intercepts can be proposed by consideration of the AUC versus dose plot that might be obtained if ω changed from 0.8 to 0.5 as dose was increased. One possible result is shown in Figure 32 where ω is 0.8 for doses from A to B, 0.5 for doses from C to D, and is changing between B and C. AUC would be proportional to dose from A to B and from C to D. The relationship would still appear linear from B to C but would exhibit a positive y-intercept. This possibility could be tested by examining AUC values over a much larger dose range than that used in the present studies. The effect of this possible explanation on the validity of bioequivalence studies will be discussed in a subsequent Discussion section.

Implications for Hydrocortisone Bioequivalence Studies

In order to examine bioequivalence of hydrocortisone tablets, an assay for hydrocortisone in plasma must be available. This assay should allow specific detection of hydrocortisone to plasma drug concentrations as low as 5 ng/ml. Hydrocortisone metabolites and dexamethasone should not cause assay interference. The assay should exhibit a high degree of repeatability in order to accurately assay a large number of plasma samples over a long period of time. All of these conditions are met by the UV assay method.

A further condition for the bioequivalence studies is that the results of the product comparisons should not be influenced by endogenous hydrocortisone concentrations. Administration of 2 mg of dexamethasone at 23:00 h prior to a blood sampling period from 08:00 h to 20:00 h, caused hydrocortisone to be suppressed to constant concentrations for the entire length of the sampling period. The suppressed concentrations were low enough to allow the elimination phase kinetics for 5 mg oral hydrocortisone doses to be characterized with negligible interference from the endogenous concentrations. Thus, using the Treatment C suppression method, bioequivalence comparisons of 10 mg and 20 mg oral hydrocortisone tablets can be made without significant influence from residual, suppressed endogenous hydrocortisone.

Bioequivalence studies normally examine parameters such as AUC, C_{max}, and t_{max} for significant differences in the rate and extent of drug absorption between different products, as compared to a standard product or formulation. Such comparisons are not possible if the parameters chosen for comparisons are not functions of dose or of the amount of absorbed drug, or if they fail to discern significant differences in the rate of drug absorption. The present studies have demonstrated a linear dose relationship in AUC and C_{max} values for oral hydrocortisone doses from 5 to 40 mg. The lack of direct proportionality to dose in these parameters does not invalidate bioequivalence comparisons, but will make differences in bioavailability more difficult to detect. In contrast, the t_{max} parameter should be relatively dose-independent, and should be more a function of drug formulation effects. The possibility of increases in t_{max} as dose is increased causes doubt as to the usefulness of this parameter in bioequivalence studies. Due to the very rapid absorption of hydrocortisone, a high frequency of early sampling times would be needed in order to make use of this parameter. The possible dose-dependence of t_{max} should be explored further. The importance of the inclusion of an absorption rate measurement in hydrocortisone

bioequivalence studies is stressed by the report of an inactive prednisone tablet formulation differing in the rate, but not the extent, of drug absorption as compared to a prednisone tablet formulation shown to be therapeutically effective (82).

If possible, bioequivalence studies should be performed in a dosage range within which drug disposition is dose- or concentration- independent. For example, an increased apparent volume of drug distribution with higher amounts of drug in the body could cause two drug formulations, with large differences in the amount of drug absorbed, to exhibit similar areas under the plasma drug concentration versus time curves. The present study has indicated that elimination rate constant dose-dependence for hydrocortisone is mainly a function of changes in the apparent volume of distribution, possibly due to concentration-dependent changes in the binding of drug to plasma proteins. Intravenous doses less than 20 mg and oral doses less then 40 mg appear to exhibit relatively constant distribution and elimination rate characteristics. The observed change in overall systemic bioavailability with the 5 mg dose indicates that oral doses greater than 5 mg should be used in the comparison studies.

Results from Study 4 indicated that a minimum of 8 subjects would be required in order to make statistically valid comparisons of pharmacokinetic parameters. Due to the possibilities of increased variability with poorer tablet products, it is suggested that the bioequivalence study be performed using a minimum of 12 subjects. All subjects should undergo complete physicals before and, if at all possible, at the end of the study. The physicals should include all of the normal liver function tests (SGOT, SGPT, alkaline phosphatase, serum bilirubin, LDH), and measurements of plasma albumin and plasma transcortin should also be included. Food and liquids should be carefully controlled. A slight modification of the present protocol to include a standardized snack at 10:00 h is suggested to help avoid any possible changes in hydrocortisone disposition due to hunger stress, even though the occurrence of such an effect is unlikely. The following modified blood sampling protocol would allow sufficient characterization of the drug absorption phase, and would also serve to examine constancy of endogenous suppression by sampling after exogenous drug has disappeared. Samples should be removed immediately prior to dosing (30 ml sample) and then at 0.17, 0.33, 0.50, 0.75, 1.0, 1.5, 2, 3, 4, 5, 6, 8, 10, 12, and 16 h (10 ml samples). The use of 20 mg

tablets, or 2-10 mg tablets, should prevent problems of dose-dependent absorption, distribution, and elimination even with tablets exhibiting much less absorption than those examined in the present study. The similarity in the results for the tablets of Study 2 and the suspension of Study 4 suggests that other brands of hydrocortisone tablets will have similar or worse relative bioavailability, and that either the Study 2 or the Study 4 product could serve as the bioequivalence standard. Data analysis should be by non-weighted non-linear regression, omitting corrected exogenous plasma hydrocortisone concentrations less than 10 ng/ml.

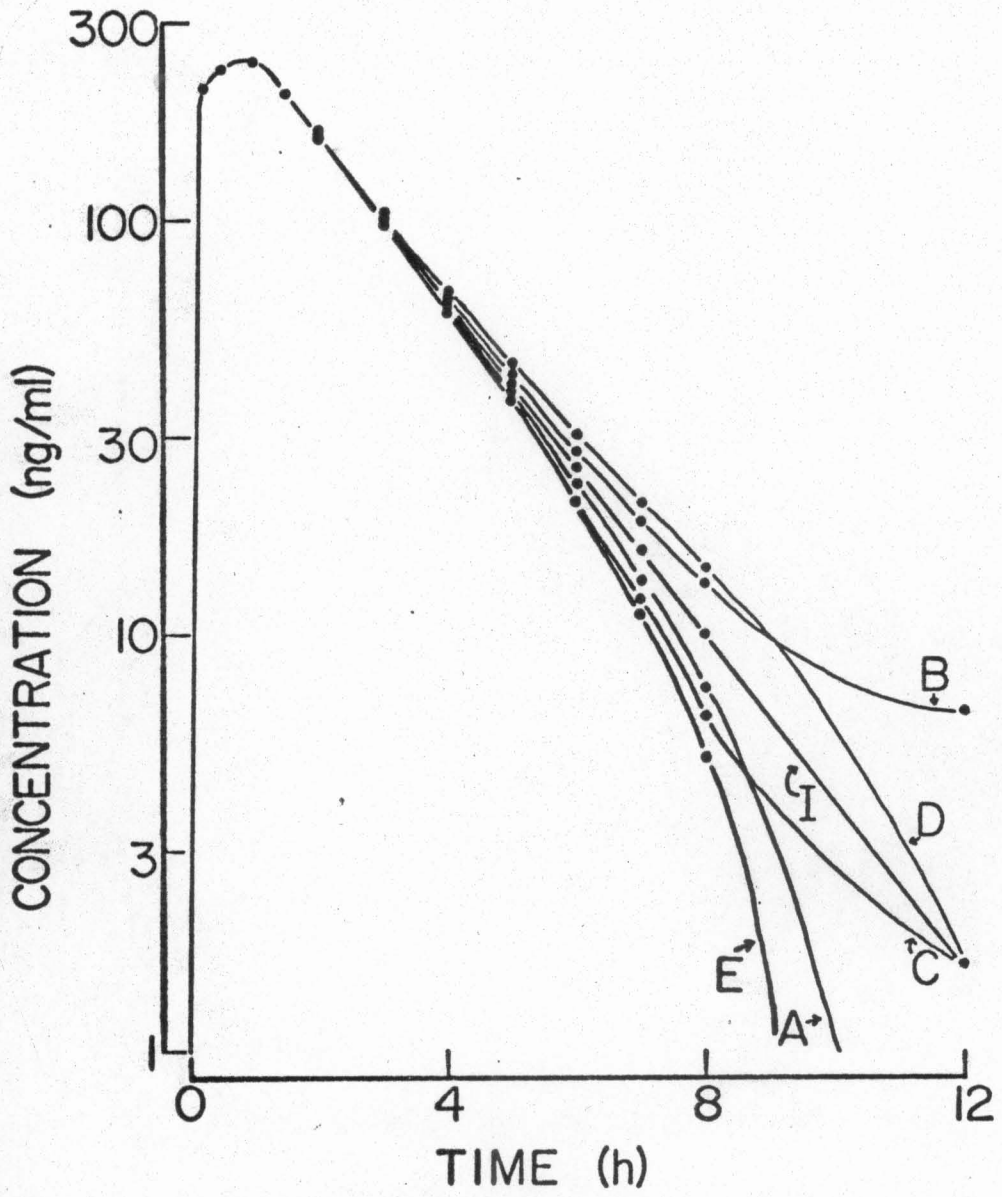


Figure 26 Simulation of the effects of 5 different endogenous plasma hydrocortisone concentration versus time profiles (A through E as described in Discussion) on an ideal (I) set of plasma hydrocortisone versus time data.

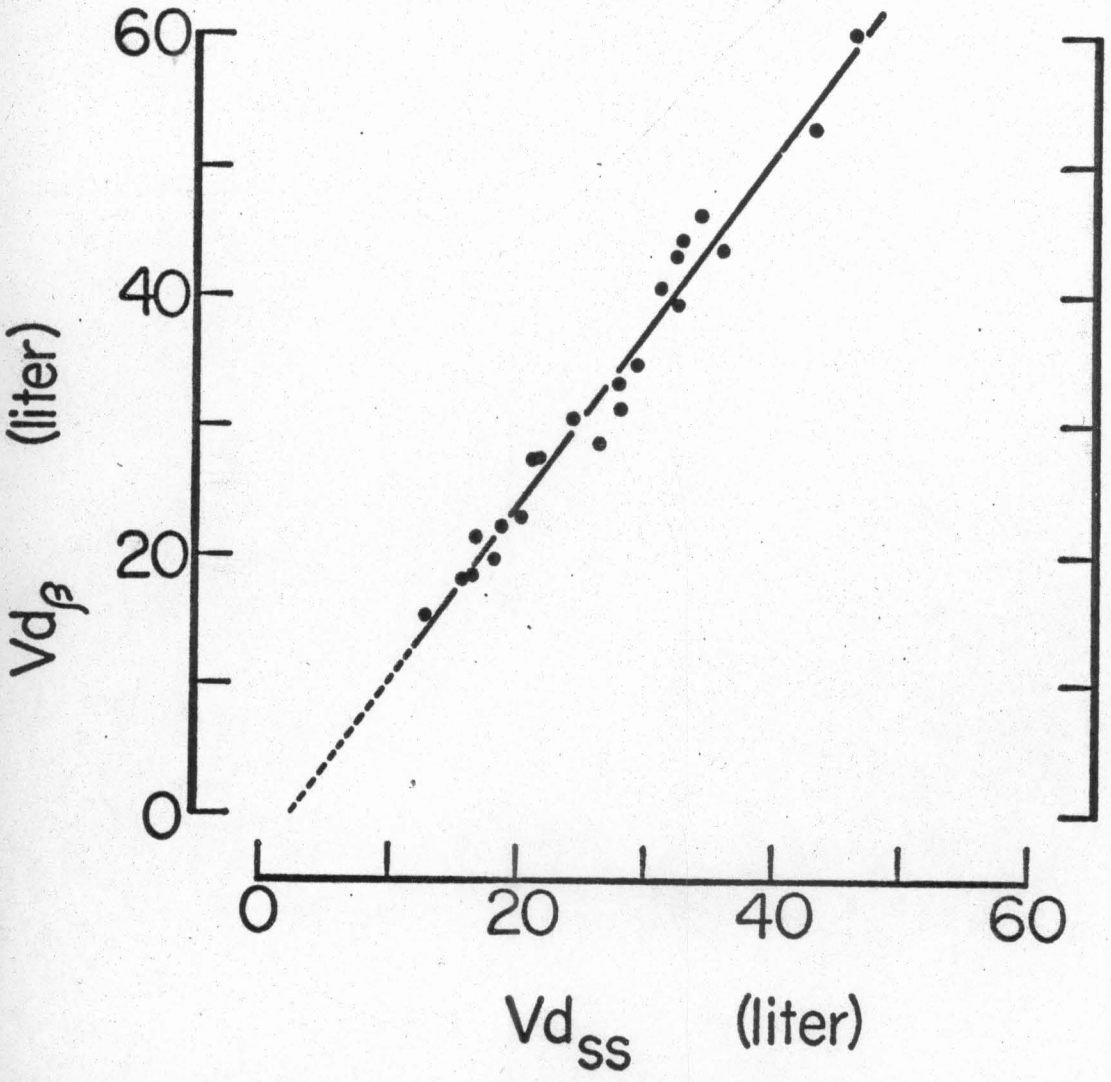


Figure 27 Relationship between apparent volume of distribution obtained by the area method, Vd_β, and the apparent volume of distribution at steady state, Vd_{ss}, for the intravenous hydrocortisone doses of Study 3.

$Vd_\beta = 1.34\ (\pm 0.05) Vd_{ss} - 2.9\ (\pm 1.4)$

$r = 0.9867$ $n = 22$

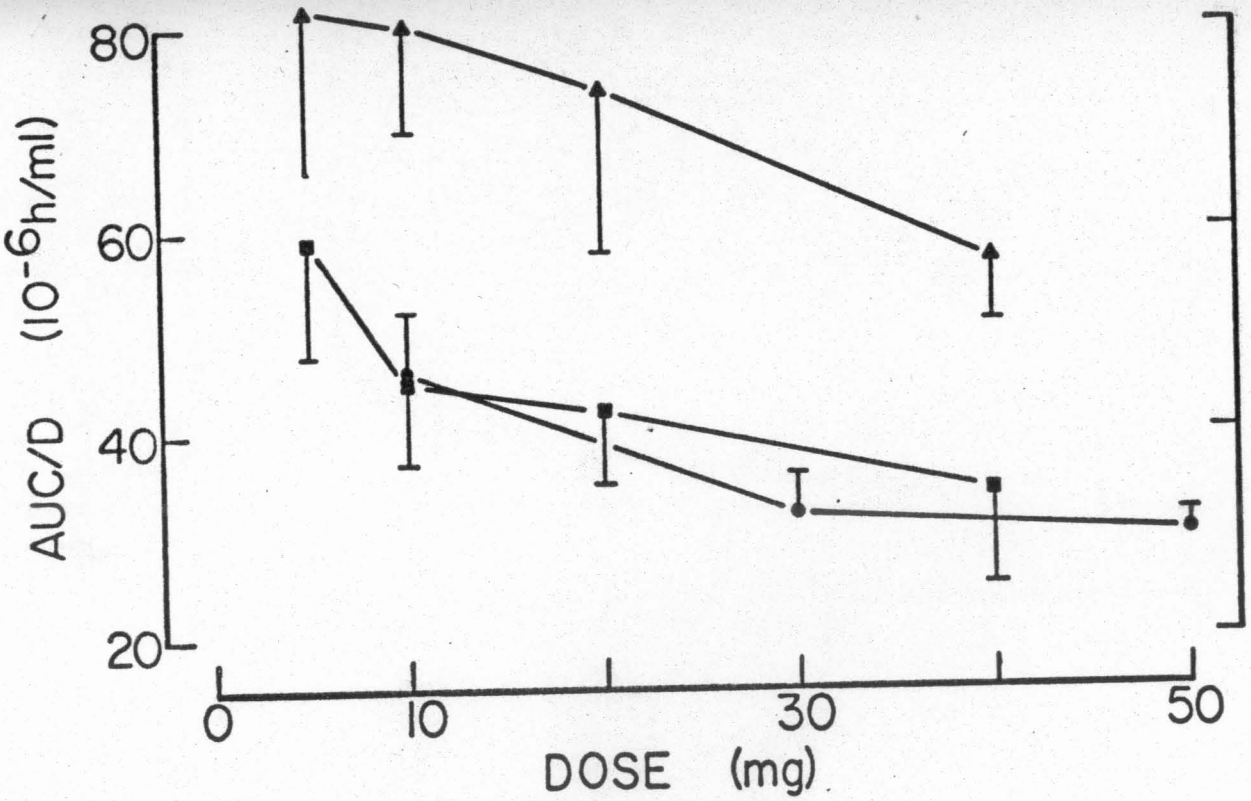


Figure 28 Mean values of $AUC_{trap}^{0\rightarrow\infty}/D$ (±1 SD) versus dose for Studies 2 (●), 3 (▲), and 4 (■).

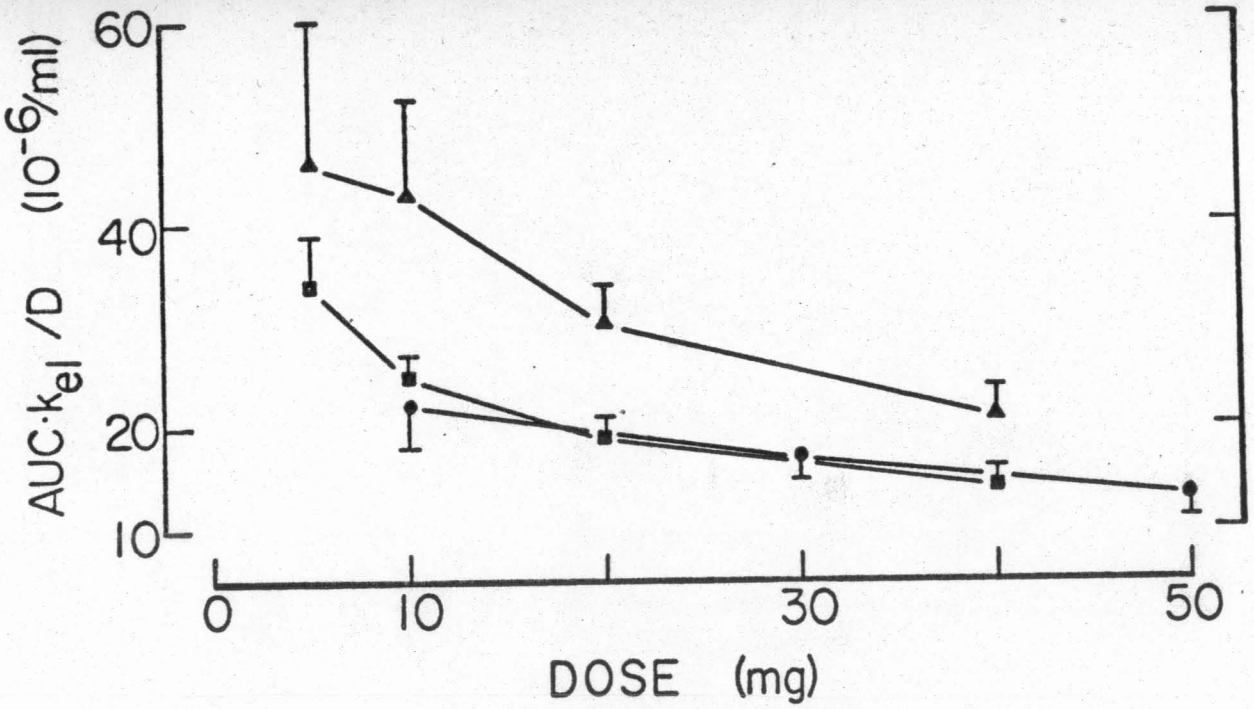


Figure 29 Mean values of $AUC_{trap}^{0\rightarrow\infty} k_{el}/D$ ($\pm$1 SD) versus dose for Studies 2 (●), 3 (▲), and 4 (■). For Study 3, $k_{el} = \beta$.

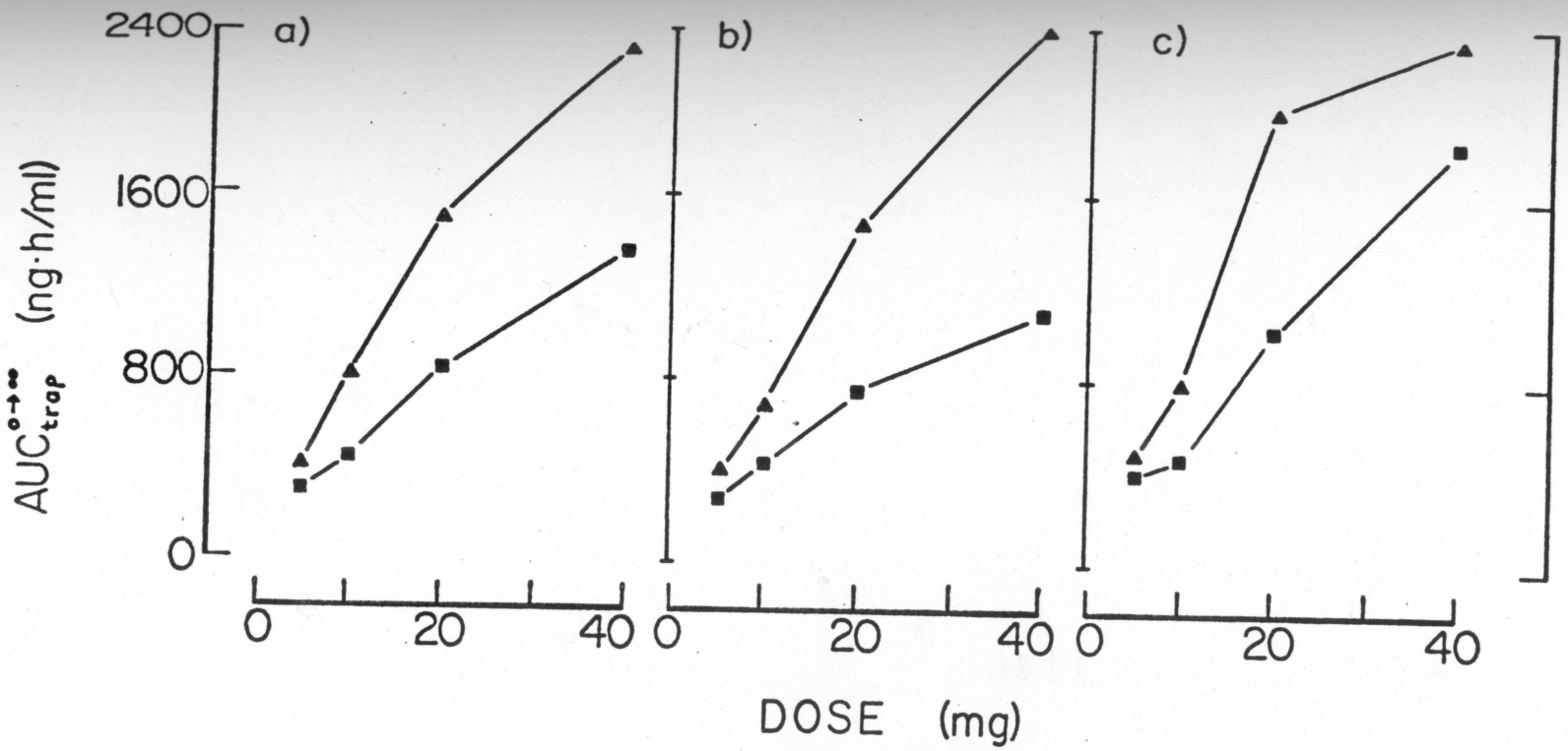


Figure 30 Trapezoidal area under the curve values from time 0 to ∞ ($AUC^{0\rightarrow\infty}_{trap}$) versus dose for Studies 3 (▲) and 4 (■).

a) Mean values b) Values for Subject 1 c) Values for Subject 3

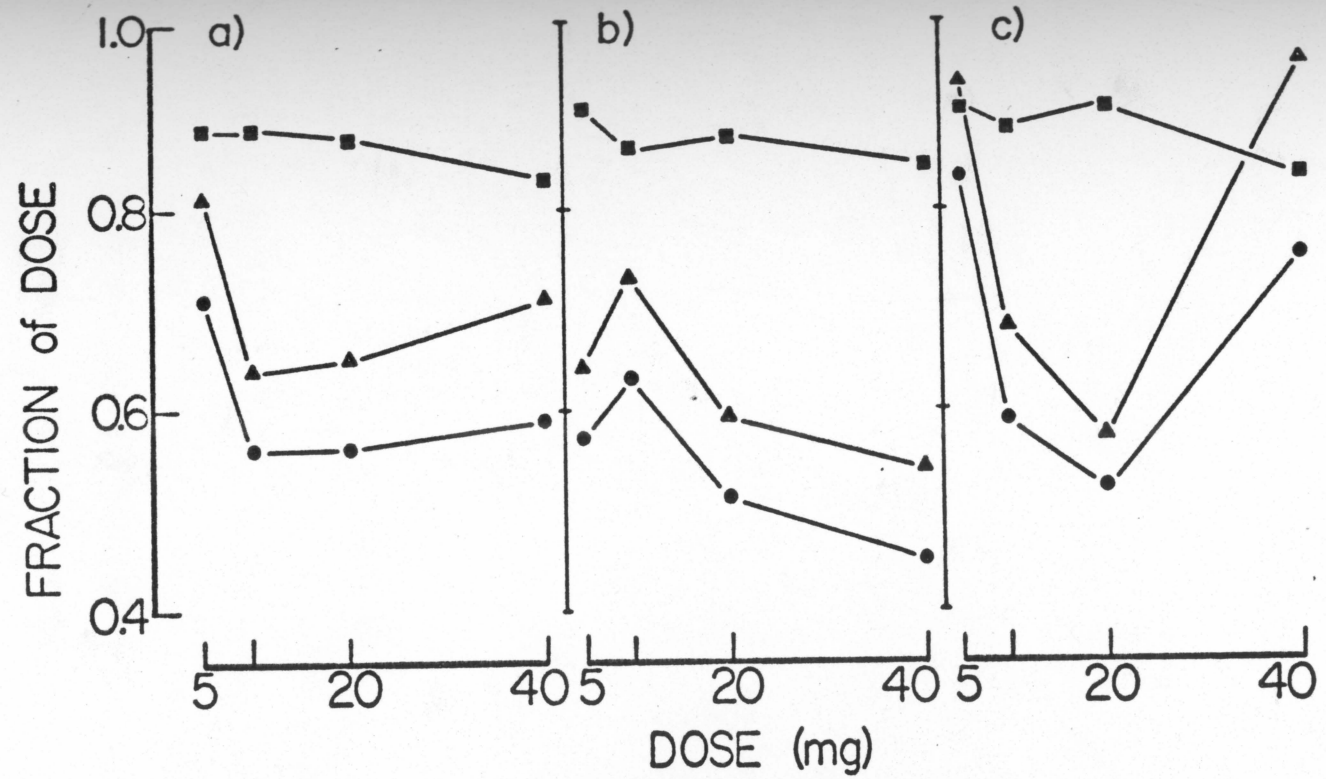


Figure 31 Dose relationships of the overall systemic bioavailability, ω (●), the fraction of dose absorbed, F (▲), and the fraction of absorbed dose reaching the systemic circulation as unchanged drug, f (■), as calculated by Equations 31, 34, and 26, respectively. a) Mean values for Studies 3 and 4, b) values for Subject 1, c) values for Subject 3.

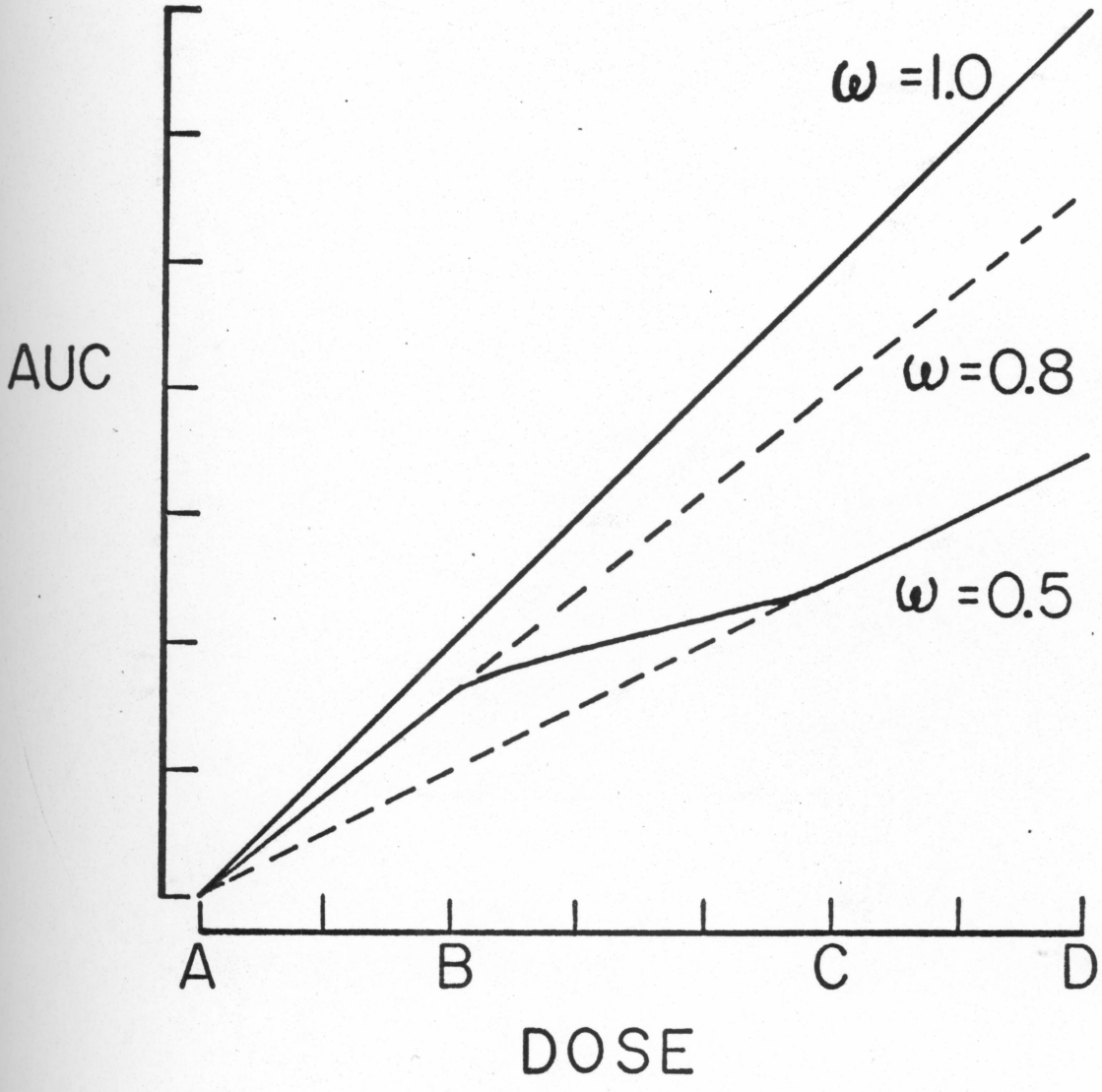


Figure 32 Hypothetical relationship between area under the plasma concentration versus time curve (AUC) and dose for a drug exhibiting a change in overall systemic bioavailability (ω) from 0.8 below B, to 0.5 above C.

Table 22 Summary of modifications to Scott assay method (41) and differences in assay results as compared to the ultraviolet method.

Factor	Method	
	Scott	Ultraviolet
Amount of NaOH	0.025 mmoles	0.1 mmoles
Internal Standard	prednisone	Δ^4-Pregnen-17α, 20β, 21-triol-3, 11-dione
Organic extractant volume	7 ml	10 ml
Length of vortex mixing	30 sec	60 sec
Centrifugation	5 min at 1300 g	15 min at 500 g
Evaporated extractant volume	5 ml	9 ml
Evaporation gas	air	N_2
Evaporation temperature	40°C	room temp
HPLC injection volume	100 µl	30 µl
HPLC injector loop	72 µl	20 µl
HPLC mobile phase	45% $CH_3OH_{(aq)}$	60% $CH_3OH_{(aq)}$
Detection wavelength	239 nm	254 nm
RESULT		
Retention time:hydrocortisone	15	9
Internal Standard	11	7
% Recovery	86%	82%
Sensitivity Limit for Plasma	11 ng/ml	4 ng/ml
Linear Assay Range	20-800 ng/ml	5-3000 ng/ml

CONCLUSIONS

1. An HPLC assay has been developed which is capable of specific quantitation of hydrocortisone in human plasma to a lower concentration limit of 5 ng/ml. The assay is free from interference caused by hydrocortisone metabolites, other endogenous steroids, and dexamethasone. Recoveries of hydrocortisone and internal standard from plasma are 82% and 83%. Response, as measured by peak height ratio is linearly related to concentration from 5 to 3000 ng/ml. As many as thirty plasma samples can be analysed per day. The assay method is suitable for use in hydrocortisone bioequivalence studies.

2. The suppression of endogenous hydrocortisone by dexamethasone, results in constant plasma hydrocortisone concentrations between 6 and 14 ng/ml. Length, but not amount, of maximal suppression is dependent on the dexamethasone dosage regimen employed. When given at 23:00 h, 1 mg of dexamethasone will keep hydrocortisone concentrations constant until about 16:00 h the following day, while an additional 1 mg of dexamethasone at 06:00 h will increase the time of maximum suppression to at least 20:00 h. Two mg of dexamethasone given at 23 h (Treatment C) will hold hydrocortisone concentrations constant until at

least 20:00 h and has less of a chance of affecting the pharmacokinetics of an 08:00 h dose of hydrocortisone than does the split 2 mg dexamethasone dose. Treatment C is ideal for use in suppressing endogenous plasma hydrocortisone concentrations during the execution of a hydrocortisone bioequivalence study.

3. Correction of exogenous plasma hydrocortisone concentrations by subtraction of suppressed endogenous concentrations obtained prior to hydrocortisone administration is based on the constant suppressed hydrocortisone concentrations observed during dexamethasone-suppression. Effects from variability in these suppressed concentrations are minimized by omitting corrected exogenous concentration data less than 10 ng/ml and by performing nonlinear regression with a weighting factor of 1.0.

4. Intravenous hydrocortisone doses of 5 to 40 mg exhibit biphasic elimination with fast and slow phase half-lives of approximately 0.1 and 1.5 h, respectively. The slow phase elimination half-life is significantly greater at 40 mg as a result of an increase in the apparent volume of drug distribution at steady state from values of about 21 l at 5 and 10 mg to 37 l at 40 mg. This change is probably

due to the concentration-dependence of the binding of hydrocortisone to plasma proteins, although verification of this as the cause would have to be done by studing the protein binding of the actual plasma samples. The relationship between AUC and dose is linear with axial intercepts of approximately zero. Pharmacokinetic disposition of intravenous hydrocortisone for doses between 5 and 20 mg is essentially independent of the administered dose.

5. Oral hydrocortisone doses from 5 to 40 mg exhibit rapid absorption with maximum plasma drug concentrations obtained within 1 h after administration. Drug undergoes first order elimination with a half-life of from 1.3 to 1.7 h. Elimination phase drug concentrations are essentially identical to those obtained from similar hydrocortisone doses given intravenously. Absolute bioavailability is about 90% for 5 mg doses and decreases to about 60% for doses from 10 to 40 mg. AUC values increase linearly as dose is increased, but exhibit large, positive y-intercepts for mean and individual plots of AUC versus dose. The lack of such an intercept for intravenous hydrocortisone suggests that the intercept is a result of dose-dependence in either the drug absorption process or the

hepatic first-pass effect. The tablet and the suspension formulations exhibited almost identical rates and extents of absorption, indicating a lack of tablet disintegration and granule dissolution effects on drug absorption for this tablet formulation. The possibility of a different absolute bioavailability at 5 mg doses indicates that doses of 10 mg or greater should be used in the bioequivalence studies.

6. The rapid rate of oral hydrocortisone absorption requires that blood samples be obtained no less frequently than 10, 20, 30, 45, and 60 minutes during the first hour after drug administration. Samples at 10, 12, and 16 h postdosing should be included to examine the constancy of endogenous hydrocortisone suppression.

7. The observed variability in the pharmacokinetic parameters for the oral doses in Study 4 indicates that a minimum number of 8 subjects is required to perform valid statistical comparisons. A subject size of 12 is suggested for future bioequivalence studies.

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APPENDICES

APPENDIX 1

Fluorescence Assay Manuscript

vation for I was ~3 kcal/mole less than that of cefazolin. This difference was manifested in the larger k_0 value at pH 9.4 for I. The results of this study are in agreement with expected effects of structural changes at R_1 on the pH–rate profiles of cephalosporin derivatives.

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ACKNOWLEDGMENTS

Abstracted in part from a dissertation submitted by E. S. Rattie to the Graduate School, Temple University, in partial fulfillment of the Master of Science degree requirements.

Fluorometric High-Pressure Liquid Chromatographic Determination of Hydrocortisone in Human Plasma

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Received February 28, 1979, from the *Division of Biopharmaceutics, Food and Drug Administration, Washington, DC 20204.* Accepted for publication May 8, 1979.

Abstract □ A highly sensitive and specific method for hydrocortisone determination in plasma is described. In this method, an internal standard (Δ^4-pregnen-17α,20α,21-triol-3,11-dione) was added to plasma, which was then extracted with a mixture of methylene chloride–ether (60:40). After separation and evaporation of the organic phase, derivatization was carried out with dansylhydrazine. Upon completion of the reaction, the excess dansylhydrazine was reacted with pyruvic acid and a second extraction and evaporation step was performed. The residue was taken up in 100 μl of the high-pressure liquid chromatographic mobile phase, and 5 μl was injected onto a microparticulate silica column. Elution was carried out with an ethylene dichloride–butanol–water (91:8.5:0.5) mobile phase. The effluent was monitored with a fluorescence detector (excitation 240 nm; emission 470-nm cutoff filter). A linear calibration curve was found from 5 to 150 ng/ml with the precision estimated to be ±7% (*CV*).

Keyphrases □ Hydrocortisone—analysis, high-pressure liquid chromatography, fluorometry, plasma, humans □ Glucocorticoids—hydrocortisone, high-pressure liquid chromatographic and fluorometric analysis, plasma, humans □ High-pressure liquid chromatography—analysis, hydrocortisone, human plasma, fluorometry

Hydrocortisone is a potent anti-inflammatory, immunosuppressive, and antiallergenic drug (1). Since the discovery of hydrocortisone, numerous synthetic glucocorticoids have been prepared. However, hydrocortisone remains an important agent in modern adrenocorticoid steroid therapy.

Various analytical methods have been used to assay hydrocortisone in plasma including fluorescence (2, 3), competitive protein binding (4), enzyme immunoassay (5), and radioimmunoassay (6). All of these methods have problems with specificity. More recently, high-pressure liquid chromatographic (HPLC) methods for hydrocortisone assay in plasma have appeared (7–10). Most HPLC methods exhibit the required specificity but do not provide the low nanogram per milliliter sensitivity needed for bioequivalence studies. This paper describes a highly sensitive and specific HPLC assay for plasma hydrocortisone that can be used in bioequivalence studies.

EXPERIMENTAL

Apparatus—The modular high-performance liquid chromatograph consisted of a constant-flow pump[1], a valve-type injector[2], a fluorescence detector[3] (excitation 240 nm; emission 470-nm cutoff filter), and a strip-chart recorder[4] (0.5 cm/min). A stainless steel column (4.6 mm i.d. × 250 mm) packed with fully porous, irregularly shaped 5-μm silica[5] was obtained commercially.

Chromatographic Conditions—The mobile phase was ethylene dichloride–butanol–water (91:8.5:0.5). A flow rate of 1.5 ml/min was established (2200 psig), and the column was equilibrated for 16 hr. The column was maintained at 19.5° by inserting it into a glass sleeve, which was immersed in a constant-temperature water bath[6].

Reagents and Materials—Hydrocortisone[7], Δ^4-pregnen-17α,-20α,21-triol-3,11-dione[7] (internal standard), pyruvic acid[8], and dansylhydrazine[9] were obtained from commercial sources. All were used as received except dansylhydrazine, which was recrystallized from chloroform prior to use. Solvents used were spectroanalyzed ethylene dichloride[6], HPLC-grade butanol, methylene chloride, and ether[10]. All other materials were reagent grade.

[1] Chromatography pump, model M6000A, Waters Associates, Milford, Mass.
[2] Sample injection valve, model U6K, Waters Associates, Milford, Mass.
[3] Model FS970, Schoeffel Instruments, Westwood, N.J.
[4] Model 9176, Varian Instruments, Palo Alto, Calif.
[5] Prepacked Hibar II column with Lichrosorb SI-60 5-μm silica, Applied Science Laboratories, State College, Pa.
[6] Model 3080, Lab-Line Instruments, Melrose Park, Ill.
[7] Sigma Chemical Co., St. Louis, Mo.
[8] Fisher Scientific Co., Pittsburgh, Pa.
[9] Regis Chemical Co., Morton Grove, Ill.
[10] Burdick & Jackson Laboratories, Muskegon, Mich.

0022-3549/79/1100-1374$01.00/0

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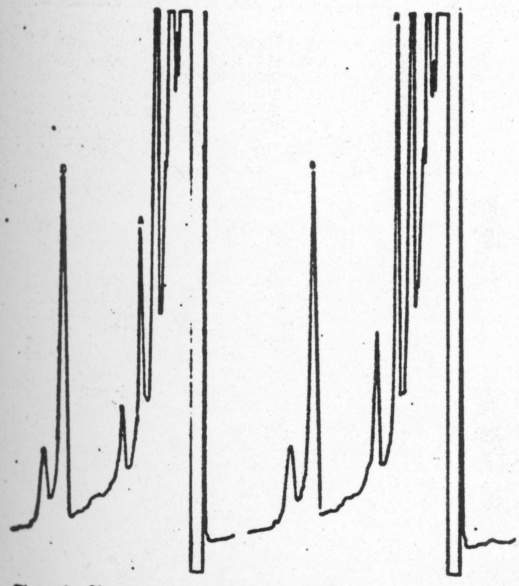


Figure 1—*Chromatogram obtained from HPLC assay of control human plasma (left) and control plasma spiked with an additional 50 ng of hydrocortisone/ml (A) (right). Both were spiked with 200 ng of internal standard/ml (B).*

Stock solutions of hydrocortisone and the internal standard were prepared individually by dissolving 10 mg of each in 100 ml of methanol and making appropriate dilutions. Plasma standards were prepared by taking small volumes of the stock hydrocortisone solutions and adding them to control plasma. The dansylhydrazine solution was prepared by dissolving 20 mg of reagent in 10 ml of absolute ethanol.

The ethanolic hydrochloric acid solution was prepared by adding 0.65 ml of concentrated hydrochloric acid to 1 liter of absolute ethanol. For the pyruvic acid solution, 40 mg of pyruvic acid was added to 10 ml of absolute ethanol. The phosphate buffer was prepared by titrating a 1.0 *M* solution of dibasic potassium phosphate to pH 8.8 with a 1.0 *M* solution of monobasic potassium phosphate.

Assay—To 1 ml of plasma in a 12-ml glass-stoppered conical centrifuge tube was added 200 ng of the internal standard (20 μl of a 10-ng/μl methanolic solution). The tubes were mixed[11], and then 6 ml of methylene chloride–ether (60:40) was added. After mixing for 30 sec, the samples were centrifuged and the organic phase was transferred to a second tube. The solvent was evaporated[12] under a gentle nitrogen stream. To the residue were added 100 μl of ethanolic hydrochloric acid and 100 μl of ethanolic dansylhydrazine.

After the solution reacted for 1 hr at room temperature, 100 μl of the pyruvic acid solution was added. After 15 min at room temperature, 2 ml of phosphate buffer and 6 ml of methylene chloride–ether (60:40) were added. The samples were mixed for 30 sec and centrifuged, and the organic phase was transferred to a third test tube. After solvent evaporation, the residue was dissolved in 100 μl of the HPLC mobile phase and 5 μl was injected.

The hydrocortisone concentrations were determined from standard curves prepared by plotting peak height ratios of the dansylhydrazones of hydrocortisone and internal standard *versus* the spiked concentrations. To correct the standard curve for endogenous hydrocortisone in control plasma, the corresponding peak height ratio was subtracted from all data points used in the standard curve.

Recovery—Plasma aliquots (1 ml) were spiked with known serial hydrocortisone quantities. After the samples were treated as described, the peak heights for the hydrocortisone dansylhydrazones (corrected for endogenous hydrocortisone) were compared to the peak heights obtained from the same amount of drug that was derivatized but not extracted. The ratio of the slopes was used to estimate percent recovery.

Precision—Normal human plasma aliquots were spiked with known amounts of hydrocortisone to give several concentrations. The samples were assayed in quadruplicate as described.

[11] Vortex-Genie, Fisher Scientific Co., Pittsburgh, Pa.
[12] N-Evap, Organomation Associates, Shrewsbury, Mass.

RESULTS AND DISCUSSION

Before bioequivalence requirements are proposed for a drug, adequate methodology should be available. Although numerous methods exist for assaying hydrocortisone, no one method has the required specificity or sensitivity.

In initial studies with the existing HPLC methods, sensitivity seemed to be the major obstacle for bioequivalence studies. Levels of 25 ng/ml or lower were difficult to measure. One approach to increase the sensitivity of these HPLC assays would be to introduce a fluorophore into the molecule. This fluorophore would allow the use of a fluorescence detector with its inherent greater sensitivity.

Derivatization of steroid keto groups has been extensively studied (12–15). In reactions with 2,4-dinitrophenylhydrazines, Reich *et al.* (12) reported that the 3-keto group reacted faster than the 17- or 20-keto group. Van Den Heuvel and Horning (15) also found that the reaction between 1,1-dimethylhydrazine and the steroid keto groups was position dependent, with the 3-keto group reacting more easily than the 20-keto group and with no reaction for the 11-keto group.

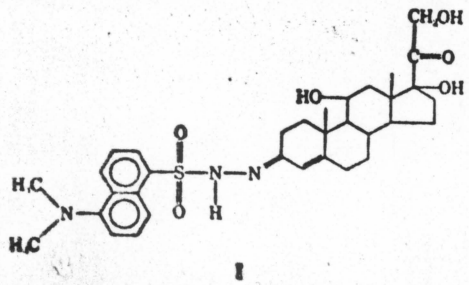
With the introduction of dansylhydrazine (11), a valuable reagent was added to the armory of derivatization reagents that could enhance chromatographic sensitivity. Dansylhydrazine was found to follow the same position dependency as the other hydrazines (13).

When the hydrocortisone dansylhydrazone (I) was prepared and subjected to HPLC analysis, two peaks were observed. These peaks were collected and analyzed by NMR. Both peaks had similar properties, pointing to the formation of *syn*- and *anti*-isomers at the 3-keto position, although no definitive conclusion could be drawn. Similar geometric isomers of the *syn*- and *anti*-types have been found for the 3-keto steroids when reacted with pentafluorobenzylhydroxylamine (14). Further evidence that the two peaks were indeed the *syn*- and *anti*-forms of the 3-keto dansylhydrazones was obtained by reacting the internal standard with dansylhydrazine.

The internal standard used in these studies is a particularly good one, since the functionality is identical, differing from hydrocortisone only in the position of the second keto group. Even though the internal standard has only one reactive keto group in the 3-position, again two peaks with similar chromatographic properties were found. These results indicate that at least with hydrocortisone and the internal standard, the 3-keto group is most reactive with dansylhydrazine and the two derivatives formed are the *syn*- and *anti*-isomers.

Attempts were made to vary the reaction conditions to change the ratio of the two peaks. No change was observed when temperature or reaction time was varied. Similar results were observed with the internal standard. The ratio was independent also of the hydrocortisone concentration. Therefore, the use of this derivatization for quantitative purposes seemed possible.

Both reversed-phase partition chromatography on a C-18 column and normal-phase adsorption chromatography on a microparticulate silica column were studied. The adsorption mode was chosen because a cleaner chromatogram was found after analysis of control plasma. The initial column stability problem was overcome by incorporating a small percentage of water in the mobile phase (16). In addition, room temperature variations affected column performance, resulting in peak shape changes



I

Table I—HPLC Method Precision

Theoretical Concentration, ng/ml	Experimental Concentration, ng/ml, Mean[a] (Range)	CV, %
10	9.8 (8.1–11)	12.3
20	19 (17–21)	8.6
40	38 (38–40)	2.3
60	60 (53–65)	8.3
80	80 (78–84)	3.0

[a] $n = 4$.

that adversely affected assay precision. Instituting column temperature control resolved this issue.

After plasma was analyzed for endogenous hydrocortisone, the resulting peaks had the same retention times and peak widths as the standard. For further confirmation that hydrocortisone was present and that no interfering peaks were hidden under the dansylhydrazone peaks, the standard addition technique was used. The peak heights were additive, and the peak widths at half-height were constant.

Chromatograms obtained in the analysis of human plasma containing only endogenous hydrocortisone and 200 ng of internal standard/ml and the same plasma spiked with an additional 50 ng of hydrocortisone/ml and 200 ng of internal standard/ml are shown in Fig. 1. Two peaks were found for each of the two compounds, as discussed previously. The peaks associated with the hydrocortisone derivatization had retention times of 8.4 and 10.0 min. The peaks associated with the internal standard derivatization had retention times of 16.8 and 18.6 min. The first of these two peaks for each compound was used in calculating peak ratios.

A standard linear plasma calibration curve was obtained in the 5–150-ng/ml range. Sensitivity studies with the hydrocortisone dansylhydrazone showed that as little as 0.25 ng can be detected. The recovery of hydrocortisone added to control plasma was studied by comparing the ratio of slopes obtained for the plasma extraction standard curve and for the direct standard curve. The recovery was calculated to be 83%.

To study method precision, known drug amounts were added to control plasma. These samples were assayed in quadruplicate as described. The coefficients of variation (CV) for the assay of hydrocortisone over the range of 10–80 ng/ml are reported in Table I.

This method was developed for planned bioequivalence studies with hydrocortisone tablets in which dexamethasone was to be used to suppress endogenous hydrocortisone. Therefore, the possibility of dexamethasone interference in the assay had to be explored. In these studies, dexamethasone was added to plasma to give a final concentration of 200 ng/ml. This concentration was well above the dexamethasone concentration expected in the study. These samples and the same control plasma were assayed for endogenous hydrocortisone. The hydrocortisone concentration in the control plasma was determined to be the same as that in the control plasma to which dexamethasone had been added. Thus, it can be concluded that dexamethasone had no effect on the HPLC assay for hydrocortisone.

Table II—Plasma Endogenous Hydrocortisone from Three Human Volunteers

Subject	Concentration, ng/ml	CV[a], %
A	83.2	5.1
B	79.6	1.2
C	80.8	4.0

[a] $n = 3$.

To demonstrate the utility of this method as a routine procedure for hydrocortisone determination, plasma from normal volunteers was assayed (Table II). Results were in the accepted concentration range for endogenous hydrocortisone (17).

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ACKNOWLEDGMENTS

The authors thank M. A. Tischler and E. B. Sheinin of the Division of Drug Chemistry, Food and Drug Administration, for the NMR analyses.

APPENDIX 2

Human Subject Consent Forms For Studies 1 Through 4

2A

Consent Form: Study 1 - Dexamethasone Suppression

Consent Form

For a Hydrocortisone Bioavailability and Pharmacokinetic Study

(Phases I and II)

A study is being conducted by the School of Pharmacy, University of Wisconsin, on the absorption of some commonly prescribed drugs from commercially marketed formulations.

There have been several reports in the medical and scientific literature that the same drug may not be absorbed as well, or may be absorbed equally as well but at different speeds, from different commercial products. For example, tablets produced by various companies may not be 'equivalent' in the way they release drug for absorption.

I will be part of a bioavailability study on hydrocortisone. Prior to beginning the hydrocortisone study, I will also participate in a short dexamethasone study (Phase I). Dexamethasone is known to suppress the normal levels of hydrocortisone in the blood. Although the exact way by which this is accomplished is unknown, it is thought that dexamethasone's action on suppressing hydrocortisone production occurs at the level of ACTH synthesis in the pituitary. On prolonged use of any of the corticosteroids, profound effects on pituitary and adrenal function have been reported. However, based upon extensive clinical experience single doses of corticosteroids can be given without harmful effects. This does not mean that there is not any risk involved. It does mean that the risk is believed to be small. This dexamethasone study is necessary so that we can be assured that sufficient suppression of normally present

hydrocortisone occurs so that it does not interfere in the bioequivalence study when tablets of hydrocortisone are given to me. In the dexamethasone phase of this study, I will be given a maximum of 2 mg of dexamethasone. This is in the normal therapeutic dosage range of 0.5-15 mg/day.

The second phase of the study (Phase II) has been designed to investigate the way in which different formulations of hydrocortisone release drug to the body after oral doses. This is done by measuring drug levels in my bloodstream at various times after I have taken an oral dose of the drug. I will be given hydrocortisone tablets of different strengths (5, 10 and 20 mg with the administered dose not to exceed 50 mg) every other week for six successive weeks. These doses are below the normal therapeutic dosage range (10-300 mg/day).

For my information, the uses (indications) and reported side effects of these drugs are as follows:

1) Uses: Hydrocortisone and dexamethasone are used for their anti-inflammatory (decrease redness and heat in tissues), immunosuppression (e.g., decrease rejection of tissue transplants) and anti-allergic activity (excessive sensitivity to certain substances). They are used to treat adrenal insufficiency (malfunctioning of adrenal gland), arthritis (inflammation of joints), rheumatic carditis (inflammation of heart muscle), bronchial asthma (lung congestion) and other related conditions.

2) Side effects: Reported side reactions of a therapeutic regimen of hydrocortisone include: sodium and fluid retention, potassium loss, increased blood pressure, muscle weakness, increased fragility of bones, peptic ulcers, skin rashes, slow wound healing, increased sweating, convulsion, vertigo, headache, decreased tolerance for carbohydrates, cataracts, glaucoma, euphoria, insomnia, increased susceptibility to infection, mood and personality changes.

The indicated side effects are not expected to occur with the single doses used in this study. However, the above information will help me recognize the side effects if they did occur.

Study Details and Important Points

1. I will have to pass a medical history and standard physical examination before I am admitted to take part in this study. This will include a laboratory examination (i.e., blood and urine tests as well as a tuberculin skin test).
2. I will not donate blood one (1) month before or one (1) month after my participation in this study.
3. I will take no drugs for two weeks before the study starts and I will take no drugs other than the ones being investigated during the study. In the event that I should require or take any medication I will report this to Dr. Welling.

4. I will refrain from any strenuous activity such as jogging, weight lifting, etc. for 24 hours prior to each dosing day and on the day of hydrocortisone administration.

5. At least two weeks before the hydrocortisone bioavailability study is to begin, eight blood samples (0.27 ounces or 8 ml each) will be drawn from my arm vein over a twelve hour period (8 AM, 10 AM, 12 noon, 2 PM, 4 PM, 6 PM and 8 PM). Then that same evening, I will receive 1 or 2 mg of dexamethasone, in the form of an elixir, with 6 ounces (180 ml) of water. The following day I may be required to take another 1 mg of dexamethasone at 6 AM. I will, however, have an additional seven blood samples (0.27 ounces or 8 ml each) drawn on that day according to the previous day's schedule. I will not eat anything after 8 PM on the day that I am given the dexamethasone, but I may drink water up until 10 PM. The day after taking the evening dexamethasone, I will not eat or drink anything until twelve noon.

6. At least two weeks after completing the dexamethasone study (see item 5), the hydrocortisone pilot study (Phase II) will be started. The evening (11 PM) before each study week's dosing day, I will be given 1 or 2 mg of dexamethasone. I may also possibly be given an additional 1 mg of dexamethasone the following day. I will not eat anything after 8 PM on the day that I am given the dexamethasone but I may drink water up until 10 PM. The next day, the hydrocortisone tablet will be administered to me at 8 AM together with 6 ounces (180 ml) of water. I will not eat any food or drink for four (4) hours after hydrocortisone administration.

7. Just prior to taking the hydrocortisone tablet, a 1.7 ounce (50 ml) control blood sample will be drawn from my forearm vein.
8. After taking hydrocortisone, 12 blood samples (0.27 ounces or 8 ml each) will be drawn from my forearm vein over a twenty-four hour period (1/4, 1/2, 1, 1-1/2, 2, 3, 4, 5, 6, 7, 8 and 12 hours). Over the approximately eight week study period, a total of 53 blood samples will be taken from me. This means that each study week a maximum of 4.9 ounces (146 ml) of blood will be taken. Over the eight week period a maximum of about 18.6 ounces (550 ml) of blood will be taken.
9. Apart from the risks already indicated for the particular drugs, I may also experience discomfort and/or risks from multiple punctures for blood specimen collection. This carries with it the possibility of soreness, bruising, bleeding, irritation of the vein wall, or infection. For blood collections a qualified person will be doing it and aseptic conditions will be used.
10. I will report any suspected side effect or any untoward feeling I may experience during any study week.
11. There will be no immediate benefit to me by taking the drugs in this study. However, depending on the outcome of the results, there may be long term benefits to me, and to others, by establishing how well the drugs are absorbed from the various formulations.
12. For my participation in the study I will be paid $3 for every blood sample taken from me. I will be paid at the end of the study.

13. I am free to withdraw my consent and to discontinue participation in the study at any time. This will in no way jeopardize my standing at the university. Additionally, my participation in the study may be terminated at any time upon the discretion of Drs. Welling and Craig.

14. I will receive a copy of this consent form, which I may retain. Also, I will receive a wallet size card which I should carry with me during the complete study. This card includes information such as the physicians and investigators' names and telephone numbers, the dates for drug administration and the study's sampling times.

15. I understand that I may not be asked to continue in the hydrocortisone bioavailability part of this study, if the dexamethasone study does not cause sufficient suppression of my endogenous hydrocortisone blood level.

16. I understand that in regard to compensation for volunteers, the policy of the University of Wisconsin is that in the event that physical injury occurs as a result of research carried out on humans, the University does not automatically provide compensation for medical care or other compensation. In the case of the study in which I am involved, the University does not take responsibility for untoward drug effects. I understand that this system has been in effect for the last seven years during which time Dr. Welling has carried out a large number of studies without any harmful effects.

17. My identity and confidentiality will be preserved if I participate in this study.

18. If there is anything I did not understand about this consent form, or the study in general, Dr. Craig or Dr. Welling will explain it to me.

Consent

I have read and understand the information provided in this consent form.

Anything I did not at first understand has been explained to me.

I hereby give my informed consent for Dr. William A. Craig, or a qualified assistant, to administer the drugs included in this study to me at such times as are indicated in the study protocol. I also consent to Dr. Craig, or a qualified assistant, to obtain venous blood samples from me at the times and using the procedures described to me.

I understand that I am free to withdraw from participation in the study at any time.

Subject_____ Witness_____

Date_____ Date_____

Physician_____ Date_____

2B

Consent Form: Study 2 - Oral Hydrocortisone Tablets

CONSENT FORM

FOR A HYDROCORTISONE BIOAVAILABILITY

AND PHARMACOKINETIC STUDY

A study is being conducted by the School of Pharmacy, University of Wisconsin, on the absorption of some commonly prescribed drugs from commercially marketed formulations.

There have been several reports in the medical and scientific literature that the same drug may not be absorbed as well, or may be absorbed equally as well but at different speeds, from different commercial products. For example, tablets produced by various companies may not be "equivalent" in the way they release drug for absorption.

I will be part of a bioavailability study on hydrocortisone. On four separate occasions separated at least by a week, I will receive a single oral dose of hydrocortisone tablets, not to exceed 50 mg. This is within the normal therapeutic dosing range for hydrocortisone of 10 to 300 mg. per day. The doses I receive on the four separate occasions will be of tablets from four different manufacturers. By sampling my blood after receiving these doses, the presence or absence of differences in absorption or "bioavailability" between the four manufacturers' products may be observed. The evening before receiving the doses, I will be given a single 2 mg. dose of dexamethasone. This is within the normal therapeutic dosage range for dexamethasone of 0.75 to 9 mg. per day. Dexamethasone is known to suppress the normal levels of hydrocortisone in the blood. Although the exact way by which this is accomplished is unknown, it is

thought to be due to a temporary reduction in the release of ACTH from the pituitary gland. ACTH is a compound thought to trigger the release of hydrocortisone into the bloodstream. Suppression of the hydrocortisone normally in the blood is necessary because the hydrocortisone doses to be given will result in blood levels of hydrocortisone similar to normal levels. These normal levels would not allow measurement of the dosed hydrocortisone.

Prolonged use of any of the corticosteroids has been reported to cause profound effects on pituitary and adrenal function. However, based on extensive clinical experience, single doses of corticosteroids can be given without harmful effects. This does not mean that there is no risk involved. It does mean that the risk is believed to be small.

For my information, the uses and reported side effects of these drugs are as follows:

1) Uses: Hydrocortisone and dexamethasone are used for their anti-inflammatory (decrease redness and heat in tissues), immunosuppresive (e.g., decrease rejection of tissue transplants), and anti-allergic activity. They are used to treat adrenal insufficiency (malfunction of the adrenal gland), arthritis (joint inflammation), rheumatic carditis (heart muscle inflammation), bronchial asthma (lung congestion), and other related conditions.

2) Side Effects: Reported side reactions of a therapeutic regimen of hydrocortisone or dexamethasone include sodium and fluid retention, potassium loss, increased blood pressure, muscle weakness,

increased fragility of bones, peptic ulcers, skin rashes, slow wound healing, increased sweating, convulsions, vertigo, headache, decreased tolerance for carbohydrates, increased susceptibility to infection, cataracts, euphoria, glaucoma, insomnia, mood and personality changes.

The indicated side effects are not expected to occur with the single doses used in this study. However, the above information will help me recognize side effects if they do occur.

PRINCIPAL INVESTIGATORS

Dr. Peter G. Welling, School of Pharmacy	263-3986
Dr. William A. Craig, School of Medicine	256-1901 Ext.261
Roger D. Toothaker, School of Pharmacy	262-5819

PLACE OF STUDY

The study will be carried out in a suitably equipped room either at the Clinical Science Center, 600 Highland Ave., Madison 53792, or in Room 5316, School of Pharmacy, 425 N. Charter St., Madison, 53706.

DOSE SIZE

Hydrocortisone, 50 mg on 4 occasions at least one week apart.

Dexamethasone, 2 mg on 4 occasions at least one week apart.

STUDY DETAILS

1) I will have to pass a medical history and standard physical examination before I will be allowed to participate in this study. This will include laboratory examination of blood and urine specimens as well as a tuberculin skin test.
2) I will not donate blood from one month before until one month after participation in this study.
3) I will take no drugs other than those received during the study for a time period from two weeks prior to the study until the end of the study. If I should require or if I do take any medication I will report this to Dr. Welling or Dr. Craig immediately.
4) I will refrain from any strenuous activity such as jogging, weight lifting, etc., during the two days each week that I receive the dexamethasone and the hydrocortisone.
5) On the day I receive dexamethasone I will not eat anything after 8 pm and I will not drink anything after 10 pm. I will be given 20 ml of dexamethasone elixer with 180 ml of water at 11 pm that same evening.
6) The following day I receive the hydrocortisone dose. I will continue to refrain completely from eating or drinking until 12 noon. I will receive hydrocortisone in tablet form with 180 ml of water at 8 am.
7) Immediately before receiving the hydrocortisone, a 50 ml pre-dose blood sample will be withdrawn from a forearm vein. After receiving hydrocortisone, additional 10 ml blood samples

will be drawn from a forearm vein at 8:15 am, 8:30, 9:00, 9:30, 10, 11, 12noon, 1, 2, 3, 4, and 8 pm for a total of 13 blood samples and approximately 170 ml of blood.

8) This pattern of dosing and sampling will be repeated three more times over the approximately 8 week long study period. This will result in a total amount of 680 ml of blood being withdrawn over the 2 month study period and a total number of 52 blood samples.

9) At 12 noon of the sampling days, I will be provided a standardized lunch. I agree to eat all components of this lunch.

10) Apart from the risks already indicated for the particular drugs, I may also experience risks and /or discomfort from multiple punctures for blood specimen collection. This includes the possibility of soreness, bruising, bleeding, vein wall irritation, and infection. These risks will be minimized by using fully qualified personnel to obtain blood samples under strict aseptic procedures.

11) I will report any suspected side effect or untoward feeling I may experience during the study to Dr. Welling or Dr. Craig.

12) I am free to withdraw from the study at any time. Additionally, my participation in the study may be terminated at any time upon the discretion of Dr. Welling or Dr. Craig.

13) There will be no immediate benefit to me from taking the drugs in this study. However, depending on the outcome of the results, there may be long-term benefits for me and for others as a result of establishing how well this drug is absorbed from various formulations.

14) If there is anything I do not understand about the study I will have one of the investigators explain it to me before I sign the consent form.

15) I will receive a copy of this consent form which I may keep. I will also be given a card which gives the names and telephone numbers of the investigators and lists the sampling times.

16) My identity and confidentiality will be preserved if I participate in this study.

17) I will be paid $3 for every blood sample taken from me. Payment will be made at the end of the study.

18) I understand that in regard to compensation to volunteers, the policy of the University of Wisconsin is that in the event that physical injury occurs as a result of research carried out on humans, the University does not automatically provide compensation for medical care or other compensation. In the case of this study, the University does not take responsibility for untoward drug effects.

Peter G. Welling, Ph.D. 608-263-3986

William A. Craig, M.D. 608-256-1901 Ext. 261

Roger D. Toothaker, M.S. 608-262-5819

All funding for this study is supplied by the United States Food and Drug Administration.

Phase II
Hydrocortisone Study
Consent Form

I have read and understand the information provided in this consent form.

Anything I did not at first understand has been explained to me.

I hereby give my informed consent for Dr. William A. Craig, or a qualified assistant, to administer the drugs included in this study to me at such times as are indicated in the study protocol. I also consent to Dr. Craig, or a qualified assistant, to obtain venous blood samples from me at the times and using the procedures described to me.

I understand that I am free to withdraw from participation in the study at any time.

In the event that physical injury occurs as a result of this research which we believe is unlikely, the University does not automatically provide reimbursement for medical care or other compensation. If physical injury is suffered in the course of this research, or for more information, please notify the investigator in charge.

Subject _____ Date _____

Witness _____ Date _____

Physician _____ Date _____

2C

Consent Form: Study 3 - Intravenous Hydrocortisone

CONSENT FORM

FOR A HYDROCORTISONE BIOAVAILABILITY

AND PHARMACOKINETIC STUDY

A study is being conducted by the School of Pharmacy, University of Wisconsin, on the absorption of some commonly prescribed drugs from commercially marketed formulations.

There have been several reports in the medical and scientific literature that the same drug may not be absorbed as well, or may be absorbed equally as well but at different speeds, from different commercial products. For example, tablets produced by various companies may not be "equivalent" in the way they release drug for absorption.

I will be part of a bioavailability study on hydrocortisone. On four separate occasions separated at least by a week, I will receive a single intravenous dose of hydrocortisone, not to exceed 40 mg. This is within the normal therapeutic dosage range for hydrocortisone of 10 to 300 mg. per day. The doses I receive on the four separate occasions will be at levels of 10, 20, 30, and 40 mg. By sampling my blood after receiving these doses, differences in the distribution of drug in the body for the different doses will be able to be detected. Also, comparison of the results with similar results for hydrocortisone given as oral tablets will allow an estimate to be made of the fraction of the oral dose of hydrocortisone that is absorbed compared to intravenous hydrocortisone. The evening before receiving the doses, I will be given a single 2 mg. dose of dexamethasone. This is within the normal therapeutic dosage range for dexamethasone of 0.75 to 9 mg. per day.

Dexamethasone is known to suppress the normal levels of hydrocortisone in the blood. Although the exact way by which this is accomplished is unknown, it is thought to be due to a temporary reduction in the release of ACTH from the pituitary gland. ACTH is a compound thought to trigger the release of hydrocortisone into the bloodstream. Suppression of the hydrocortisone normally in the blood is necessary because the hydrocortisone doses to be given will result in blood levels of hydrocortisone similar to normal levels. These normal levels would not allow measurement of the dosed hydrocortisone.

Prolonged use of any of the corticosteroids has been reported to cause profound effects on pituitary and adrenal function. However, based on extensive clinical experience, single doses of corticosteroids can be given without harmful effects. This does not mean that there is no risk involved. It does mean that the risk is believed to be small.

For my information, the uses and reported side effects of these drugs are as follows:

1) Uses: Hydrocortisone and dexamethasone are used for their anti-inflammatory (decrease redness and heat in tissues), immunosuppresive (e.g., decrease rejection of tissue transplants), and anti-allergic activity. They are used to treat adrenal insufficiency (malfunction of the adrenal gland), arthritis (joint inflammation), rheumatic carditis (heart muscle inflammation), bronchial asthma (lung congestion), and other related conditions.
2) Side Effects: Reported side reactions of a therapeutic regimen of hydrocortisone or dexamethasone include sodium and fluid retention, potassium loss, increased blood pressure, muscle weakness, increased

fragility of bones, peptic ulcers, skin rashes, slow wound healing, increased sweating, convulsions, vertigo, headache, decreased tolerance for carbohydrates, increased susceptibility to infection, cataracts, euphoria, glaucoma, insomnia, mood and personality changes.

The indicated side effects are not expected to occur with the single doses used in this study. However, the above information will help me recognize side effects if they do occur.

PRINCIPAL INVESTIGATORS

Dr. Peter G. Welling, School of Pharmacy	263-3986
Dr. William A. Craig, School of Medicine	256-1901 ext. 261
Roger D. Toothaker, School of Pharmacy	262-5819

PLACE OF STUDY

The study will be carried out in a suitably equipped room either at the Clinical Science Center, 600 Highland Avenue, Madison, WI 53792, or in Room 5316, School of Pharmacy, 425 N. Charter Street, Madison, WI 53706.

DOSE SIZE

Hydrocortisone--10, 20, 30, and 40 mg. on 4 separate occasions at least one week apart.

Dexamethasone--2 mg. on 4 occasions at least one week apart.

STUDY DETAILS

1) I will have to pass a medical history and standard physical examination before I will be allowed to participate in this study. This will include laboratory examination of blood and urine specimens as well as a tuberculin skin test.
2) I will not donate blood from one month before until one month after participation in this study.
3) I will take no drugs other than those received during the study for a time period from two weeks prior to the study until the end of the study. If I should require or if I do take any medication I will report this to Dr. Welling or Dr. Craig immediately.
4) I will refrain from any strenuous activity such as jogging, weight lifting, etc., during the two days each week that I receive the dexamethasone and the hydrocortisone.
5) On the day I receive dexamethasone I will not eat anything after 8 pm and I will not drink anything after 10 pm. I will be given 20 ml of dexamethasone elixer with 180 ml of water at 11 pm that same evening.
6) The following day I receive the hydrocortisone dose. I will continue to refrain completely from eating or drinking until 12 noon. I will receive hydrocortisone in injection form at 8 am.
7) Immediately before receiving the hydrocortisone, a 50 ml pre-dose blood sample will be withdrawn from a forearm vein. After receiving hydrocortisone, additional 10 ml blood samples

will be drawn from a forearm vein at 8:10 am, 8:20, 8:30, 8:45, 9:00, 9:30, 10:00, 11:00, 12 noon, 1:00, 2:00, and 4:00 pm for a total of 13 blood samples and approximately 170 ml of blood.

8) This pattern of dosing and sampling will be repeated three more times over the approximately 4 week long study period. This will result in a total amount of 680 ml of blood being withdrawn over the 1 month study period and a total number of 52 blood samples.

9) At 12 noon of the sampling days, I will be provided a standardized lunch. I agree to eat all components of this lunch.

10) Apart from the risks already indicated for the particular drugs, I may also experience risks and/or discomfort from multiple punctures for blood specimen collection. This includes the possibility of soreness, bruising, bleeding, vein wall irritation, and infection. These risks will be minimized by using fully qualified personnel to obtain blood samples under strict aseptic procedures.

11) I will report any suspected side effect or untoward feeling I may experience during the study to Dr. Welling or Dr. Craig.

12) I am free to withdraw from the study at any time. Additionally, my participation in the study may be terminated at any time upon the discretion of Dr. Welling or Dr. Craig.

13) There will be no immediate benefit to me from taking the drugs in this study. However, depending on the outcome of the results, there may be long-term benefits for me and for others as a result of establishing how well this drug is absorbed from various formulations.

Phase II
Hydrocortisone Study
Consent Form

I have read and understand the information provided in this consent form.

Anything I did not at first understand has been explained to me.

I hereby give my informed consent for Dr. William A. Craig, or a qualified assistant, to administer the drugs included in this study to me at such times as are indicated in the study protocol. I also consent to Dr. Craig, or a qualified assistant, to obtain venous blood samples from me at the times and using the procedures described to me.

I understand that I am free to withdraw from participation in the study at any time.

In the event that physical injury occurs as a result of this research which we believe is unlikely, the University does not automatically provide reimbursement for medical care or other compensation. If physical injury is suffered in the course of this research, or for more information, please notify the investigator in charge.

Subject_____ Date _____

Witness_____ Date _____

Physician_____ Date _____

2D

Consent Form: Study 4 - Oral Hydrocortisone Suspension

Participant Consent Form

For a Hydrocortisone Bioavailability Study

PURPOSE:

The purpose of the study is to determine how much and how fast hydrocortisone is absorbed into the blood from four different dose levels.

Participation will require that I take 5 to 40 mg of hydrocortisone as a solution in orange juice on four separate occasions. There will be a two week interim period between each hydrocortisone dosing. At 11 PM the evening before receiving the doses, I will take 2 mg of dexamethasone as an elixir. Dexamethasone is known to lower the normal levels of hydrocortisone in the blood. It is necessary that I take the dexamethasone so that the normal levels of hydrocortisone in my blood may be sufficiently reduced. Without this reduction of normal levels of hydrocortisone, it would be difficult to interpret the blood level data. I will submit to venipuncture following each administration of hydrocortisone. The following has been clearly explained to me by Dr. Craig.

BENEFITS:

I will receive no medical or academic benefit from my participation in this study.

PRECAUTIONS:

I have been told that I cannot enter this investigation if I have a history of kidney, liver, gastrointestinal, cardiovascular or blood disease. I agree to inform Dr. Craig of any medical problems I have or have had. I have been informed that I must not take any medication or drugs including vitamins or aspirin for a period of 14 days before the investigation starts, during, and for 24 hours following my participation in this investigation. I also agree not to take part in any strenuous physical activities such as tennis, basketball or football on the two days each week that I receive the dexamethasone and hydrocortisone.

RISKS:

To help prevent the possibility of anemia, I have not donated blood for eight weeks prior to the start of the study and I agree not to donate blood for eight weeks following the investigation.

In rare instances I may experience some reactions to hydrocortisone and dexamethasone. Some of these reactions are sodium and fluid retention, potassium loss, increased blood pressure, muscle weakness, increased fragility of bones, peptic ulcers, skin rashes, slow wound healing,

increased sweating, convulsions, vertigo, headache, decreased tolerance for carbohydrates, increased susceptibility to infection, cataracts, euphoria, glaucoma, insomnia, mood and personality changes. Dr. Craig will explain to me about these possibilities before I agree to enter this study. The blood samples will be taken by venipuncture which may cause bleeding, pain or in rare cases, infection.

PROCEDURE:

Before I enter the study, I must pass a medical history and standard physical examination. This will include laboratory analyses of blood and urine specimens as well as a tuberculin skin test.

On the day I receive dexamethasone I will not eat anything after 8 PM and I will not drink anything after 10 PM. I will receive 2 mg of dexamethasone with 180 ml of water at 11 PM. The following day I will receive the hydrocortisone dose and I will continue to fast until 12 noon.

Immediately before receiving the first hydrocortisone dose, a 50 ml blood sample (about 2 ounces) will be withdrawn from a forearm vein. On subsequent weeks, an 8 ml blood sample (about 1/4 ounce) will be drawn prior to drug administration. After receiving hydrocortisone at 8:00 AM, additional 8 ml blood samples (about 1/4 ounce) will be drawn from a forearm vein at 8:15, 8:30, 9:00, 9:30, 10, 11, 12 noon, 1, 2, 3, 4 and 8 PM. This pattern of dosing and sampling will be repeated three more times over the approximately eight week study period. This means that each study week a maximum of 146 ml of blood (about 5 ounces) will be taken. Over the eight week period, 458 ml of blood (about 1 pint) will be withdrawn and a total of 52 blood samples taken.

At 12 noon of the blood sampling days, I will be provided a standardized lunch. I agree to eat all components of this lunch.

For my protection I agree to report any side effects I have to Dr. Craig. If I have a side effect, Dr. Craig may, for my protection, stop my participation in the investigation. If I should require emergency medical treatment and I cannot contact Dr. Craig, I will try to contact my personal physician. Failing to contact either physician I will report to the Emergency Room of the University Hospital or nearest emergency room. The emergency medical treatment will be provided without cost to me. No funds are available to pay for any other expenses I might have because of side effects such as loss of wages or permanent injury.

I will receive a copy of the consent form which I may keep. I will also be given a card which gives the names and telephone numbers of the investigators and lists the sampling times.

I understand that I can ask questions at any time and can also withdraw from this investigation at any time for any reason. If I decide to withdraw from this investigation at any time, I understand that such a decision will not affect my receiving any needed emergency care to treat side effects that resulted from my participation.

I will receive $4 for every blood sample taken from me. Payment will be made at the end of the study.

I understand that my medical records will be retained in the files of the principal investigator and may be made available to the FDA according to Federal regulations.

I have read and understand the information given above and agree to enter this investigation.

_____ _____

Participant Date

_____ _____

Witness Date

_____ _____

Investigator Date

APPENDIX 3

Subject Eligibility, Record, and Data Forms Used in Studies 1 through 4

SUBJECT INTERVIEW
ELIGIBILITY QUESTIONAIRE

Study Applied For: _____

Subject Name: _____

SS Number: _____

Home Address: _____

_____

Phone Number: Work/Day _____

Home/Night _____

Age ____ Sex ___ Weight _____ Height _____

	YES	NO
1) Subject is not receiving chronic medication. if so, what medication(s): _____ _____	☐	☐
2) Subject is in good health without evidence of cardiovascular, renal, gastrointestinal, metabolic, hepatic, or other diseases.	☐	☐
3) Subject has received no drug for 1 week/2 weeks (circle) prior to the start of the study.	☐	☐
4) Subject does not suffer from any allergies. If so, what allergies: _____ _____	☐	☐
5) Subject is willing to submit to a complete physical examination.	☐	☐
6) Subject is willing to sign an Informed Consent Form.	☐	☐

SUBJECT INTERVIEW
ELIGIBILITY QUESTIONAIRE

	YES	NO
7) Subject is willing to consume all meals provided or to fast as directed by the study supervisor.	☐	☐
8) Subject is willing to follow all directions of the study supervisor as outlined in the instruction forms to be provided. (Can be omitted until instruction form has been read).	☐	☐
9) Subject understands he/she may discontinue particitation in the study at any time and will be paid for the amount of the study completed at the end of the study.	☐	☐

_____ _____

Signature - Subject Date

_____ _____

Signature - Investigator Date

SUBJECT RECORD

Study applied for: _____

Subject Name: _____

S S Number: _____

Home Address: _____

_____

Phone Numbers: Work/Day _____

Home/Night _____

Age ____ Sex ____ Weight _____p, _____kg Height _____in., _____cm

	YES	NO
1) Subject has read and signed the Informed Consent Form.	☐	☐
2) Subject has read and understands Experimental Directions.	☐	☐
3) All Laboratory Examination values for the subject are within normal ranges and/or are acceptable.	☐	☐
4) Subject has passed the physical examination.	☐	☐
5) Subject has completed all other criteria as listed below:	☐	☐
a) _____		
b) _____		
c) _____		
6) Subject fulfills all criteria and qualifies for participation in the specified study.	☐	☐

_____ _____

Investigator Date

_____ _____

Physician Date

SUBJECT DATA SHEET

Name:_____ Number:_____ Study:_____

Age:____ Weight:_____ Height:_____ Sex:___ BSA:_____

Health:_____

Blood Pressure:_____ Temperature:_____ Pulse:_____

Serum Creatinine:_____ Creatinine Clearance:_____

Concomitant Drug Therapy:_____

_____

Study Drug Schedule

Drug	Dose	Route	Duration or Tablets	Date	Time	Interval
1)						
2)						
3)						
4)						
5)						

Adverse Reactions:_____

SAMPLING

TIME			CONCENTRATION		
Theoretical	Actual	E.T.		Urine/Vol/pH	Saliva/pH

SUBJECT DATA SHEET

SAMPLING

TIME			CONCENTRATION		
Theoretical	Actual	E.T.		Urine/Vol/pH	Saliva/pH

APPENDIX 4

Summaries of Directions Given to Human Subjects Prior to Participation in Studies

Summary of Directions for Volunteers in Phase 1 Hydrocortisone Study

The directions, outlined in more detail in the consent form you have received, are summarized here for your convenience.

1) Drugs, other than those given in the study, should not be taken from 2 weeks prior to the study until the end of the study without informing Dr. Welling.

2) Strenuous activities (jogging, etc.) should not be performed the day before sampling or during the sampling days.

3) Sampling Day 1- Eat normally until 8 p.m., drink normally until 10 p.m. No eating after 8 p.m., no drinking after 10 p.m.

   Blood samples must be drawn as close to 8 a.m., 10a.m., 12 noon, 2p.m., 4p.m., 6p.m., and 8p.m. as possible. Please come to 5316 Chamberlin five minutes before these times.

   Dexamethasone elixer will be given in 5316 Chamberlin at 11 p.m.:

   Group A: 10 ml. elixer with 180 ml. water
   Group B: 20 ml. elixer with 180 ml. water
   Group C: 10 ml. elixer with 180 ml. water

   Please come to 5316 Chamberlin 5 minutes before 11 p.m.

4) Sampling Day 2- No eating or drinking before 12 noon. You may eat and drink normally after 12 noon.

   Group C (only) will receive 10 ml. Dexamethasone elixer with 180 ml. water at 6 a.m. in 5316 Chamberlin. Please come 5 minutes early.

   Blood samples will be drawn at 8a.m., 10a.m., 12 noon, 2p.m., 4p.m., 6p.m., and 8p.m. Please come to 5316 Chamberlin 5 minutes before these times.

5) You will receive $3 per blood sample at the end of the study.

Thank you very much for your cooperation.

SUMMARY OF DIRECTIONS
FOR VOLUNTEERS IN
PHASE II HYDROCORTISONE STUDY

The directions, outlined in more detail in the consent form you have received, are summarized here for your convenience.

1) Drugs, other than those given in the study, should not be taken from 2 weeks prior to the study until the end of the study without informing Dr. Welling.

2) Strenuous activities (jogging, etc.) should not be performed the day before sampling or during sampling days.

3) DAY 1 - Eat normally until 8 pm, drink normally until 10 pm. No eating after 8 pm, no drinking after 10 pm.

You will receive 10 ml. of dexamethasone elixer with 180 ml. water at 11 pm. Please come to 5316 Chamberlin at least 5 minutes before 11 pm.

4) DAY 2 - No eating or drinking before 12 noon. You may eat and drink normally after 12 noon.

You will receive 10 ml. dexamethasone elixer with 180 ml. water at 6 am. Please come to 5316 Chamberlin at least 5 minutes before 6 am.

Blood samples will be taken at the University Hospital. Please be in the lobby of the Veterans Administration (VA) Hospital by 7:15 am. A 50 ml. blood sample will be taken before 8 am. You will receive an hydrocortisone tablet with 180 ml. water at 8 am. and blood samples will then be taken at 8:15, 8:30, 9:00, 9:30, 10, 11, 12, 1, 2, 3, 4, and 8 pm. You will be required to remain at the University Hospital during the sampling period for observation.

5) This study will be done three times:

Day 1	Day 2
Monday, September 24	Tuesday, September 25
Monday, October 8	Tuesday, October 9
Monday, October 22	Tuesday, October 23

6) You will receive $3 per blood sample at the end of the study. Thank you for your cooperation!

HYDROCORTISONE IV STUDY

Dexamethasone(2mg oral):11pm,5316 Pharmacy, 6/15,6/22,6/29,and7/6.

Hydrocortisone(10-40mg IV):8am,J5 218 CSC, 6/16,6/23,6/30,and7/7.

Blood samples:J5 218 CSC, 6/16,6/23,6/30,and7/7 at 7:30,8:10,8:20,8:30,8:45,9,9:30,10,11,12,1,2,and 4.

No food after 8pm,no liquid after 10pm the night of Dex. until noon the next day.

Meet at K entrance(Nielson tennis court side)of CSC.

Roger D. Toothaker, M.S. 262-5819/255-2374

Peter G. Welling Ph.D. 263-3986/273-3615

HYDROCORTISONE SOLUTION STUDY

Dexamethasone(2mg oral): 11pm, 5316 Pharmacy, 9/4, 9/18, 10/2 and 10/16.

Hydrocortisone(5-40mg oral): 8am J5 218 CSC, 9/5, 9/19, 10/3 ,and 10/17.

Blood samples: J5 218 CSC, 9/5, 9/19, 10/3,and 10/17 at 7:30,8:15,8:30,9,9:30,10,11,12,1, 2,3,4,and 8.

No food after 8pm, no liquid after 10pm the night of Dex. until noon the next day.

Meet at K entrance (Nielson tennis court side) of CSC.

Roger D. Toothaker,MS. 2625819/2552374

Peter G. Welling,Ph.D. 2633986/2733615

APPENDIX 5

Programs and Instructions for Calculations Performed on Casio FX502P Programmable Calculator

One Compartment Oral

PKics #111
Location: Tape 1, 180-6

Curve Stripping, Initial Estimates,
Final Parameter Values.

Output: k_a, K_{el}, $t^a_{\frac{1}{2}}$, $t^{el}_{\frac{1}{2}}$, FD/V, C_{max}, t_{max}, AUC

STEP	OPERATION	DISPLAY	
1	Mode [1]	0	
2	PO	3	Indicates number of next step
3	C_0 exe	4	
4	C^{el}_x exe	5	
5	t^{el}_x exe	6	
6	(C^a_0 exe, t^a_0 exe)	C^a_0, r_0	
	⋮	⋮	
	(C^a_n exe, t^a_n exe)	C^a_n, r_n	
7	Go to [2]	8	Indicates number of next step
8	r_0 exe	9	
9	r_y exe	10	
10	t_y exe	11	
11	Go to [3] exe	1...k_a	Marker number briefly precedes value.
12	exe	2...k_{el}	
13	exe	3...$t^a_{\frac{1}{2}}$	
14	exe	4...$t^{el}_{\frac{1}{2}}$	
15	exe	5...FD/V	
16	exe	6...C_{max}	
17	exe	7...t_{max}	
18	exe	8...AUC	
19	Go to [1]	3] start next data step at step 3.	

(To repeat answers only, start at step 11
Hit Go to [3] only, do not hit exe

MEMORY CONTENTS

1	C_0	.1	t_y
2	log C_0	.2	k_a
3	log C^{el}_x	.3	$t^a_{\frac{1}{2}}$
4	t_x	.4	k_a-k_{el}
5	k_{el}	.5	FD/V
6	$t^{el}_{\frac{1}{2}}$	.6	2.303
7	C^a_y	.7	.693
8	r_0	.8	C_{max}
9	log r_0	.9	t_{max}
0	log r_y	.0	AUC
F	P1:I	.F	P3:I

Instructions

1. Plot C vs t data.
2. Draw post-absorption line.
3. Get C_o (intercept) and some C_x^{el} and t_x (on post abs. line)
4. Do step 1→5.
5. At step 6, enter C^a, t^a pairs until $C^a \geq C^{el}$ (or until $r_i \leq 0$)
   Record r_i's and plot r vs t.
6. Get r_0 (intercept, should be = to C_0) and some r_y and t_y.
7. Do steps 7→10.
8. Do steps 11→18 recording the values as they appear.
9. To repeat answers only, do step 11 (hit [Go to] [3] only, do not hit [exe]).
   For next data set, do step 19 (hit [Go to] [1]) and start at step 3.

EQUATIONS:

$$m = \frac{C_1 - C_2}{t_1 - t_2} \qquad k = -2.303\,m \qquad t_{\frac{1}{2}} = \frac{0.693}{k}$$

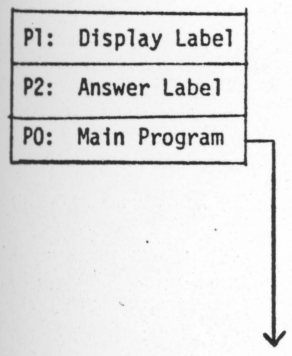
$$C_t = \frac{FD}{V}\left(\frac{k_a}{k_a - k_{el}}\right)\left(e^{-k_{el}t} - e^{-k_a t}\right)$$

$$C_o = \frac{FD}{V}\left(\frac{k_a}{k_a - k_{el}}\right) \qquad \frac{FD}{V} = \frac{C_o(k_a - k_{el})}{k_a}$$

$$t_{max} = \frac{2.303}{(k_a - k_{el})} \log\left(\frac{k_a}{k_{el}}\right) \qquad AUC = \frac{FD}{V k_{el}}$$

PKics #111

		STEPS
P1: Display Label	1, M+F, MRF, halt,	4 + 1
P2: Answer Label	1, M+.F, MR.F, Inv Pause,	4 + 1
P0: Main Program		151 + 1
	Total steps: 162	



Lbl 1, Inv MAC, ., 6, 9, 3, Min.7, 2, ., 3, 0, 3, Min.6, 2, Min F, GSBP1, Min 1,
log, Min 2, GSBP1, log, Min 3, GSBP1, Min 4, MR3, -, MR2, =, ÷,
MR4, X, MR.6, ±, ÷, Min 5, MR.7, Inv x→y, =, Min 6, GSBP1,
Lbl 4, Min 7, halt, x, MR5, ±, =, Inv e^x, x, MR1, =, -, MR7, =, halt, Go to 4,
Lbl 2, 1, M+F, GSBP1, Min 8, log, Min 9, GSBP1, log, Min 0, GSBP1, Min.1,
MR0, -, MR9, =, ÷, MR.1, x, MR.6, ±, ÷, Min.2, MR.7, Inv x→y, =,
Min.3, GSBP1,
Lbl 3, GSBP2, MR.2, halt, GSBP2, MR5, halt, GSBP2, MR.3, halt, GSBP2, MR6, halt,
MR.2, -, MR5, =, Min.4, x, MR1, ÷, MR.2, =, Min.5, GSBP2, MR.5, halt, MR5, ÷, MR.2,
=, Inv $x^{y}_{(100)}$, (, MR5, ÷, MR.4,), =, x, MR.5, =, Min.8, GSBP2, MR.8, halt, ÷, MR5, =,
log, x, MR.6, ÷, MR.4, =, Min.9, GSBP2, MR.9, halt, MR.5, ÷, MR5, =, Min.0, GSBP2,
MR.0, halt

PKics #201

Location: Tape 1,20-6

2 Compartment IV

Curve Stripping and Initial Estimates

Output: $\alpha, \beta, t_{\frac{1}{2}}^{\alpha}, t_{\frac{1}{2}}^{\beta}, K_{12}, K_{21}, K_2, A, B, D/V_1$

STEP	OPERATION	DISPLAY	
1	Mode [1]	0	
2	[PO]	3	Indicates number of next step.
3	B [exe]	4	
4	C_x^{β} [exe]	5	
5	t_x [exe]	6	
6	(C_0^{α} [exe]	C_0^{α}	
	t_0 [exe])	r_0	
:	:	:	
	(C_n^{α} [exe]	C_n^{α}	
	t_n [exe])	r_n	
7	Go to [4]	8	Indicates number of next step.
8	r_0 [exe]	9	
9	r_y [exe]	10	
10	t_y [exe]	11	
11	Go to [5]	1...α	Marker number briefly precedes value.
12	[exe]	2...β	
13	[exe]	3...$t_{\frac{1}{2}}^{\alpha}$	
14	[exe]	4...$t_{\frac{1}{2}}^{\beta}$	
15	[exe]	5...K_{12}	
16	[exe]	6...K_{21}	
17	[exe]	7...K_2	
18	[exe]	8...A	
19	[exe]	9...B	
20	[exe]	10...D/V	
21	Go to 1	3] start next data step at step 3	

MEMORY CONTENTS

1	B	.1	t_y
2	log B	.2	α
3	log C_x^{β}	.3	$t_{\frac{1}{2}}^{\alpha}$
4	t_x	.4	K_{21}
5	β	.5	K_2
6	$t_{\frac{1}{2}}^{\beta}$	.6	K_{12}
7	C_y^{α}	.7	D/V_1
8	r_0	.8	
9	log r_0	.9	
0	log r_y	.0	
F		.F	

(to repeat answers only, start at step 11)

PKics #201

INSTRUCTIONS

1. Plot C vs t data
2. Draw β line
3. Get B and some C_x^{β} and t_x
4. Do steps 1 thru 5
5. At step 6, enter C^{α}/t pairs until $C^{\alpha} \leq C^{\beta}$
   Record r_n's and plot r vs t ($\propto$ line)
6. Get r_0 and some r_y and t_y
7. Do steps 7 thru 10
8. Do steps 11 thru 21 recording the values as they appear
9. Repeat for next data set

PKics #201

P0

192 steps

Lbl 1, AC, Inv MAC,

Lbl 2, 3, =, halt, Min 1, log, Min 2, 4, =, halt, log, Min 3, 5, =, halt, Min 4, MR3, -, MR2, =, ÷, MR4, x, 2, ., 3, 0, 3, ±, ÷, Min 5, ., 6, 9, 3, Inv x→y, =, Min 6, 6, =, halt,

Lbl 3, Min 7, halt, x, MR5, ±, =, Inv e^x, x, MR1, =, ±, +, MR7, =, halt, Go to 3,

Lbl 4, 8, =, halt, Min 8, log, Min 9, 9, =, halt, log, Min 0, 1, 0, =, halt, Min.1, MR0, -, MR9, =, ÷, MR.1, x, 2, ., 3, 0, 3, ±, ÷, Min.2, ., 6, 9, 3, Inv x→y, =, Min.3, 1, 1, =, halt,

Lbl 5, MR8, x, MR5, +, MR1, x, MR.2, =, ÷, ⌐, MR8, +, MR1, =, Min.4, MR.2, x, MR5, ÷, MR.4, =, Min.5, ±, +, MR.2, +, MR5, -, MR.4, =, Min.6, MR8, +, MR1, =, Min.7, 1, =, Inv Pause, MR.2, halt, 2, =, Inv Pause, MR5, halt, 3, =, Inv Pause, MR.3, halt, 4, =, Inv Pause, MR6, halt, 5, =, Inv Pause, MR.6, halt, 6, =, Inv Pause, MR.4, halt, 7, =, Inv Pause, MR.5, halt, 8, =, Inv Pause, MR8, halt, 9, =, Inv Pause, MR1, halt, 1, 0, =, Inv Pause, MR.7, halt

PKics #202
Location: Tape 40-6

2 Compartment IV Bolus

Parameter calculation from computer printout

Output: $t_{\frac{1}{2}}^{\alpha}$, $t_{\frac{1}{2}}^{\beta}$, $t_{\frac{1}{2}}^{el}$, A, B, V_1, V_1/kg, Vd_{ss}, Vd_{ss}/kg, V_1K_2, $V_1K_2/1.73m^2$, AUC, $AUC(K_2)$

STEP	OPERATION	DISPLAY	
1	Mode 1	0	
2	PO	3	Indicates number of next step
3	K_{12} exe	4	
4	K_{21} exe	5	
5	K_2 exe	6	
6	D/V_1 exe	7	
7	α exe	8	
8	β exe	9	
9	D exe	10	
10	wt exe	11	
11	BSA exe	12	
12	exe	1...$t_{\frac{1}{2}}^{\alpha}$	Marker number briefly preceeds value
13	exe	2...$t_{\frac{1}{2}}^{\beta}$	
14	exe	3...$t_{\frac{1}{2}}^{el}$	
15	exe	4...A	
16	exe	5...B	
17	exe	6...V_1	
18	exe	7...V_1/kg	
19	exe	8...Vd_{ss}	
20	exe	9...Vd_{ss}/kg	
21	exe	10...V_1K_2	
22	exe	11...V_1K_2 (BSA normalized.)	
23	exe	12...AUC	
24	exe	13...$AUC(K_2)$	
25	Go to 1	3	start next data step at step 3

MEMORY CONTENTS

1	$(K_{12})(V_1K_2)_{norm}$	.1	Vd_{ss}
2	$(K_{21})(V_1K_2)$	.2	AUC
3	K_2	.3	.693
4	$(D/V_1)(AUCK_2)$	.4	$t_{\frac{1}{2}}^{\alpha}$
5	α	.5	$t_{\frac{1}{2}}^{\beta}$
6	β	.6	$t_{\frac{1}{2}}^{el}$
7	BSA	.7	A
8	D	.8	B
9	wt	.9	V_1/kg
0	V_1	.0	Vd_{ss}
F	AUC	.F	Vd_{ss}/kg

PKics #202

INSTRUCTIONS

1. Tabulate K_{12}, K_{21}, K_2, D/V_1, α, and β from 1110 printout.
2. List Dose (mg), weight (kg), and BSA (m^2) for each subject.
3. Do steps 1→11.
4. Do steps 12→25 recording the values as they appear.
5. a) To repeat answers only, hit [Go to] [2], readout will be result of step 12.
   b) To repeat calculations on same data set, hit [Go to] [3], readout will be result of step 11.
   c) For new data set (subject), (do step 25 - hit [Go to] [11] and start at step 3.

PKics #202

Total of 221 steps

Lbl 1, AC, Inv MAC, 3, =, halt, Min 1, 4, =, halt, Min 2, 5, = halt, Min 3,
6, =, halt, Min 4, 7, =, halt, Min 5, 8, =, halt, Min 6, 9, =, halt, Min 8,
1, 0, =, halt, Min 9, 1, 1, =, halt, Min 7, 1, 2, =, halt,

Lbl 3, ., 6, 9, 3, Min.3, ÷, MR5, =, Min.4, MR.3, ÷, MR6, =, Min.5, MR.3,
÷, MR3, =, Min.6, MR5, -, MR2, =, ÷, [, MR5, -, MR6,], x, MR4, =,
Min.7, MR2, -, MR6, =, ÷, [, MR5, -, MR6,], x, MR4, =, Min.8, MR8, ÷,
MR4, =, Min 0, ÷, MR9, =, Min.9, MR1, ÷, MR2, +, 1, =, x, MR0, =, Min.0, ÷,
MR9, =, Min.F, MR.7, ÷, MR5, +, [, MR.8, ÷, MR6, =, Min F, MR0, x,
MR3, x, 1, 0, 0, ÷, 6, =, Min 1, ÷, MR7, x, 1, ., 7, 3, =, Min 2, MRF, x, MR3, =, Min 4,

Lbl 2, 1, =, Inv Pause, MR.4, halt, 2, =, Inv Pause, MR.5, halt, 3, =, Inv Pause,
MR.6, halt, 4, =, Inv Pause, MR.7, halt, 5, =, Inv Pause, MR.8, halt,
6, =, Inv Pause, MR0, halt, 7, =, Inv Pause, MR.9, halt, 8, =, Inv Pause,
MR.0, halt, 9, =, Inv Pause, MR.F, halt, 1, 0, =, Inv Pause, MR1, halt, 1, 1, =,
Inv Pause, MR2, halt, 1, 2, =, Inv Pause, MRF, halt, 1, 3, =, Inv Pause, MR4, halt

PKics #204
Location: Tape 1, 80-6

2 Compartment IV Model Equation

Input: A, α, B, β, t

Output: C_t

$$C_t = Ae^{-\alpha t} + Be^{-\beta t}$$

STEP	OPERATION	DISPLAY	
1	Mode 1	0	
2	P4	3	Indicates number of next step
3	A	4	
4	α	5	
5	B	6	
6	β	7	
7	t_1	C_1	
8	t_2	C_2	
9	t_3	C_3	
⋮	⋮	⋮	
n+6	t_n	C_n	
n+7	Go to 1	3	start next data set at step 3

1. List A, α, B, and β for each subject
2. Pick desired times, t_i
3. Do steps 1→ n+6 recording C_i as it appears
4. For new data set, do step n+7 (i.e., hit Go to 1 and start at step 3)

P4 49 + 1 = 50 total steps

Lbl 1, Inv MAC, 2, =, halt, Min (A) 1, 3, =, halt, Min (α) 2, 4, =, halt, Min (B) 3, 5, =, halt, Min (β) 4, 6, =, halt, (t_i)

Lbl 2, Min 5, MR1, x, [, [, MR2, x, MR5, +/-,], Inv e^x,], +, MR3, x, [, [, MR4, x, MR5, +/-,], Inv e^x,], =, halt, Go to 2

MEMORY CONTENTS

1	A	.1
2	α	.2
3	B	.3
4	β	.4
5	t_i	.5
6		.6
7		.7
8		.8
9		.9
0		.0
F		.F

Stats #445
Location: Tape 1,

Linear Regression

Input: $(x,y)_1$

Optional Input: ΣX, ΣX^2, ΣY, ΣY^2, ΣXY, n

Output: m, b, S_m, S_b, r, r^2

STEP	OPERATION	DISPLAY
1	Mode 1	0
2	Inv P9	0
3	X_1 exe	X_1
4	Y_1 exe	1
⋮	⋮	⋮
n+2	X_n exe	X_n
n+3	Y_n exe	n
n+4 (10)	Go to 4	m
n+5 (11)	exe	b
n+6 (12)	exe	S_m
n+7 (13)	exe	S_b
n+8 (14)	exe	r
n+9 (15)	exe	r^2

Start next data set at Step 2 or optional step 2.

OPTIONAL:

STEP	OPERATION	DISPLAY
1	Mode 1	0
2	Inv P9	0
3	Go to 3	0
4	ΣX exe	X
5	ΣX^2 exe	X^2
6	ΣY exe	Y
7	ΣY^2 exe	Y^2
8	ΣXY exe	XY
9	n exe	n

MEMORY CONTENTS

1	ΣX	.1	S_{Y2}
2	X_1	.2	
3	ΣX^2	.3	
4	ΣY	.4	
5	Y_1	.5	
6	ΣY^2	.6	
7	ΣXY	.7	
8	S_{XY}	.8	
9	S_{XX}	.9	
0	S_{YY}	.0	
F	n	.F	

Stats #445

INSTRUCTIONS

1. Enter data through steps 1 to n+3 (or through 1 to 9 if these values are already available).
2. Do steps n+4 to n+9 (10 to 15) recording answers as they appear.
3. Start next data set at step 2 or optional step 2.
4. To repeat answers, hit [Go to] [4] .

EQUATIONS

$$m = \frac{S_{xy}}{S_{xx}} \qquad b = \frac{\Sigma y}{n} - \frac{m \Sigma x}{n} \qquad S_m = \sqrt{\frac{s_y^2}{S_{xx}}}$$

$$S_b = \sqrt{s_y^2 \left[\frac{S_{xx} n + (\Sigma x)^2}{S_{xx} n^2}\right]}$$

$$r = \frac{n \Sigma xy - \Sigma x \Sigma y}{\sqrt{[n \Sigma x^2 - (\Sigma x)^2][n \Sigma y^2 - (\Sigma y)^2]}}$$

$$S_{xx} = \Sigma x^2 - \frac{(\Sigma x)^2}{n} \qquad S_{yy} = \Sigma y^2 - \frac{(\Sigma y)^2}{n}$$

$$S_{xy} = \Sigma xy - \frac{\Sigma x \Sigma y}{n} \qquad s_y^2 = \frac{S_{yy} - \frac{(S_{xy})^2}{S_{xx}}}{(n-2)}$$

Stats #445

Inv P9 Main Program

1 + 162 = 163 total steps

Lbl 1, Inv MAC,

Lbl 2, halt, (ΣX) m+1, (X) Min 2, Inv X^2, (ΣX^2) m+3, MR2, halt, (ΣY) m+4, (Y) Min 5, Inv X^2, (ΣY^2) m+6,

MR5, X, MR2, =, (ΣXY) m+7, (n) 1, m+F, MRF, Go to 2,

Lbl 3, halt, (ΣX) Min 1, halt, (ΣX^2) Min 3, halt, (ΣY) Min 4, halt, (ΣY^2) Min 6, halt, (ΣXY) Min 7, halt, (n) Min F, halt,

Lbl 4, MR7, -, [, MR1, X, MR4, ÷, MRF,], =, (S_{XY}) Min 8, MR3, -, [, MR1, Inv X^2, ÷, MRF,

], =, (S_{XX}) Min 9, MR6, -, [, MR4, Inv X^2, ÷, MRF,], =, (S_{YY}) Min 0, -, [, MR8, Inv X^2, ÷,

MR9,], =, ÷, [, MRF, -, 2,] =, (S_Y^2) Min .1, MR8, ÷, MR9, =, m halt, MR4, ÷, MRF,

-, [, MR8, ÷, MR9, X, MR1, (100) ÷, MRF,], =, b halt, MR.1, ÷, MR9, =, Inv $\sqrt{X}$,

S_m halt, MR9, X, MRF, +, MR1, Inv X^2, =, ÷, MR9, ÷, MRF, Inv X^2, X, MR.1,

=, Inv $\sqrt{X}$, S_b halt, [, MR7, X, MRF, -, MR1, X, MR4,], ÷, [, [, MR3, X,

MRF, -, MR1, Inv X^2,], X, [, MR6, X, MRF, -, MR4, Inv X^2,],], Inv $\sqrt{X}$,

=, r halt, Inv X^2, r^2 Go to 1,

PKics #541
Location: Tape 1, 120-6

Trapezoidal Area Under the Curve ($AUC^{0\to\infty}$)

Input: n (= # data points), β, C_o, $t_0, \ldots C_{n-1}$, t_{n-1}

Output: $AUC^{0\to\infty}$

$$AUC^{0\to\infty} = \sum_{i=0}^{n-1} \frac{(t_{i+1}-t_i)(C_{i+1}+C_i)}{2} + \frac{C_{i+1}}{\beta \text{ (or } k_{el})}$$

STEP	OPERATION	DISPLAY
1	Mode 1	0
2	PO	-3
3	n exe	-4
4	k_{el} or β exe	-5
5	C_0 exe	-6
6	t_0 exe	-7
7	C_1 exe	-8.
8	t_1 exe	-9
⋮	⋮	⋮
2n+3	C_{i+1} exe	-(2n+4)
2n+4	t_{i+1} exe	-(2n+5)
2n+5	exe	AUC (answer)
2n+6	Go to 1	3

Indicates number of next step

Start next data set at step 3.

MEMORY CONTENTS

1	β or k_{el}	.1	
2	$(C_0)(C_1)\ldots(C_{n-2})$	.2	
3	$(t_0)(t_1)\ldots(t_{n-2})$	.3	
4	$(C_1)(C_2)\ldots(C_{n-1})$	.4	
5	$(t_1)(t_2)\ldots(t_{n-1})$	.5	
6		.6	
7		.7	
8		.8	
9		.9	
0		.0	P2:I
F	2n+5	.F	Σ

PKics #541

INSTRUCTIONS:

1. Find β or k_{el} (h^{-1}) for each subject
2. List $C_0 \rightarrow C_{n-1}$ and $t_0 \rightarrow t_{n-1}$ for each subject and count number of data points, n.
3. Do steps 1 → 2n+5 and record answer
4. If a mean and standard deviation for a set of AUC's are desired, hit X_0 before hitting [Go to] [1] (while AUC is still displayed). $\overline{AUC}$ will then be at [Inv] [$\bar{x}$] and standard deviation will be at [Inv] [σ_{n-1}]. Hit [Inv] [SBC] before starting next group of AUC's.
5. For new data set (subject), do step 2n+6 (hit [Go to] [1]) and start at step 3.

PKics #541

[Po: Main Program] 38 steps + 1 **Total steps = 65**

Lbl 1, 0, Min.F, 3, ±, Min.0, =, halt, x, 2, +, 5, =, ±, Min F, GSBP2, Min 1, GSBP2, (2n+5) (β)

Min 2, GSBP2, Min 3, GSBP3, (C_0) (t_0)

Lbl 2, Min 4, GSBP2, Min 5, GSBP1, GSBP3, GSBP2, Inv x = F, Go to 3, Go to 2, (C) (t)

Lbl 3, MR2, ÷, MR1, =, M+.F, MR.F, halt,

[P1: Sum Calculation] 15 steps + 1

MR5, -, MR3,], Inv Abs, X, [, MR2, +, MR4,], ÷, 2, =, M+.F, (Σ)

[P2: Display Label] 4 steps + 1

1, M-.0, MR.0, halt,

[P3: Memory Transfer] 4 steps + 1

MR4, Min 2, MR5, Min 3

APPENDIX 6

Published Manuscript for Study 1

Suppression of Endogenous Hydrocortisone with Dexamethasone

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Abstract □ A newly developed high-pressure liquid chromatographic method was used to study the optimum dosage regimen needed to suppress endogenous hydrocortisone. Nine volunteers were randomly placed in three groups. Each group received 1 mg of dexamethasone at 11 pm (Treatment A), 2 mg of dexamethasone at 11 pm (Treatment B), or 1 mg at 11 pm and an additional 1 mg at 6 am the following day (Treatment C). Analysis of multiple blood samples obtained the day before and the day after drug administration showed suppression in all three groups. Although the duration and extent of this suppression varied, adequate suppression to permit bioavailability studies was observed for Treatments B and C.

Keyphrases □ Hydrocortisone—suppression of endogenous hydrocortisone with dexamethasone □ Dexamethasone—suppression of endogenous hydrocortisone

The principal natural glucocorticoid in humans is hydrocortisone (1). Although numerous synthetic glucocorticoids have been prepared, hydrocortisone remains an important agent in modern adrenocorticosteroid therapy, and many generic formulations have appeared on the market. However, the bioequivalence of these products has not been established. To conduct bioequivalence studies, assay methodology must be available with the required sensitivity, specificity, accuracy, and precision. A number of apparently adequate high-pressure liquid chromatographic (HPLC) methods have been reported recently (2–9).

BACKGROUND

Hydrocortisone production follows a circadian rhythm, so suppression of hydrocortisone production is necessary to simplify the interpretation of bioavailability data. Therefore, in addition to an appropriate analytical procedure, a method that will adequately suppress the endogenous hydrocortisone levels is needed. Without this suppression, it is not possible to quantitate the bioavailability of the administered hydrocortisone dosage forms. One way to achieve this suppression is to administer dexamethasone. This procedure was used in studies on prednisone (10, 11) and prednisolone (12, 13). In these cases, dexamethasone administration was necessary since endogenous steroids cross react with the antiserums used in these radioimmunoassays.

Dexamethasone is thought to act by blocking the release of endogenous ACTH (14). However, no extended detailed quantitative study has been performed to determine the amount of dexamethasone required to suppress endogenous steroids. The duration of such suppression also is not clearly established.

In one study (15), hydrocortisone levels were determined at 2 and 4 hr after administration of 1 mg of dexamethasone. During this period, hydrocortisone levels dropped to 14% of their basal values. In another study (16), the degree of suppression of endogenous hydrocortisone was related to the time of day the dexamethasone was administered. In a third study (17), 8 mg of dexamethasone was given for 2 days. The mean plasma hydrocortisone level for the subjects was 6.8 ng/ml; the blood sampling time was not given.

The present paper explores the extent and duration of dexamethasone suppression of circulating endogenous hydrocortisone using a newly developed, highly sensitive and specific HPLC assay.

EXPERIMENTAL

The nine normal male volunteers (22–45 years old) involved in this study had normal vital signs and selected laboratory parameters. The subjects, 63–80 kg, were randomly assigned to three groups of three each (Table I). Group A received 1 mg of dexamethasone, Group B received 2 mg of dexamethasone, and Group C received 2 mg of dexamethasone in two 1-mg divided doses administered 7 hr apart. Dexamethasone was given as an oral elixir[1] (0.5 mg/5 ml) in all cases.

Table I—Subject Data

Identification	Group	Height, cm	Weight, kg	Age, years
1	A	185	73	28
2	A	180	80	22
3	A	170	63	25
4	B	168	74	37
5	B	170	77	31
6	B	178	66	28
7	C	185	75	39
8	C	180	75	22
9	C	178	64	45

Basal values for plasma hydrocortisone levels were determined from heparinized blood samples (8 ml each) obtained at 8 am, 10 am, 12 noon, 2 pm, 4 pm, 6 pm, and 8 pm on Day 1 of the study. Prior to receiving the evening dexamethasone dose, the subjects were required to fast after 8 pm and to maintain that fast until noon the following day. At 11 pm on the same evening, dexamethasone (Groups A and C, 1 mg; and Group B, 2 mg) was administered to all volunteers followed by 180 ml of water. The following morning (Day 2), subjects in Group C received an additional 1 mg of dexamethasone followed by 180 ml of water at 6 am. Heparinized blood samples then were taken from all volunteers beginning at 8 am and continuing at 10 am, 12 noon, 2 pm, 4 pm, 6 pm, and 8 pm. All samples were collected, and the plasma was separated and frozen until it was assayed for hydrocortisone.

All plasma samples were assayed using a previously reported HPLC method (9) with minor modifications. An internal standard (4-pregnene-17α, 20α,21-triol-3,11-dione) was added to the plasma, which then was extracted with methylene dichloride–ether (60:40). After separation and evaporation of the organic phase, derivatization was carried out with dansylhydrazine. Upon completion of the reaction, the excess dansylhydrazine was reacted with pyruvic acid and a second extraction was performed.

The samples were placed in a water bath at room temperature and were evaporated just to dryness under a gentle nitrogen stream. The residue was immediately taken up in 200 μl of the HPLC mobile phase. Five microliters of this solution was injected[2] later onto a well-conditioned microparticulate silica column[3]. Elution[4] was carried out with an ethylene dichloride–butanol–water (93:6.6:0.4) mobile phase (1.5 ml/min). Conditioning of the column was achieved by pumping the mobile phase through the column for 1–2 days. The effluent was monitored with a fluorescence detector[5] [excitation, 240 nm; emission, 470 nm (cutoff filter)]. A linear calibration curve was found for hydrocortisone concentrations from 3 to 1000 ng/ml.

A special glassware cleaning procedure was adopted to eliminate extraneous chromatographic peaks that sometimes were observed. The procedure consisted of rinsing the glassware with distilled water (no soap was used), placing it into a commercial oven[6], which then was carried through a self-cleaning cycle, and rinsing all of the items with distilled water and methanol.

The hydrocortisone levels present in the clinical plasma samples were

[1] Decadron, lot A3240, Merck Sharp and Dohme.
[2] Model U6K sample injection valve, Waters Associates, Milford, Mass.
[3] Prepacked Hibar II column with LiChrosorb SI-60 5-μm silica, Applied Science Laboratories, State College, Pa.
[4] Model M6000A pump, Waters Associates, Milford, Mass.
[5] Model FS970, Schoeffel Instruments, Westwood, N.J.
[6] Kenmore model 42.3 self-cleaning oven, Sears, Roebuck and Co., Chicago, Ill.

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Table II—Basal Plasma Hydrocortisone Levels on Day 1 of Study

Time	Hydrocortisone Concentration, ng/ml								
	Group A			Group B			Group C		
	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5	Subject 6	Subject 7	Subject 8	Subject 9
8 am	116	182	150	114	162	212	208	138	78
10 am	148	218	102	126	120	182	210	164	98
Noon	56	108	108	100	94	130	92	92	86
2 pm	76	164	128	76	144	138	74	122	86
4 pm	94	120	90	62	60	100	78	88	42
6 pm	102	148	148	28	94	96	88	126	58
8 pm	44	102	24	14	46	68	42	104	28

Table III—Plasma Hydrocortisone Levels on Day 2 after Dexamethasone Administration

Time	Hydrocortisone Concentration, ng/ml								
	Group A			Group B			Group C		
	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5	Subject 6	Subject 7	Subject 8	Subject 9
8 am	7	8	12	10	10	7	16	8	16
10 am	12	7	4	7	9	7	9	6	8
Noon	24	8	18	6	9	10	7	8	11
2 pm	10	8	12	10	11	10	11	10	14
4 pm	13	7	9	11	8	8	7	7	12
6 pm	40	82	36	10	7	9	8	9	7
8 pm	14	44	14	8	36	9	8	19	9

determined from standard curves prepared by plotting the peak height ratios of the dansylhydrazones of hydrocortisone and the internal standard *versus* the concentrations of the known standards. To correct the standard curve for endogenous levels of hydrocortisone present in the control plasma, the peak height ratio of the control plasma was subtracted from all data points used in the standard curve.

RESULTS AND DISCUSSION

To establish the regimen for dexamethasone administration that would sufficiently suppress endogenous hydrocortisone production, three dexamethasone regimens were evaluated. The basal plasma levels of hydrocortisone in all nine subjects on Day 1 are recorded in Table II. There was a general trend, but not a smooth progression, of decreasing plasma levels from morning to evening. This trend agrees with the observation that plasma hydrocortisone levels follow a circadian rhythm.

The plasma levels of hydrocortisone on Day 2 are given in Table III. Substantial suppression was seen in all subjects. The plasma levels in subjects on Treatment C did not exceed 16 ng/ml from 8 am to 6 pm. Volunteers receiving Treatment B had plasma levels below 11 ng/ml throughout the same period. The plasma levels achieved with both Treatments B and C were quite stable. Suppression of endogenous hydrocortisone was not as complete in the subjects receiving Treatment A and showed greater variations. All three volunteers in Group A had plasma levels of at least 36 ng/ml by 6 pm on Day 2.

Since stress is known to affect the plasma levels of endogenous hydrocortisone (18), concern existed about the possibility of endogenous hydrocortisone levels fluctuating after suppression had been achieved with dexamethasone administration. In this study, the plasma level fluctuations seen during the control period were virtually absent in the postadministration period. This finding is important since any significant variations in the suppressed plasma hydrocortisone levels would seriously hinder the interpretation of bioavailability data of hydrocortisone dosage forms.

A profile analysis (19) using an F test for the average treatment effects, time effects, treatment with time effects, and subject effects was performed on the basal and suppressed plasma hydrocortisone levels. The data were analyzed by comparing Treatment A with Treatments B and C as well as Treatment B with Treatment C. The statistical analysis of basal plasma levels indicated that there were no significant differences due to what were evaluated as treatment effects ($p = 0.52$ and 0.90) and no significant differences due to treatment with time effects ($p = 0.29$ and 0.56). However, there were significant differences due to time ($p = 0.0001$) and intersubject variability ($p = 0.0001$), which could be expected. For the suppressed plasma hydrocortisone levels, the analysis indicated Treatment A was significantly different from Treatments B and C ($p = 0.007$). Treatments B and C were not significantly different ($p = 0.97$). A test of the treatment with time effects likewise indicated that Treatment A was significantly different from Treatments B and C ($p = 0.0001$) but that Treatments B and C were not ($p = 0.98$).

As shown in the preceding analysis, the extent and duration of the suppression of endogenous plasma hydrocortisone were similar with Treatments B and C. Therefore, selection of either regimen for subsequent bioavailability studies would be reasonable. However, since Treatment C requires an evening and a morning dose, Treatment B is more convenient. In addition, the possibility cannot be ruled out that an early morning dose of dexamethasone may affect the kinetics of orally administered hydrocortisone.

A study is underway to investigate the kinetics of hydrocortisone following a single oral dose; Treatment B administration of dexamethasone is being used.

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APPENDIX 7

Submitted Manuscript for Study 2

"Oral Hydrocortisone Pharmacokinetics: A Comparison of Fluorescence and Ultraviolet HPLC Assays for Hydrocortisone in Plasma:"

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KEY WORDS: Hydrocortisone, Pharmacokinetics, HPLC, Fluorescence, Ultraviolet, Oral

ABSTRACT

In order to evaluate two HPLC assay methods for hydrocortisone, three fasted, male subjects received single 10, 30, and 50 mg oral doses of hydrocortisone tablets on three separate occasions. Endogenous hydrocortisone was suppressed by giving 2 mg dexamethasone 9h prior to dosing. Plasma samples obtained serially for 8h after dosing hydrocortisone were assayed by reverse phase HPLC with UV detection, and by normal phase HPLC with fluorescence detection of the dansylhydrazine derivative of hydrocortisone. The two assay methods gave equivalent plasma hydrocortisone concentrations as determined by paired t-test and linear correlation. Pharmacokinetic parameters obtained using data from either assay method were equivalent. Metabolite interference was apparently absent in both assay methods. Drug concentrations in plasma from all 3 doses were best described by the one-compartment open model with first order absorption and elimination, incorporating an absorption lag time. Values of C_{max}, FD/V, and AUC did not increase in proportion to the dose. Mean C_{max} values of 192, 373, and 396 ng/ml were obtained at 0.61, 0.78, and 1.4h following the 10, 30 and 50 mg doses, respectively. The non-proportional increase in plasma hydrocortisone levels with increasing dose size may be due to reduced gastrointestinal absorption, changes in binding of drug to plasma proteins, a greater first-pass effect, or a combination of these.

INTRODUCTION

Hydrocortisone, the principal endogenous glucocorticoid in humans, (1), is widely used as a therapeutic agent for many disorders. Bioequivalency of the large number of generic hydrocortisone tablets available has not been established. Two factors preventing such bioequivalence studies have been the lack of a sufficiently sensitive and specific assay for hydrocortisone in plasma, and the presence of significant and variable endogenous hydrocortisone blood concentrations, which follow a circadian rhythm (2).

A number of high pressure liquid chromatographic (HPLC) assays for hydrocortisone in biological fluids have recently appeared in the literature. Most of these employ ultraviolet (UV) detection of underivatized hydrocortisone (3-9), while others are based on detection of a fluorescent hydrocortisone derivative (10, 11).

A method to decrease endogenous levels of hydrocortisone to relatively constant, non-interfering values has recently been published (12). Administration of 2 mg dexamethasone at 23:00h suppressed endogenous hydrocortisone levels from a normal range of 40 - 200 ng/ml, to 6 - 14 ng/ml until at least 18:00h the following day. This allows sufficient time for blood concentrations of exogenous hydrocortisone to be measured with minimal interference from endogenous hydrocortisone. The small variation in suppressed endogenous hydrocortisone levels also permits the subtraction of a single baseline concentration value for endogenous hydrocortisone from all exogenous hydrocortisone concentrations obtained during the suppression period.

In this report two assay methods, one using normal phase HPLC with fluorescence detection of the dansyl derivative of hydrocortisone, and the other using reverse phase HPLC with UV detection of hydrocortisone, were compared

by assaying serial plasma samples obtained during an 8 hr period from three healthy volunteers who had ingested hydrocortisone tablets. The plasma concentration versus time data permits preliminary description of the pharmacokinetics of exogenous hydrocortisone.

EXPERIMENTAL

Materials

Materials used in the fluorescence HPLC assay have been described previously (10). For the UV HPLC assay, standard hydrocortisone (Sigma Chemical Co., St. Louis, MO 63178), was dried to constant weight before use. A 75 µg/ml hydrocortisone solution in methanol was diluted to obtain a series of working standard solutions of 25, 125, 250, 500, 1000, 2000, 2500, and 3500 ng/ml hydrocortisone in 60% aqueous methanol. Plasma samples for the standard curve were obtained by adding 0.2 ml of standard solution to 1 ml of dexamethasone-suppressed plasma to give plasma hydrocortisone concentrations ranging from 5 to 700 ng/ml. The internal standard for both the UV and the fluorescent assays was Δ^4 - Pregnen - 17α, 20β, 21 - triol - 3,11 - dione (Sigma), which was used as received. The sodium hydroxide used in the UV assay was reagent grade. Dichloromethane (Aldrich Chemical Co., Milwaukee, WI 53233) was distilled prior to use. Methanol (Burdick and Jackson Laboratories, Muskegon, MI 49442) was used as received. The distilled water used throughout the experiment was further purified using a Barnstead PCS water purification system. Plasma for standard curves was obtained between 07:00 and 09:00 h subsequent to

administration of 2 mg dexamethasone at 23:00h the previous evening. Hydrocortisone concentrations in suppressed plasma samples were approximately 5 ng/ml. Hydrocortisone:internal standard peak height ratios in suppressed plasma were subtracted from the peak height ratios from plasma containing known concentrations of hydrocortisone during preparation of standard curves. Methanolic solutions were stored at -10°C and aqueous solutions were kept at 5°C. All solutions were brought to room temperature before use.

Drugs

Dexamethasone[1] contained 0.1 mg dexamethasone per ml of elixir. Hydrocortisone[2] was administered as 10 mg tablets.

Subjects

Three normal male volunteers (22-45 years old) underwent complete physical examinations including blood and urine analyses after giving informed consent. Vital signs and laboratory values for all subjects were normal. The subjects weighed 64 to 75 kg, and their heights ranged from 178 to 180 cm.

Protocol

Hydrocortisone was administered at 08:00h together with 180 ml H_2O. Each subject received doses of 10, 30, and 50 mg at least one week apart in a randomized design. Subjects received 20 ml (2 mg) dexamethasone elixir together with 180 ml H_2O at 23:00h the previous evening. Subjects were fasted from 20:00h on the day prior to dosing until 12:00h on the dosing day. No other liquid was allowed from 22:00h on the day prior to dosing until the hydrocortisone

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[1]Decadron elixir, Merck Sharp and Dohme, Lot A3240

[2]Merck Sharp and Dohme, Lot V2478

dose was administered. Heparinized blood samples (10 ml) were taken from a forearm vein just prior to hydrocortisone administration, and then at 0.25, 0.5, 1.0, 1.5, 2, 3, 4, 5, 6, 7, and 8h postdosing. Subjects were ambulatory during blood sampling. Blood samples were centrifuged immediately and the plasma divided into two portions which were frozen at -20°C. One plasma portion was packed in dry ice and sent by air freight to the Food and Drug Administration laboratories for fluorimetric analysis. The other portion was stored in this laboratory at -20°C until assayed by the HPLC - UV method.

Fluorimetric Assay

The fluorimetric HPLC assay of plasma samples was carried out at the laboratories of the Food and Drug Administration using a previously published method (10).

UV Assay

The UV HPLC assay used was a modified version of a previously described method (3). Plasma (1 ml) containing 200 ng internal standard and 0.1 ml of 2N aqueous sodium hydroxide was extracted with 10 ml dichloromethane, vortex-mixed for 1 min, and then centrifuged for 15 minutes at 500 x g in a polyethylene-stoppered, ground glass centrifuge tube. Plasma and the creamy interface were aspirated off, and the organic layer transferred to a clean tube and evaporated to dryness under nitrogen, with the tube immersed in a room temperature water bath. Tube walls were rinsed with 1 ml dichloromethane and evaporated as before. The residue was reconstituted with 100 µl of the HPLC mobile phase (60% aqueous methanol), vortexed for 1 minute, and centrifuged for 5 minutes. A 30 µl aliquot of this solution was injected into

the HPLC through a Rheodyne 7125 injector fitted with a 20 µl fixed volume loop. After injection the sample passed first through a 2.1 x 70 mm precolumn (Co Pell ODS, 30-8 µm) and then through a 4.6 x 250 mm Lichrosorb C18, 10 µm analytical (Altex) HPLC column. Mobile phase was pumped at 1.0 ml/min using an Altex 110A constant flow pump, resulting in a pump pressure of 700 psi. Effluent was monitored at 254 nm with a Waters 440 fixed wavelength detector. Elution times for hydrocortisone and internal standard were 10 min and 8 min, respectively. For plasma samples that yielded hydrocortisone: internal standard peak height ratios of less than 0.1 (hydrocortisone concentrations less than 25 ng/ml), a second injection was performed and the peak height ratios obtained from the two injections were averaged.

Pharmacokinetic and Statistical Analysis

Hydrocortisone plasma concentration data from all three doses, obtained using both assay procedures, were adequately described by the one-compartment open pharmacokinetic model with first order absorption and elimination, and an absorption lag time. Thus, the hydrocortisone concentration C_t in plasma at any time t after dosing was described by Equation 1 (13)

$$C_t = \frac{FD}{V}\left(\frac{k_a}{k_a - k_{el}}\right)\left(e^{-k_{el}(t - t_{lag})} - e^{-k_a (t - t_{lag})}\right) \qquad (1)$$

where k_a and k_{el} are the first order absorption and elimination rate constants, t_{lag} is the absorption lag time, F is the fraction of dose D absorbed, and V is the apparent distribution volume of drug in the body.

Equation parameters were obtained by nonlinear regression of individual hydrocortisone concentration data sets using the program NREG (14) on a Univac 1100 digital

computer. Absorption half-time, $t1/2^{a}$, and elimination half-life, $t1/2^{el}$, were obtained from the respective rate constants by $t1/2 = \ln 2/k$. The maximum drug concentration in plasma, C_{max}, and the time at which this maximum occurred, t_{max}, for each data set were calculated from Equations 2 and 3, respectively.

$$C_{max} = \frac{FD}{V}\left(\frac{k_a}{k_{el}}\right)^{\frac{k_a}{k_{el} - k_a}} \qquad (2)$$

$$t_{max} = \frac{2.303}{(k_{el} - k_a)} \log\left(\frac{k_{el}}{k_a}\right) + t_{lag} \qquad (3)$$

Model independent values of the area under the drug concentration versus time curve (AUC) from 0 to 8h and from 0 to infinite time were calculated by Equations 4 and 5

$$AUC^{0\to 8h} = \sum_{i=1}^{n-1} \frac{(C_{i+1} + C_i)(t_{i+1} - t_i)}{2} \qquad (4)$$

$$n = 12$$

$$AUC^{0\to\infty} = AUC^{0\to 8h} + \frac{C^{8h}}{k_{el}} \qquad (5)$$

where C^{8h} is the plasma concentration of drug at 8h.

Comparison of the concentration values obtained by the two assay procedures was by paired t-test. Pharmacokinetic parameters were analyzed for dose, subject, and assay effects by three-way analysis of variance (ANOVA). Factors shown to have a significant effect by ANOVA were further analyzed using Tukey's significant difference test (15).

RESULTS

Figure 1 shows two representative chromatograms from the UV HPLC assay. Figure 1a) illustrates the low level of endogenous hydrocortisone in suppressed plasma. During analysis, the same amount of internal standard was used over the entire hydrocortisone concentration range. Recorder attenuation was adjusted to give nearly full scale response for internal standard with hydrocortisone concentrations below 200 ng/ml, and detector sensitivity was decreased from 0.005 AUFS to 0.01 AUFS for hydrocortisone concentrations greater than 200 ng/ml.

An absorbance scan showed hydrocortisone to have an absorption maximum at 243 nm in 45% aqueous methanol. Absorbance at 254 nm was sufficiently close to the maximum to permit the use of a fixed wavelength detector with a mercury lamp source.

Recovery of hydrocortisone and internal standard from plasma was determined at three concentrations for hydrocortisone and at one concentration for internal standard. Recovery was calculated as the ratio of compound peak height for extracted compound compared to a direct on-column injection. Recovery of hydrocortisone was 82% and was concentration independent over the 50 to 500 ng/ml range studied. Recovery of internal standard at 200 ng/ml was 66%.

The results of six replications of the standard curve for hydrocortisone concentrations between 5 and 700 ng/ml are shown in Figure 2. Response was linear at these concentrations . The coefficient of variation ranged

from 1.6% (200 ng/ml) to 8.0% (10 ng/ml) for between day replicates as shown in Table I. The limit of detection for a signal-to-noise ratio of 2.5 was approximately 4 ng/ml.

Assay Comparison

Mean hydrocortisone plasma concentrations for the three subjects at the three dosage levels, obtained by both assay methods, are shown in Figure 3. Concentrations obtained by the two assay methods at each sample time within each dosage level (11 sample times at each dose level) were compared using the paired t-test. A significant difference at the 95% confidence level between the two assays was shown at only 2 out of the 33 sampling times. Figure 4 shows all concentrations obtained by the UV assay plotted against those obtained using the fluorescence method. Assay equivalence is indicated by a slope not significantly different from 1.0, and an intercept not significantly different from 0.0. The possibility of metabolite interference in the UV assay was explored by plotting the ratio of the concentrations obtained by the fluorescence and UV methods, versus time. The resulting plot shown in Figure 5 shows no significant time-dependent effect on the fluorescence:UV concentration ratio.

Pharmacokinetics

Representative plots of individual data sets at each dose level using both assays are shown in Figure 6 along with the corresponding computer-generated lines of best fit. The adequacy of the proposed pharmacokinetic model to describe the data is clear in each case. Mean values of pharmacokinetic parameters are shown in Table II, and the results of statistical analysis of these parameters in Table III. Intersubject variability was quite high, with a significant effect on all parameters except k_a. The dose-related parameters, FD/V, AUC,

and C_{max}, were dependent on dose size with the exception that C_{max} failed to show a significant difference between the 30 and 50 mg doses. However, the dose-normalized F/V and C_{max}/D values were not constant between doses, with F/V decreasing from a mean value of 23.5×10^{-6} ml^{-1} from the 10 mg dose to 13.7×10^{-6} ml^{-1} from the 50 mg dose. A similar effect was observed for C_{max}/D. Parameters which were expected to be dose-independent (k_{el}, t_{max}, and t_{lag}) were significantly affected by dose size. The elimination rate constant was significantly smaller for the 50 mg dose resulting in a longer elimination half-life than that obtained for the 10 and 30 mg doses. The mean time of peak hydrocortisone plasma levels, t_{max}, increased from 0.61 - 0.66 h from the 10 mg dose, to 1.4h from the 50 mg dose. The lag time increased from 0.13 - 0.16 h following the 50 mg dose to 0.26 - 0.29 following the 30 mg dose, while the 10 mg dose yielded intermediate values.

DISCUSSION

The lack of a sensitive and specific assay for hydrocortisone in biological fluids has previously prevented the examination of the pharmacokinetics and bioavailability of hydrocortisone products. It has also been difficult to study therapeutic and endogenous levels of hydrocortisone as a function of time due to the absence of a fast, convenient assay method.

As many substances in plasma absorb in the ultraviolet region of the spectrum, there was some doubt regarding the specificity of the UV assay for hydrocortisone. The excellent agreement in the results obtained by the UV assay as compared to the fluorescence assay in this study shows that the two methods are equally specific. The sensitivity of these procedures permits examination, not only of exogenous and endogenous hydrocortisone plasma concentrations, but also of endogenous hydrocortisone levels achieved during dexamethasone suppression.

Mean elimination half-life values for oral hydrocortisone obtained in this study from the 10, 30, and 50 mg doses were respectively 90, 77, and 97 min by the UV method and 93, 80, and 98 min by the fluorescence method. Previous studies with intravenous hydrocortisone have reported drug half-life values in plasma between 58 min and 161 min, and mean values ranging from 99 min to 138 min (16-20). Previously reported t_{max} values range from 40 min to 90 min after oral hydrocortisone doses from 3 to 50 mg (21, 22) which agree with t_{max} values of 37, 47, and 62 min at 10, 30, and 50 mg obtained in this study.

A large intersubject variability in hydrocortisone plasma levels has been previously reported, and is seen also in this study. However, the limited data reported here suggest that hydrocortisone pharmacokinetics may also be dose-dependent as drug levels in plasma do not appear to increase in direct proportion to the size of the administered dose.

There are a number of possible explanations for the apparent dose-dependent pharmacokinetics of hydrocortisone. Drug absorption may be inhibited at higher doses due to limited tablet dissolution, or as a result of capacity-limited transport across the gastrointestinal epithelium.

The non-proportionality of hydrocortisone levels with respect to dose may also, however, be the result of changes in drug protein binding and/or altered distribution. Hydrocortisone is cleared from plasma mainly by hepatic biotransformation. Changes in protein binding may therefore affect the rate and extent to which drug gains access to the hepatic metabolizing enzymes. Plasma hydrocortisone binds to two different plasma proteins. At concentrations below 200 ng/ml hydrocortisone is strongly and specifically bound to transcortin (16, 23), and *in vitro* and *in vivo* studies have shown that the presence of transcortin greatly reduces the biotransformation rate of hydrocortisone by liver cells (16, 24). At hydrocortisone concentrations greater than 200 ng/ml, transcortin binding sites are saturated and hydrocortisone becomes loosely and nonspecifically

bound to albumin, which does not compete with liver enzymes due to its low affinity for hydrocortisone (24). The 10 mg dose in the present study yielded hydrocortisone plasma concentrations below 200 ng/ml, while concentrations above this value were achieved with the 30 and 50 mg doses.

The apparent volume of distribution of hydrocortisone may increase with larger doses due to the predominance of the lower affinity binding to albumin over that to transcortin. This would cause the value of FD/V to undergo a smaller increase with increasing doses than might be expected.

The lower binding affinity of hydrocortisone at higher doses may also increase the degree of hepatic clearance during the first-pass resulting in a smaller percentage of higher doses reaching the general circulation. However, the lower binding affinity at higher doses may also cause partial saturation of the biotransformation capacity of the liver, and this is consistent with the smaller elimination rate constant obtained following the 50 mg dose compared to the 10 mg and 30 mg doses. Enzyme saturation may also be responsible for slight downward curvature in the plot of the logarithm of hydrocortisone levels versus time observed between 1.5h and 5h following the 50 mg dose (Figure 3c), compared to the linearity of the curve following the 30 mg dose (Figure 3b), and the slight upward curvature following the 10 mg dose (Figure 3a). However, more data are needed to confirm this.

While the relationship between the AUC values in Table II and dose size can be described adequately by a straight line, a significant, positive y-intercept is obtained. This suggests that circulating hydrocortisone levels following drug administration are not proportional to dose. A recent study (25) of hydrocortisone concentrations in plasma following oral cortisone acetate in humans has shown a similar linear AUC-dose relationship with a positive y-intercept. The apparent nonlinear pharmacokinetics of hydrocortisone observed in these studies warrant futher examination as they may have important therapeutic implications, and may also influence conclusions drawn from bioavailability studies.

ACKNOWLEDGEMENT

Funds for the above study were provided by Contract number 223-78-3006 from the United States Food and Drug Administration.

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TABLE I. UV Assay Between-Day Variability

Theoretical Conc. (ng/ml)	Experimental Conc., ng/ml Mean	(S.D.)	n	Coefficient of Variation
5	7.2	(0.5)	6	7.6%
10	11.7	(0.9)	6	8.0%
25	25.5	(0.5)	6	1.9%
50	51	(3)	6	5.7%
100	96	(3)	6	2.9%
200	193	(3)	6	1.6%
400	402	(17)	5	4.1%
700	695	(17)	6	3.9%

Table II Mean pharmacokinetic parameters for hydrocortisone obtained from both the fluorescence (F) and ultraviolet (U) assay results.

Parameter			k_a	k_{el}[a]	$t_{1/2a}$	$t_{1/2el}$	t_{max}	t_{lag}	$\frac{FD}{V}$	C_{max}	$\frac{F}{V}$	$\frac{C_{max}}{D}$	AUC_{eqn}[b]	$AUC_{trap}^{0\to 8c}$	$AUC_{trap}^{0\to\infty c}$	r^2
Units			h^{-1}	h^{-1}	h	h	h	h	$\frac{ng}{ml}$	$\frac{ng}{ml}$	$\frac{10^{-6}}{ml}$	$\frac{10^{-6}}{ml}$	$\frac{ng\ h}{ml}$	$\frac{ng\ h}{ml}$	$\frac{ng\ h}{ml}$	--
Dose	Assay													467		
10 mg	F	Mean	7.7	0.58	0.16	1.21	0.66	0.17	264	196	26.4	19.6	458	464[d]	488	0.984
		SD	7.3	0.08	0.13	0.17	0.29	0.04	20	25	2.0	2.5	34	0.7	24	0.024
	U	Mean	23	0.47	0.12	1.50	0.61	0.18	235	192	23.5	19.2	503	458	474	0.980
		SD	32	0.06	0.10	0.17	0.30	0.07	36	34	3.6	3.4	32	50	48	0.017
30 mg	F	Mean	26	0.52	0.18	1.33	0.74	0.29	520	411	17.3	13.7	1301	977	998	0.991
		SD	28	0.04	0.28	0.10	0.49	0.15	48	91	1.6	3.0	343	78	85	0.012
	U	Mean	7.7	0.54	0.22	1.29	0.78	0.26	510	373	17.0	12.4	1071	943	958	0.986
		SD	6.3	0.04	0.26	0.09	0.52	0.14	75	79	2.5	2.6	106	111	113	0.019
50 mg	F	Mean	1.7	0.43	0.59	1.63	1.44	0.16	680	391	13.6	7.8	1587	1476	1544	0.986
		SD	1.3	0.05	0.37	0.17	0.55	0.18	121	77	2.4	1.5	129	139	133	0.013
	U	Mean	1.4	0.43	0.55	1.62	1.40	0.13	785	396	13.7	7.9	1591	1491	1559	0.988
		SD	0.5	0.03	0.18	0.09	0.36	0.23	75	55	1.5	1.1	128	140	142	0.001

a: k_{el} obtained by least squares linear regression

b: $AUC_{eqn} = FD/Vk_{el}$

c: AUC_{trap} by trapezoidal method

d: n = 2, C_{8h} for subject 1 was not detectable

TABLE III. Results of Three-way ANOVA for Dose, Assay, and Subject Effects and the Tukey Multiple Comparison

All differences between assay values were found to be insignificant at the 95% confidence level.

	DOSE EFFECT		SUBJECT EFFECT	
	P value from ANOVA	Tukey Differences	P value from ANOVA	Tukey Differences
k_a	>.05	- [a]	>.05	-
k_{el}	<.01	50 < 10,30	<.05	3U,1U,3F<2U,2F,1F [b]
FD/V	<.01	10 < 30 < 50	<.01	1U<1F,3F,3U,2U,2F
C_{max}	<.01	10 < 30, 50	<.01	1U,2F,2U,1F<3U,3F
t_{max}	<.01	10 < 30 < 50	<.01	3F,3U<1F,1U,2U,2F
Trap AUC	<.01	10 < 30 < 50	<.01	1U<1F<2F,2U<3F,3U
t_{lag}	<.05	50 < 10 < 30	<.01	2U,2F,3U,3F<1U,1F
r^2	>.05	-	>.05	-

a: Not Calculated

b: U = ultraviolet assay, F = fluorescence assay

FIGURE LEGENDS:

Fig. 1. Representative Chromatograms from UV HPLC assay of hydrocortisone (2) and internal standard (1) extracted from human plasma.

a) Dexamethasone-suppressed plasma containing 200 ng/ml internal standard and 11 ng/ml hydrocortisone (endogenous). Range = 0.005

b) Sample plasma containing 200 ng/ml internal standard and 149 ng/ml hydrocortisone (dosed + endogenous) Range = 0.005

Fig. 2. Standard Curve for analysis of hydrocortisone in plasma by the UV HPLC method. (6 replicates) 0.00497 ($\pm$ 0.00003)x - 0.014 ($\pm$ 0.010) r = 0.9989 (HC : hydrocortisone , IS : internal standard)

Fig. 3. Mean hydrocortisone plasma concentration versus time for 3 subjects ($\pm$ 1 S.D.) at dosages of a) 10 mg, b) 30 mg, and c) 50 mg.

▲ ——————— ▲ UV ● - - - - - - - ● Fluorescence

Fig. 4. Hydrocortisone plasma concentrations as obtained by UV detection (C^U) versus those obtained by fluorescence detection (C^f).

Y = 0.992($\pm$ 0.018)x - 2($\pm$ 3), n = 94, r = 0.986

Fig. 5. Ratio of hydrocortisone plasma concentrations obtained by the fluorescence and UV methods versus time. Y = -0.009($\pm$ 0.007)x + 1.02($\pm$ 0.03)

Fig. 6. Representative individual plots of hydrocortisone plasma concentration data versus time with computer-generated curve for a) 10 mg, b) 30 mg, and c) 50 mg doses in Subject 3.

▲ ——————— ▲ UV ● - - - - - - - - ● Fluorescence

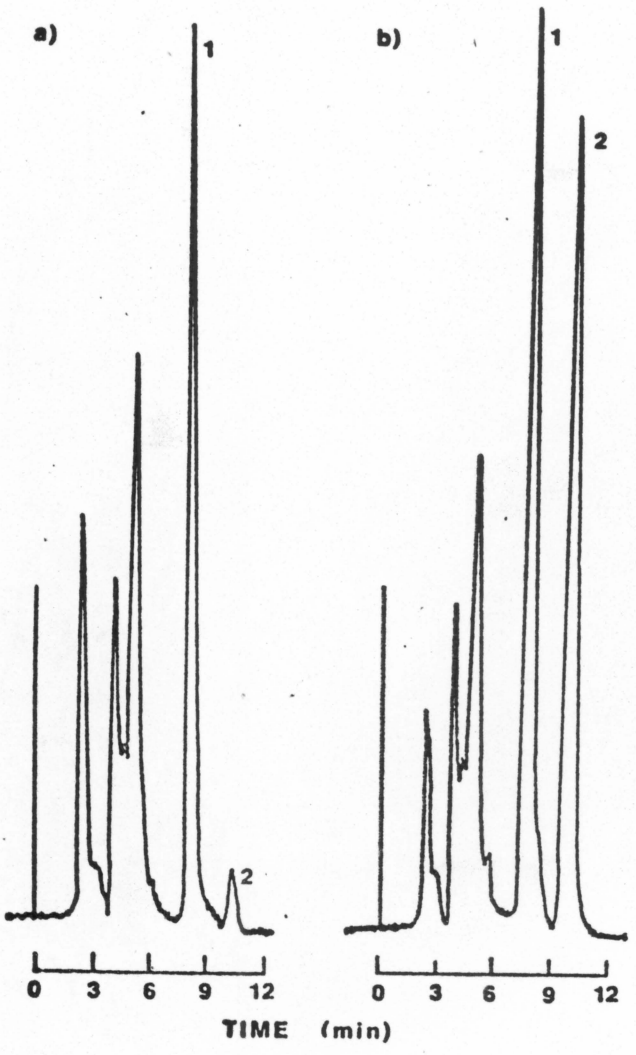

Figure 1

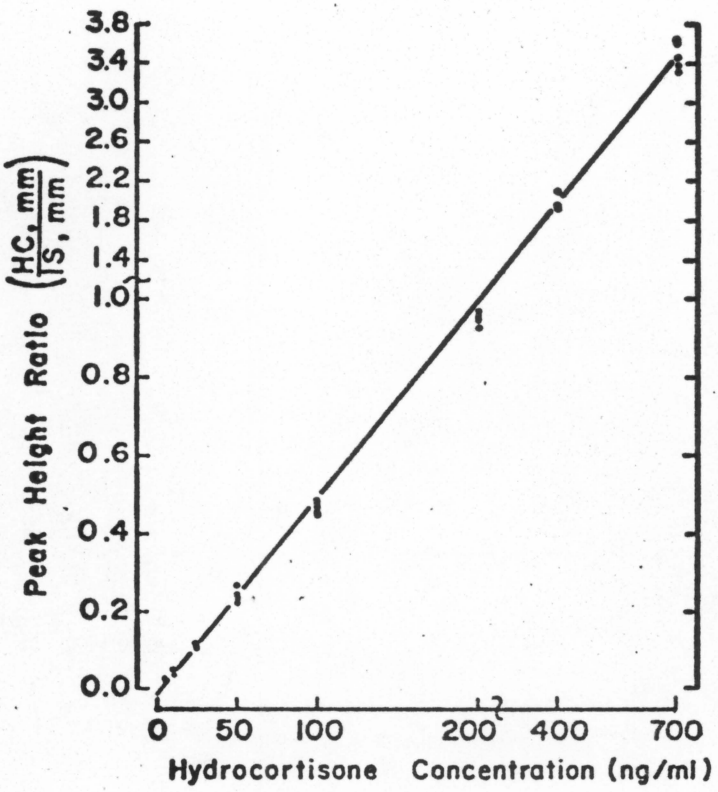
Peak Height Ratio (HC, mm / IS, mm)
3.8
3.4
3.0
2.6
2.2
1.8
1.4
1.0
0.8
0.6
0.4
0.2
0.0
0
50
100
200
400
700
Hydrocortisone Concentration (ng/ml)

Figure 2

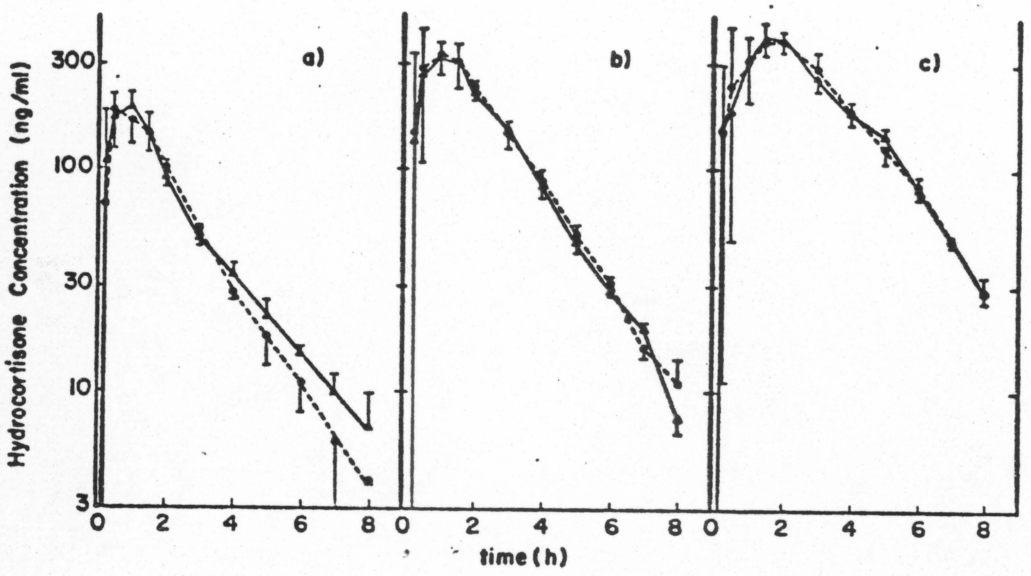
Hydrocortisone Concentration (ng/ml)
300
100
30
10
3
a)
b)
c)
0
2
4
6
8
time (h)

Figure 3

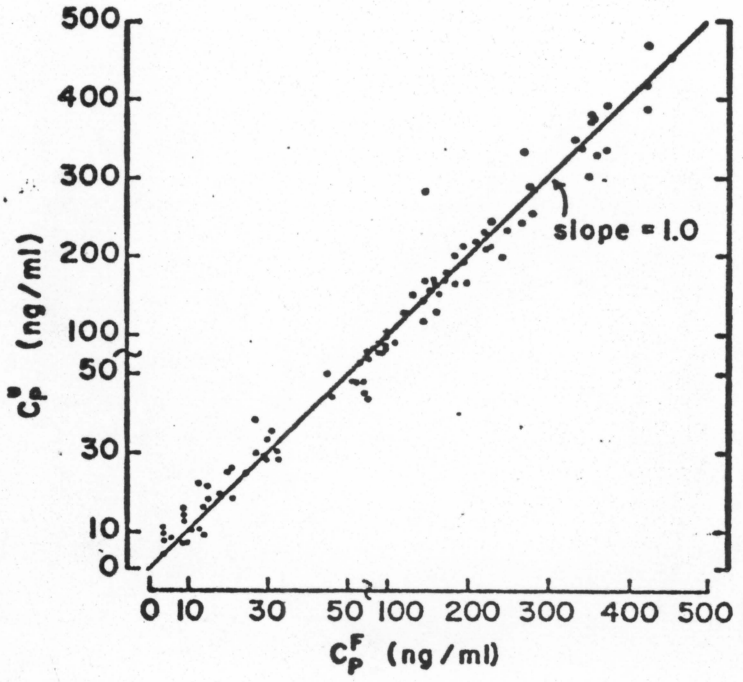
500
400
300
200
100
50
30
10
0
C_p^B (ng/ml)
slope = 1.0
0 10 30 50 100 200 300 400 500
C_p^F (ng/ml)

Figure 4

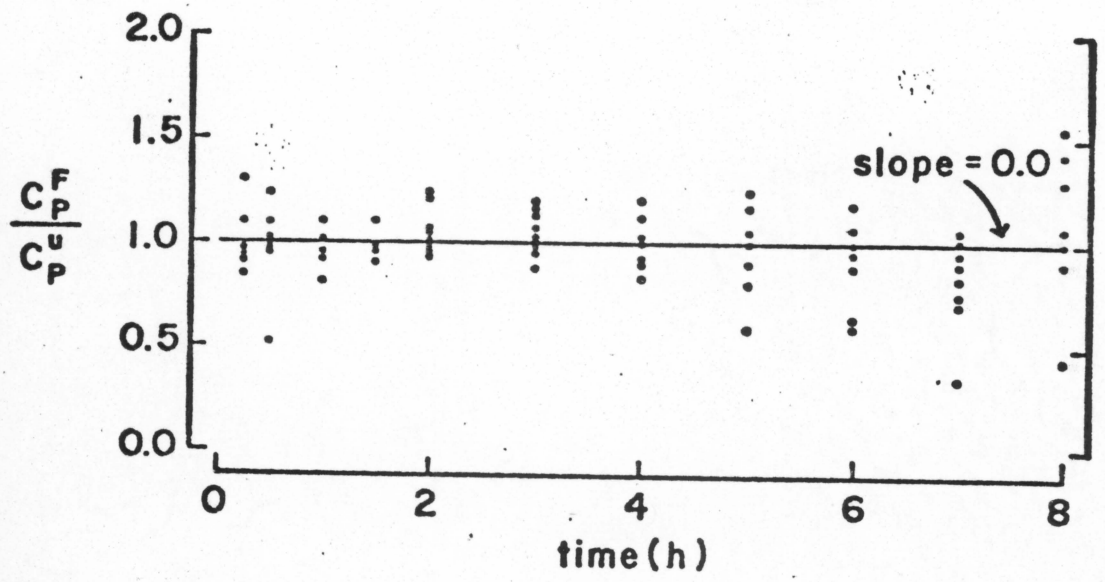

Figure 5

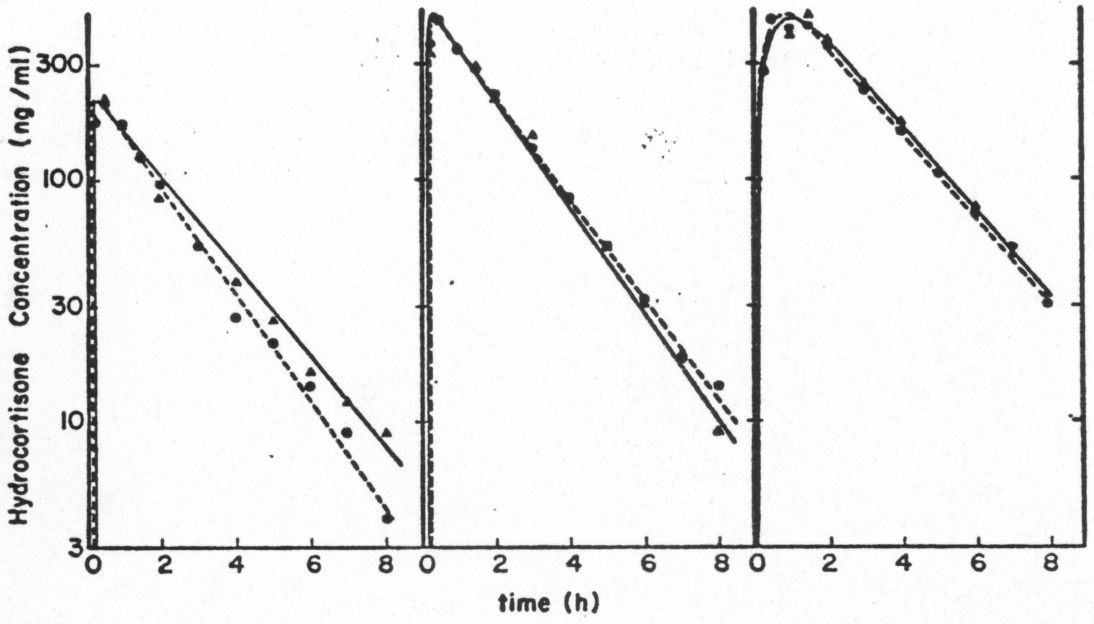
Hydrocortisone Concentration (ng/ml)
300
100
30
10
3
0
2
4
6
8
time (h)

Figure 6

APPENDIX 8

Manuscript in Preparation for Study 3

EFFECT OF DOSE SIZE ON THE PHARMACOKINETICS OF INTRAVENOUS HYDROCORTISONE DURING ENDOGENOUS HYDROCORTISONE SUPPRESSION

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Key words: Intravenous hydrocortisone, Blood concentrations, Pharmacokinetics, Endogenous hydrocortisone suppression

ABSTRACT

The pharmacokinetics of hydrocortisone were examined following single intravenous doses of 5, 10, 20, and 40 mg doses of hydrocortisone, as the sodium succinate salt, to healthy male volunteers. Endogenous hydrocortisone was suppressed by administration of 2 mg dexamethasone the night before hydrocortisone injection. Plasma samples obtained serially during 8 h after hydrocortisone injection were assayed by reverse-phase HPLC using a fixed wavelength (254 nm) ultraviolet detector.

Initial concentrations of hydrocortisone in plasma were proportional to dose size. The subsequent decline in hydrocortisone concentrations was biphasic, and individual data sets were adequately described in terms of the pharmacokinetic two-compartment open model. Values of pharmacokinetic parameters were similar from the 5 mg, 10 mg, and 20 mg doses. Following the 40 mg dose, the overall elimination rate constant decreased, while the distribution volume, V_{dss}, and plasma clearance increased, in comparison with the values obtained from lower doses.

Changes in the pharmacokinetics of hydrocortisone at high doses may be related to drug concentration-dependent changes in the binding of hydrocortisone to plasma proteins. Previously reported dose-dependent changes in some pharmacokinetic parameters following oral hydrocortisone are attributed to absorption rather than distribution or elimination effects.

INTRODUCTION

Hydrocortisone, the principal endogenous glucocorticoid in man, is used therapeutically for a variety of indications (1). Two factors had hitherto prevented accurate description of hydrocortisone pharmacokinetics; the insufficient sensitivity and specificity of analytical methods, and also interference due to endogenous circulating hydrocortisone.

High pressure liquid chromatography (HPLC) methods have recently been described, using both ultraviolet absorption and fluorescence detectors, which are capable of selectively measuring plasma hydrocortisone concentrations as low as 3 ng ml^{-1} (2-7). Circulating levels of endogenous hydrocortisone, which vary diurnally between 40 and 200 ng/ml, have recently been shown to be suppressed to concentrations between 6 and 14 ng ml^{-1} following oral administration of dexamethasone (8). A 2 mg dose of dexamethasone given at 11 pm results in uniform suppression of endogenous circulating hydrocortisone until at least 8 pm the following day.

A previous study in this laboratory (7) suggested a possible lack of dose-proportionality in plasma hydrocortisone concentrations following oral does of 10, 30, and 50 mg hydrocortisone. Similar observations have been reported following oral cortisone acetate (9). Linear plots of areas under plasma hydrocortisone profiles against dose in both studies yielded positive Y-intercepts. The distribution and elimination characteristics of hydrocortisone may also be dose-dependent (7).

In order to carry out meaningful bioavailability and pharmacokinetic studies with oral hydrocortisone formulations, it is necessary first to establish whether the distribution and elimination characteristics of hydrocortisone exhibit dose-dependency following parenteral doses, i.e., in the absence of absorption effects.

In the present study, the pharmacokinetics of hydrocortisone were examined following single 5, 10, 20, and 40 mg doses to healthy male volunteers.

EXPERIMENTAL

Materials

Standard solutions of 0.025-15 μg ml^{-1} hydrocortisone (Sigma Chemical Co., St. Louis, MO) were prepared in 60% aqueous methanol. The internal standard for the HPLC assay, Δ^4-pregnen-17α,20β,21-triol-3,11-dione (Sigma Chemical Co.) was prepared as a 4 μg ml^{-1} solution in methanol. Dichloromethane and methanol (Burdick and Jackson Laboratories, Muskegon, MI 49442) were used as received. Distilled water was further purified by means of a Barnstead PCS water purification system (Barnstead Sybron Corp., Boston, MA 01232). Methanolic and aqueous solutions were stored at -10°C and 5°C, respectively, and were brought to room temperature before use. All solvents and chemicals were reagent grade quality, and were used as supplied.

Subjects

After giving informed consent, six male volunteers (age 21-29 years, height 175-196 cm, weight 64-82 kg) underwent physical examinations which included blood and urine analysis. All vital signs and laboratory values were within normal limits.

Protocol

Each subject received single intravenous doses of 5, 10, 20, and 40 mg hydrocortisone sodium succinate (A-Hydrocort, Lot # 11-573-AF, Abbott Laboratories, Illinois). The solution for injection contained 50 mg hydrocortisone equivalents per ml. Accurate delivery of dosage volumes of 0.1-0.8 mg was assured by injecting from a 1.0 ml tuberculin syringe, during a 15 second period.

The different dosages were administered to each subject at least one week apart, according to a randomized block design. Subjects received no solid food from 8 pm the previous day until 12 noon on the day of hydrocortisone administration. No liquids were permitted from 11 pm the previous day until 8 am the next day, when 180 mg of water were ingested immediately following the hydrocortisone injection. No further fluids were permitted until 12 noon on that day. All subjects received 20 ml of dexamethasone elixir containing 0.1 mg dexamethasone per ml (Decadron elixir, Lot # A 3240, Merck Sharp and Dohme, West Point, PA 19486) together with 180 mg of water at 11 pm on the day preceding each hydrocortisone dose.

Hydrocortisone sodium succinate was injected into a forearm vein. Blood samples (10 ml) were taken from the contralateral forearm vein into heparinized vacuum tubes (Vacutainer, Becton-Dickinson, Rutherford, NJ 07070) immediatedly before and then serially from 10 min until 8 h after dosing. Subjects were ambulatory throughout the sampling period. Plasma was obtained by centrifugation, and stored at -20°C until assayed. Assays were routinely carried out within one month and no degradation of hydrocortisone occurred during this period.

The concentrations of hydrocortisone in plasma were determined by the HPLC-UV method recently developed in this laboratory (7). The concentrations of endogenous hydrocortisone in suppressed plasma were subtracted from observed levels for the construction of standard curves, and concentrations of endogenous hydrocortisone in predose plasma samples were subtracted from all postdose values in order to obtain circulating drug levels due to administered hydrocortisone. The mean recovery efficiency for hydrocortisone from plasma in 82% and the coefficients of variation for replicate determinations for hydrocortisone concentrations between 5 and 3000 ng ml^{-1} are uniformly

within 10%.

Data Analysis

Initial estimates of hydrocortisone pharmacokinetic parameters were obtained from individual data sets using standard graphical procedures. Improved estimates of parameter values were obtained by nonlinear regression analysis of individual non-weighted data using the program NRCG (10) on a Univac 1110 digital computer. Model-independent estimates of areas under plasma drug concentration versus time curves from zero to infinite time ($AUC^{0\to\infty}$) were calculated using the trapezoidal method (11), with end correction where appropriate.

Pharmacokinetic parameter values were examined for dose and subject effects by means of two-way analysis of variance (ANOVA). Particular dose effects were examined by means of Tukey's significance difference test (12)

RESULTS

The mean plasma levels of hydrocortisone resulting from the four different doses are described in Figure 1. Similar hydrocortisone plasma profiles were obtained from each of the doses. Drug levels delcined rapidly until 0.5-1.0 h after dosing, and subsequently at a slower rate. During the slower rate of decline the individual slopes were clearly monoexponential.

Initial hydrocortisone levels were closely related to dose size; mean peak values from the 5, 10, 20, and 40 mg doses, obtained at 10 min after dosing, were respectively 312, 573, 1095, and 1854 ng ml^{-1}.

Following the slower rate of decline, mean plasma hydrocortisone levels from the 10, 20, and 40 mg doses had fallen to 6, 14, and 36 ng ml^{-1}, respectively, by 8 h. Hydrocortisone levels from the 5 mg dose could not generally be detected beyond the 6 h sampling time.

The biphasic decline in plasma hydrocortisone levels is consistent with

the pharmacokinetic two-compartment open model, and individua data sets were therefore analyzed in terms of Equations 1 and 2 (13).

$$C = D/V_1(\alpha-\beta)[(k_{21}-\beta)e^{-\beta t} - (k_{21}-\alpha)e^{-\alpha t}] \quad (1)$$

$$\begin{matrix}\alpha \\ \beta\end{matrix} = 0.5[(k_{12}+k_{21}k_2) \pm [(k_{12}+k_{21}+k_2)^2 - 4k_{21}k_2]^{1/2}] \quad (2)$$

Where D is the administered dose, V_1 is the apparent distribution volume of the central body compartment, and k_{12}, k_{21}, and k_2 are first-order rate constants for drug transfer between the central and peripheral compartments (k_{12}, k_{21}) and for drug elimination, respectively.

The results of pharmacokinetic analysis are given in Table I. The high coefficients of determination, r^2, indicate excellent description of the observed data by the model. The plots obtained from computer analysis are superimposed on the means of the observed hydrocortisone levels in Figure 1.

Among the rate constants determined, the values of α, k_{12}, and k_2 were independent of dose size, while β and k_{21} were significantly reduced following the 40 mg dose compared to the 5 mg and 10 mg doses. The overall elimination half-life, $t_{1/2\beta}$, was also significantly longer following the 40 mg dose compared to the 5 mg and 10 mg doses. No differences were observed in the values of any rate constants between the 5, 10, and 20 mg doses. The mean elimination half-lives from these doses, 1.3-1.7 h, are similar to previously reported values (14-17).

Areas under hydrocortisone plasma profiles were dose-related. Mean rations of $AUC^{0\to\infty}$ values between the 10 mg and 5 mg, and the 20 mg and 10 mg doses were 2.0 and 1.95, respectively. Doubling the dose from 20 mg to 40 mg however, yielded a mean $AUC^{0\to\infty}$ ratio between these doses of 1.6.

After correction for the administered dose, area values from the 5, 10, and 20 mg doses, and also those from the 20 and 40 mg doses, were indistinguishable, whereas the corrected area from the 40 mg dose was significantly smaller than those from the 5 and 10 mg doses.

Dose-related changes in corrected area values are reflected in the apparent distribution volumes of hydrocortisone. The mean volumes of the central compartment V_1 ranged from 6.9 L to 8.8 L, and were independent of dose. However, while the values of V_{dss} were significantly indistinguishable from the 5, 10, and 20 mg doses, mean values ranging from 20.7 L to 26.0 L, V_{dss} for the 40 mg dose was significantly increased to 37.5 L. The high correlation between V_{dss} and $t_{1/2\beta}$ is shown in Figure 2.

The effect of the increase in the apparent overall distribution volume, V_{dss}, of hydrocortisone following the 40 mg dose on the plasma clearance was greater than that of the decrease in the value of β, and resulted in a significant increase in plasma clearance to a mean value of 294 ml min^{-1}, compared to 209-239 ml min^{-1} from the 5, 10, and 20 mg doses.

DISCUSSION

The results of a previous study involving oral doses of hydrocortisone (7) have suggested that the pharmacokinetics of hydrocortisone may exhibit dose-dependency in the therapeutic range.

Doses of 10, 30, and 50 mg hydrocortisone yielded mean peak plasma levels of hydrocortisone ranging from +92 ng ml^{-1} following the 10 mg dose to 396 ng ml^{-1} following the 50 mg dose. Lack of dose-proportionality in the plasma levels of hydrocortisone led to the suggestion that the absorption of hydrocortisone may decrease, the distribution of hydrocortisone in the body may increase, or both of these may occur, with increasing doses.

In the present study, intravenous doses of 5, 10, 20, and 40 mg hydro-

cortisone gave rise to mean maximum plasma hydrocortisone levels much higher than those following the oral doses, ranging from 312 ng ml^{-1} from the 5 mg dose to 1854 ng ml^{-1} from the 40 mg dose. The peak level that was previously obtained following the 50 mg oral dose (7) was thus intermediate between the maximum level following the 5 mg and 10 mg intravenous doses.

Evidence of dose nonproportionality following intravenous hydrocortisone occurred only following the 40 mg dose, and there ws no evidence of dosage effects following the 5, 10, and 20 mg intravenous doses.

These observations lead us to conclude that the distribution and elimination characteristics of hydrocortisone were unaffected by the oral doses that were employed in the previous study (7). Observed nonlinearity in the pharmacokinetic parameters of oral hydrocortisone is attributable therefore to dose-dependent changes in drug bioavailability.

Drug concentration-dependent changes in the binding of hydrocortisone to plasma proteins were considered to be a possible explanation for observed changes in hydrocortisone pharmacokinetics following oral doses (7). The results obtained here indicate that this is unlikely. However, the same phenomenon may explain the apparent increase in V_{dss} following the 40 mg intravenous hydrocortisone dose. At concentrations below 200 ng ml^{-1} plasma hydrocortisone is tightly bound to transcortin, and the free fraction of circulating drug is only 5 percent (18). At plasma hydrocortisone concentrations greater than 200 ng/ml, a greater proportion of compound is loosely bound to albumin and the free fraction increases to approximately 25 percent.

Following the 5 mg and 10 mg intravenous doses, plasma levels of hydrocortisone were generally less than 200 ng ml^{-1} for almost the entire post-distributive, or β, phase. The 20 mg dose yielded plasma hydrocortisone levels greater than 200 ng ml^{-1} for approximately 1.5 h, while the 40 mg dose

resulted in levels exceeding 200 ng ml^{-1} for 3-4 h postdosing. Insufficient data points were taken to detect any change in drug distribution or elimination characteristics as levels dropped below 200 ng ml^{-1} following the 20 mg and 40 mg doses. Indeed, the excellent fit of all individual post-distributive data to a monoexponential function suggests that no such change occurs. However, discernable changes are probably obscured by the compeurating effects of increased binding which in turn possibly gives rise to reduced tissue distribution and also less efficient biotransformation of lower drug concentrations.

The longer plasma half-life of hydrocortisone at higher drug concentrations is clearly related to an increase in the apparent distribution volume, as shown in Figure 3. However the increase in hydrocortisone plasma clearance following the 40 mg dose, compared to other doses, suggests that the increase in distribution volume is the major pharmacokinetic change taking place. It is likely that while reduced binding to plasma proteins at high drug concentrations permits more hydrocortisone to enter extravascular fluids, it also facilates access of drug to the hepatocyte and hence undergo biotransformation. The low affinity binding of hydrocortisone to plasma albumin has been shown to be less inhibitory to hepatic uptake and biotransformation than the high affinity binding to transcortin (19).

The results of this study show that the distribution and elimination characteristics of hydrocortisone in healthy male volunteers are independent of dose size after intravenous doses of 5, 10 and 20 mg, but are dose-dependent after a intravenous dose of 40 mg. In view of the low circulating levels of hydrocortisone obtained after oral doses compared to intravenous doses, dose-dependent changes in hydrocortisone distribution or elimination are unlikely to occur following single oral doses of 5-50 mg. This conclusion

does not extend to possible dose-related changes in the first-pass metabolism of orally administered hydrocortisone, which has yet to be investigated.

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FIGURE CAPTIONS

Figure 1 Mean (± 1 SE) plasma concentrations of hydrocortisone following intravenous doses of 5 mg (○), 10 mg (●), 20 mg (▲), and 40 mg (■) hydrocortisone sodium succinate. The drawn lines are constructed from Equation 1 using the means of individual pharmacokinetic parameters from each dose, following nonlinear regression analysis.

Figure 2 Relationship between the overall elimination half-life, $t_{1/2\beta}$, of hydrocortisone versus the apparent volume of distribution at steady state, Vd_{ss}, for hydrocortisone doses of 5 mg (○), 10 mg (●), 20 mg (▲), and 40 mg (■). The linear regression equation for the line is (SD in parentheses):

$$t_{1/2\beta} = 0.029\ (\pm 0.005)\ VD_{ss} + 0.79\ (\pm 0.14) \quad r = 0.771$$

TABLE 1

Mean values of pharmacokinetic parameters for hydrocortisone at four dose levels. Standard deviations are shown in parentheses. The p-value indicated the significance level of treatment effects, calculated by ANOVA. Differences between individual treatments are calculated by Tukey's significant difference test.

Parameter	Value (n=6) 5 mg	10 mg	20 mg	40 mg	P	Tukey's test
α (h^{-1})	6.3 (1.5)	8.7 (2.7)	7.6 (1.1)	6.8 (0.5)	>0.05	- [a]
β (h^{-1})	0.56 (0.18)	0.55 (0.08)	0.46 (0.08)	0.37 (0.02)	<0.05	5 10 20 40
k_{12} (h^{-1})	3.0 (0.9)	4.9 (1.9)	4.3 (0.8)	3.8 (0.3)	>0.05	-
k_{21} (h^{-1})	2.3 (0.7)	2.3 (0.6)	1.5 (0.2)	1.2 (0.2)	<0.025	5 10 20 40
k_2 (h^{-1})	1.6 (0.4)	2.2 (0.6)	2.2 (0.6)	2.2 (0.4)	>0.05	-
$t_{½el}$ (h)	0.46 (0.11)	0.38 (0.11)	0.33 (0.07)	0.33 (0.04)	>0.05	-
$t_{½\beta}$ (h)	1.3 (0.3)	1.3 (0.2)	1.7 (0.2)	1.9 (0.1)	<0.005	40 20 10 5
V_1 (L)	8.5 (2.1)	7.0 (2.2)	6.9 (1.9)	8.8 (1.8)	>0.05	-
V_{dss} [b] (L)	20.7 (7.3)	20.8 (4.3)	26.0 (4.1)	37.5 (5.8)	<0.001	40 20 10 5
V_{pl} [c] (ml min^{-1} m^{-2})	209 (42)	218 (23)	239 (44)	294 (34)	<0.001	40 20 10 5
r^2 [d]	0.998 (0.002)	0.999 (0.007)	0.999 (0.004)	0.999 (0.003)	-	-
$AUC^{0\to\infty}$ (ng h ml^{-1})	410 (80)	790 (100)	1480 (310)	2290 (260)	<0.001	40 20 10 5
AUC^{*}_{β} (ng ml^{-1})	230 (70)	430 (80)	630 (90)	840 (110)	<0.001	40 20 10 5
AUC/D (10^{-3} kg h ml^{-1})	6.1 (0.1)	5.9 (0.8)	5.4 (0.6)	4.3 (0.4)	<0.025	5 10 20 40

a: not calculated

b: $V_{dss} - V_1 (1 + k_{12}/k_{21})$

c: plasma clearance = $D/AUC^{0\to\infty}$

d: $r^2 = Eob^2/(Eobs^2 - Edev^2)$

Figure 1

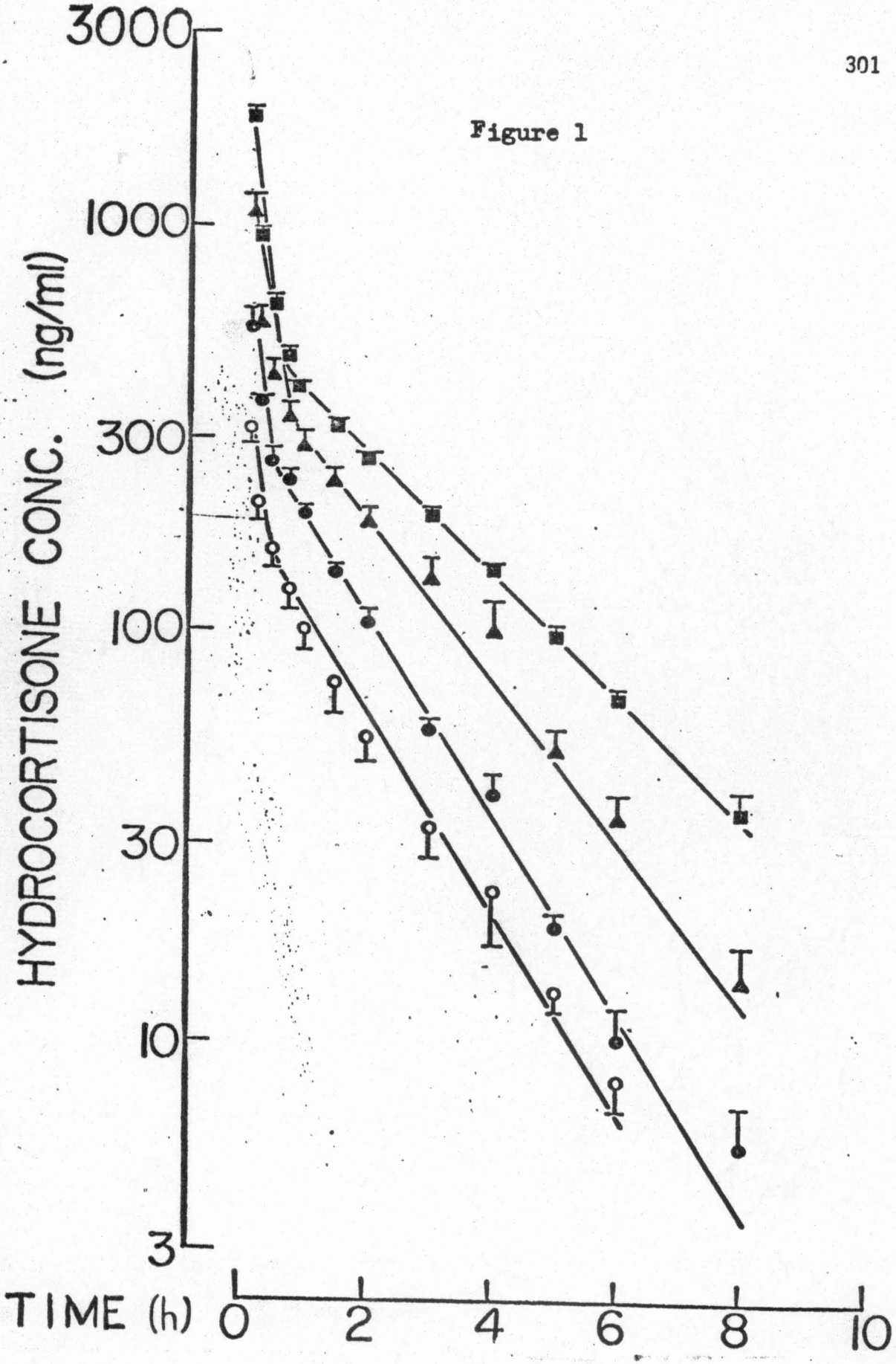
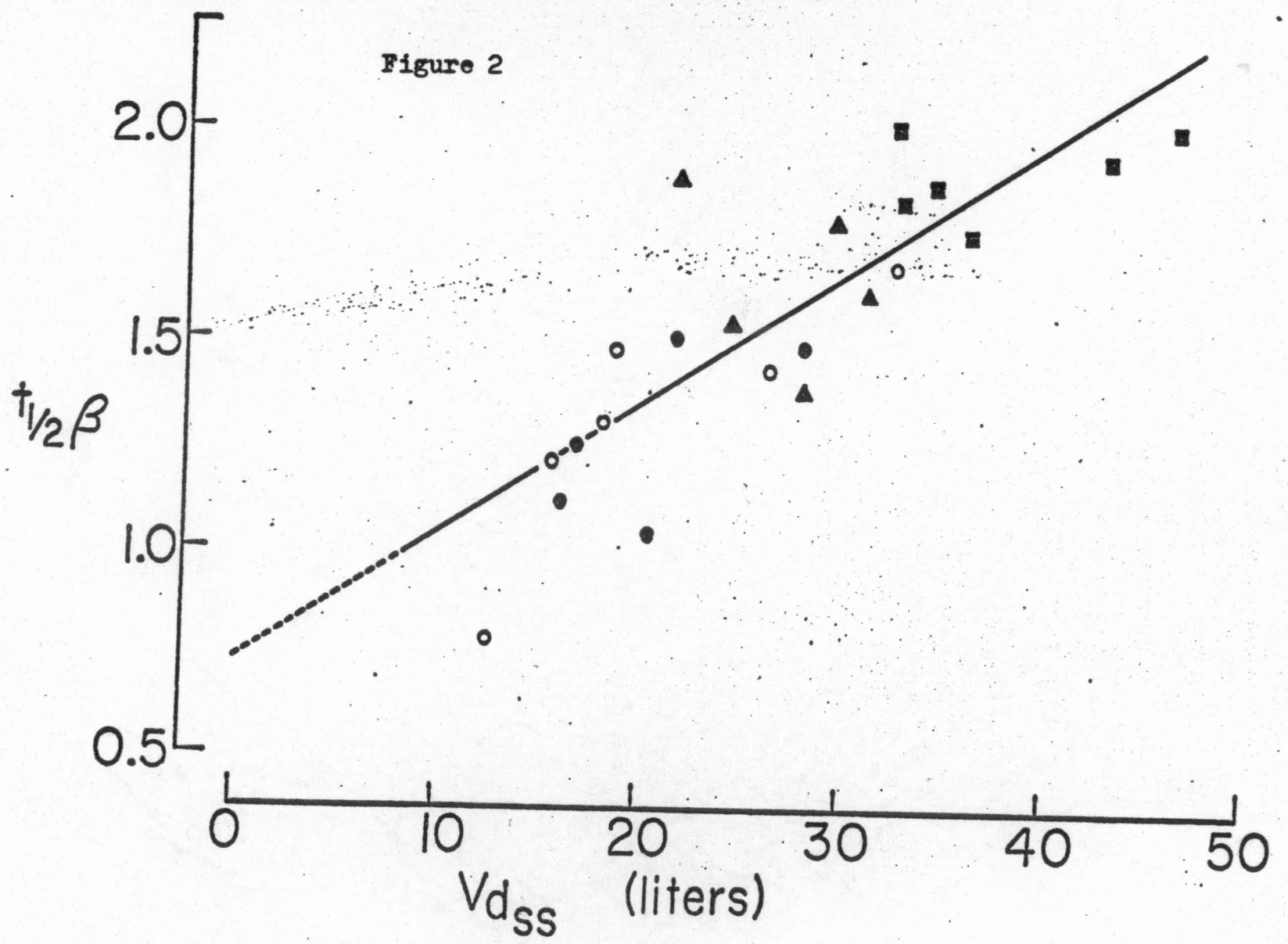
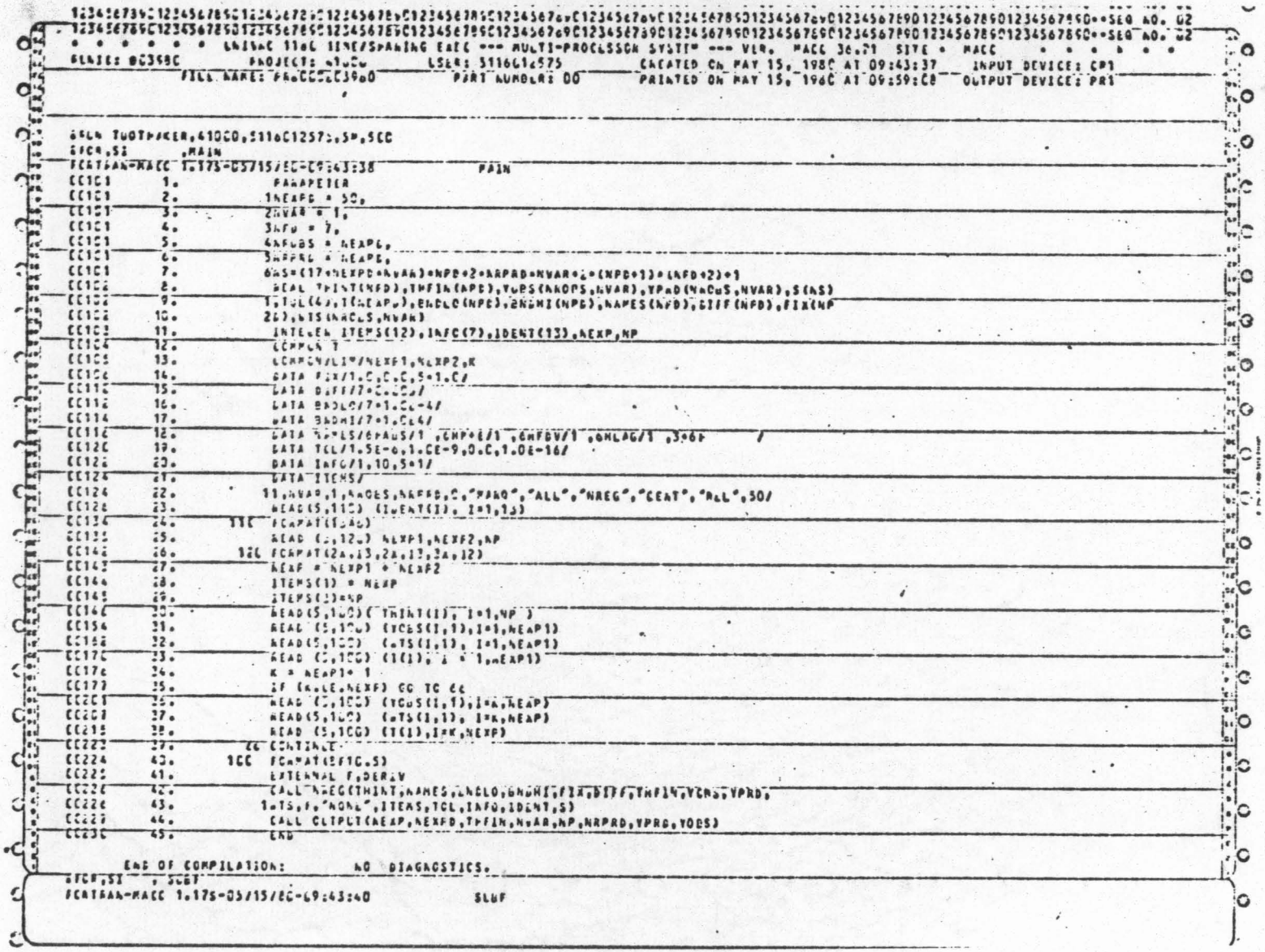

3000
1000
300
100
30
10
3
HYDROCORTISONE CONC. (ng/ml)
TIME (h)
0
2
4
6
8
10

Figure 2



APPENDIX 9

NREG Program and Sample Printout for Study 2



```
1234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890--SEQ NO. 02
1234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890--SEQ NO. 02
* * * * * * LAISAC 1106 LINE/SPARING EXEC *** MULTI-PROCESSOR SYSTEM *** VER.  MACC 36.21  SITE *  MACC     * * * * * *
$LABEL: 0C369C        PROJECT: ANSCW        USER: 5116G14575        CREATED ON MAY 15, 1980 AT 09:43:37    INPUT DEVICE: CP1
              FILE NAME: PROGRAM39A0        PART NUMBER: 00        PRINTED ON MAY 15, 1980 AT 09:59:08    OUTPUT DEVICE: PR1


$LGO TWOTRACER,41000,5116C12575,SP,SCC
$FOR,SI    ,MAIN
FORTRAN-MACC 1.17S-05/15/80-09:43:38            MAIN
CC101     1.          PARAMETER
CC101     2.         1NEAPS = 50,
CC101     3.         2NVAR = 1,
CC101     4.         3NFD = 7,
CC101     5.         4NPOBS = NEAPS,
CC101     6.         5NPRD = NEAPS,
CC101     7.         6NS=(17+NEXPO+NVAR)+NPO+2+NPRD+NVAR+2+(NPO+1)+(NFD+2)+1
CC102     8.          REAL THINT(NFD),THFIN(NPE),YOBS(NNOPS,NVAR),YPRD(NNOBS,NVAR),S(NS)
CC102     9.         1,TOL(4),T(NEAPS),ENDLO(NPE),ENDHI(NPE),NAMES(NFD),DIFF(NFD),FIX(NF
CC102    10.         2D),WTS(NNOBS,NVAR)
CC103    11.          INTEGER ITEMS(12),INFO(7),IDENT(13),NEXP,NP
CC104    12.          COMMON T
CC105    13.          COMMON/LIM/NEXP1,NEXP2,K
CC106    14.          DATA FIX/1.0,0.0,5*1.0/
CC110    15.          DATA DIFF/7*0.0D0/
CC113    16.          DATA ENDLO/7*1.0E-4/
CC114    17.          DATA ENDHI/7*1.0E4/
CC116    18.          DATA NAMES/6HAUS/1 ,6HPOE/1 ,6HFBV/1 ,6HLAG/1 ,5*6H      /
CC120    19.          DATA TOL/1.5E-6,1.0E-9,0.0,1.0E-16/
CC121    20.          DATA INFO/1,10,5*1/
CC124    21.          DATA ITEMS/
CC124    22.         11,NVAR,1,NAGES,NEXPO,0,"PARO","ALL","NREG","GEAT","RAL",50/
CC126    23.          READ(5,110) (IDENT(I), I=1,13)
CC134    24.      110 FORMAT(13A6)
CC135    25.          READ (5,120) NEXP1,NEXP2,NP
CC142    26.      120 FORMAT(2A,13,2A,13,3A,12)
CC143    27.          NEAP = NEXP1 + NEXP2
CC144    28.          ITEMS(1) = NEXP
CC145    29.          ITEMS(3)=NP
CC146    30.          READ(5,100)( THINT(I), I=1,NP )
CC154    31.          READ (5,100) (YOBS(I,1),I=1,NEAP1)
CC162    32.          READ(5,100)  (YTS(I,1), I=1,NEAP1)
CC170    33.          READ (5,100) (T(I), I = 1,NEAP1)
CC176    34.          K = NEAP1+ 1
CC177    35.          IF (K.LE.NEXP) GO TO 66
CC201    36.          READ (5,100) (YOBS(I,1),I=K,NEAP)
CC207    37.          READ(5,100)  (YTS(I,1), I=K,NEAP)
CC215    38.          READ (5,100) (T(I),I=K,NEXP)
CC222    39.       66 CONTINUE
CC224    40.      100 FORMAT(8F10.5)
CC225    41.          EXTERNAL F,DERIV
CC226    42.          CALL NREG(THINT,NAMES,ENDLO,ENDHI,FIX,DIFF,THFIN,YOBS,YPRD,
CC226    43.         1.TS,F,"NONE",ITEMS,TOL,INFO,IDENT,S)
CC237    44.          CALL OUTPUT(NEAP,NEXPO,THFIN,NVAR,NP,NPRPD,YPRD,YOBS)
CC236    45.          END

      END OF COMPILATION:        NO  DIAGNOSTICS.
$FOR,SI    , SCET
FORTRAN-MACC 1.17S-05/15/80-09:43:40            SLNF
```

```
CC154     31.          J = I - 1
CC155     32.        2C Y(I) = Y(J) + DMAX
CC157     33.           IF ( MP.EQ.1 ) GO TO 160
CC161     34.           GO TO 170
CC162     35.        16C NEAR = NEXPT
CC163     36.           NEAR1 = NEXPB
CC164     37.           K = NEXPB
CC165     38.           GO TO 180
CC166     39.        17C CONTINUE
CC167     40.           NEAR1 = 1
CC170     41.           K = 1
CC171     42.           NEAR = NEXPB
CC172     43.        18C CONTINUE
CC173     44.           CALL PLTMPIN,YPAR,NEAR1,NEAR,NVAR,NP,NRPRO)
CC174     45.           NEAR1 = NEAR
CC175     46.           DO 2C I = 1,NEAR1
CC176     47.        2C YMAX = AMAX1(YMAX,YPAR(I,1))
CC202     48.           YSCLE = YMAX/5.E
CC203     49.           YSCLE = YMAX/5.0
CC204     50.           CALL GRAPHCT,6HLINEAR,YPAR,6HLINEAR,6HEXPO,15,5HBLANK,"TIMES$",7U
CC204     51.          1ACTION VALUES$",4HAXES,"PLOT OF OBSERVED,O""S AND PREDICTED,P""S
CC20A     52.          2VALUES$",12.0,1E.0)
CC205     53.           CALL GRAPHNET,"SCALE",NOBS,"SCALE",LU,14,5HBLANK)
CC206     54.           IF (NEXP2.EQ.0) GO TO 66
CC210     55.           IF(MP.EQ.1) GO TO 2CC
CC212     56.        6C CONTINUE
CC213     57.           RETURN
CC214     58.           END

      END OF COMPILATION:        NO  DIAGNOSTICS.
$PAP
PAP 1.4R1+E RLIGIS 05/15-09:42:45
AFCM STATUS OF OUTPUT ELEMENT=UNKNOWN

BANKNAME(LEN)        ADDRESS LIMITS
$BLANK(4)            CC1CCC 111657     36784 BLANK WORDS DECIMAL
$BLANK(5)            111CCC 141561     12658 BLANK WORDS DECIMAL
STARTING ADDRESS  111435


                SEGMENT NAME          CC1CCC 111657     111CCC 141561

BRITBI/U.CCC1           $(1)    CC1CCC CC1165
PLCC4C/GSPV3E           $(1)    CC1166 CC1463        $(C)   111CCC 111C32
BRITIS/FC610            $(1)    CC1464 CC2527        $(C)   111C34 111251
BRIMC/MAGNLIBREGC4      $(1)    CC2530 CC3273        $(C)   111252 111774
BRIMEC/MACALIBREGDE     $(1)    CC3274 CC3562        $(C)   111775 112C45
                                                     $(2)   BLANK$COMMON
BRIS.P/MAGBLIBREGDE     $(1)    CC3563 CC4206        $(C)   112C46 112113
                                                     $(2)   BLANK$COMMON
BERRES/MACFORFUN12      $(1)    CC42C7 CC4436        $(2)   112114 112173
SYMBLL/GSPV3S           $(1)    CC4437 CC4625        $(C)   112174 112213
                                                     $(2)   GSPCTL
PCORE/GSPV54            $(1)    CC4626 CC465C
```

```
MADISON ACADEMIC COMPUTING CENTER - SUBROUTINE AREG                    09/13/80    TRANSMITTAL NO.  803960    CALL  1    PAGE  1
CRE CONPT GRAL HYDROCORTISONE SUBJECT 1 DOSE IC ASSAY FLOOR CORR

                                        S U M M A R Y   O F   T H E   D A T A

                                                    ITEMS

ITEMS(1): NEXP  =    10  NUMBER OF EXPERIMENTS          ITEMS(7): METHOD = "MARQ"   MARQUARDT'S METHOD
ITEMS(2): NVAR  =     1  NUMBER OF VARIABLES            ITEMS(8): WTS    = "ALL"    WEIGHTING BY OBSERVATION
ITEMS(3): NP    =     4  NUMBER OF PARAMETERS           ITEMS(9): ADERIV = "AREG"   AREG CALCULATED DERIVATIVES
                                                       ITEMS(10): KSYM  = "CENT"   CENTRAL DIFFERENCES
ITEMS(4): NAGES =    10  ROW DIMENSION OF ARRAY YOBS    ITEMS(11): KDIFF = "REL"    RELATIVE DIFFERENCES
ITEMS(5): NAPRD =    10  ROW DIMENSION OF ARRAY YPRD
ITEMS(6): NWTS  =    10  ROW DIMENSION OF ARRAY WTS     ITEMS(12): MAXIT =    50    LIMIT ON NUMBER OF ITERATIONS

                    TOLERANCES                                          SUNDRIES

TOL(1) = 1.5000000-06  REL. CHANGE IN A PARAMETER          NUMBER OF ACTIVE PARAMETERS =  3
TOL(2) = 1.0000000-06  REL. CHANGE IN SUM OF SQUARES       NUMBER OF CELLS OF SCRATCH REQUIRED =  189
TOL(3) = 0.0000000     RATIO TO INITIAL SUM OF SQUARES
TOL(4) = 1.0000000-10  PIVOT TOLERANCE

                                                  PARAMETERS

  NO.      NAME          INITIAL VALUE      FRACTION FOR          LOWER BOUND          UPPER BOUND
                                            DERIV. ESTIMATE

    1      ABS/1         1.0900000+00       5.0000000-03         1.0000000-04         1.0000000+04
F   2      MSE/1         7.7956599-01       5.0000000-03         1.0000000-04         1.0000000+04
    3      FSV/1         3.4230000+02       5.0000000-03         1.0000000-04         1.0000000+04
    4      LAG/1         1.0000000-01       5.0000000-03         1.0000000-04         1.0000000+04

                        S I N G U L A R   V A L U E S   A N D   V E C T O R S   A T   I N I T I A L   P O I N T

SING. VALUES              3.4164837+02        9.4395563+01        5.6837218-01

PAR.    1  ABS/1          -.2455635           .9690082            -.0056645
      F 2  MSE/1            ***                 ***                 ***
        3  FSV/1          -.0000099           .0056901             .9999836
        4  LAG/1           .9690246           .2465629            -.0000142

SETUP TIME =      .044  SECONDS
```

CHE COMPT GRAL HYDROCONTISCAE SUBJECT 1 BOSE 10 ASSAY FLUOR CORR

THE ITERATIONS

ITERATION NO. 1	BASE POINT		TEST POINT
SUP OF SQUARES	5.3676616+03		4.4225576+03
L	0		0
LAMBDA	0.0000000		0.0000000
GAMMA	1.0000000+00		1.0000000+00
ANGLE IN DEGREES	.000		89.2228
PAR. PIVOT REDUCTION	1.0000000+00		4.9324549-01
PAR. 1 ABS/1	1.0000000+00		1.6564561+00
2 P+E/1	7.7999999-01	F	7.7999999-01
3 REV/1	3.4600000+04	S	3.0456251+02
4 LAG/1	1.0000000-01		2.2329096-01

CUMULATIVE NO. OF FUNCTION CALLS = 2 ITERATION TIME = .024 SECONDS CUMULATIVE TIME = .067 SECONDS

THE LAST ITERATION

ITERATION NO. 8	BASE POINT		TEST POINT
SUP OF SQUARES	3.6213952+03		3.6213954+03
L	4		8
LAMBDA	8.2032746+04		2.9339350+03
GAMMA	1.0000000+00		1.0000000+00
ANGLE IN DEGREES	82.0000		45.7035
PAR. PIVOT REDUCTION	8.9852957-01		8.3043730-01
PAR. 1 ABS/1	1.0462126+00		1.0462154+00
2 P+E/1	7.7999999-01	F	7.7999999-01
3 REV/1	3.1576970+02		3.1576970+02
4 LAG/1	1.9768964-01	S	1.9768976-01

CUMULATIVE NO. OF FUNCTION CALLS = 16 ITERATION TIME = .036 SECONDS CUMULATIVE TIME = .176 SECONDS

ITERATION TERMINATES:
PAR. RELATIVE CHANGE IN A PARAMETER .LT. TOL(1) = 1.5000000-06

CAL COMPT GRAL HYDROCLASTICAE SUBJECT 1 0056 10 ASSAY FLOR CORR

FINAL PARAMETER VALUES

FINAL VALUE SUM OF SQUARES = 3.6213553+03

PR.	1	ABS/1	1.849.126+00
P	2	P*E/1	7.7595559-01
	3	FBV/1	3.157E270+02
	4	LAG/1	1.976c764-01

ITERATION SUMMARY

ITER NO.	SUM OF SQUARES	L	LAMBDA	GAMMA	ANGLE	NO. PIVOT FAILURES	NO. BOUNDS FAILURES	MAX. PIVOT REDUCTION	TIME IN SECONDS
C	5.367661E+03	C	0.0000000	1.0000000+00	-.0040	C	0	1.0000000+00	.000
1	4.442c578+03	0	0.0000000	1.0000000+00	85.2248	0	0	4.9324549-01	.024
2	3.6213135+03	C	0.0000000	1.0000000+00	89.6435	0	0	6.3307910-01	.025
3	3.6214053+03	C	0.0000000	1.0000000+00	88..516	0	0	6.4412456-01	.012
4	3.6213857+03	C	0.0000000	1.0000000+00	89.6019	0	0	6.4892478-01	.040
5	3.6213955+03	C	0.0000000	1.0000000+00	88.2866	0	0	6.5017870-01	.019
6	3.6213954+03	0	0.0000000	1.0000000+00	89.0944	0	0	6.4896579-01	.002
7	3.6213953+03	4	8.2c521e6+00	1.0000000+00	62.0960	C	0	6.9252957-01	.013
E	3.6213956+03	8	2.0035350+03	1.0000000+00	45.7035	C	0	8.3043730-01	.038

SINGULAR VALUES AND VECTORS AT TERMINAL POINT

SING. VALUES 5.729.198+02 5.2447651+01 7.3502114-01

PAR.	1	ABS/1	-.0615266	.9980309	-.0099745
P	2	P*E/1	***	***	***
	3	FBV/1	-.0002270	.0065776	.9999562
	4	LAG/1	.9981807	.0619258	-.0003913

NORMALIZING ELEMENTS AND CORRELATION MATRIX

PARAMETER NO.			1	2	3	4
NORM. ELTS.			2.3376619-02	***	1.3604333+00	2.1706076-03
PAR.	1	ABS/1	1.0000000			
F	2	P*E/1	***	***		
	3	FBV/1	-.5c0.232	***	1.0000000	
	4	LAG/1	.5215223	***	-.2451641	1.0000000

CONFIDENCE LIMITS ON LINEAR HYPOTHESIS

			LOWER LIMIT	FINAL VALUE	UPPER LIMIT
PAR.	1	ABS/1	7.8c0c220-01	1.8492120+00	2.9123607+00
F	2	P*E/1	***	7.7999999-01	***
	3	FBV/1	2.5298199+02	3.1576670+02	3.7765541+02
	4	LAG/1	9.8636128-02	1.6768964-01	2.9044015-01

EXPLORATION

			LOWER TEST	SUM OF SQUARES	LINEAR EST. OF SUM OF SQUARES	UPPER TEST	SUM OF SQUARES	LINEAR EST. OF SUM OF SQUARES
PAR.	1	ABS/1	1.5834.50+00	3.9411265+03	3.6038757+03	2.1150002+00	3.8533667+03	3.8149526+03
F	2	P*E/1	7.7999569-01	***	***	7.7999999-01	***	***
	3	FBV/1	3.0029702+02	3.8203070+03	3.8162564+03	3.3124039+02	3.8203055+03	3.8162564+03
	4	LAG/1	1.7300201-01	3.8077142+03	3.6214017+03	2.2237727-01	3.8346157+03	3.6214017+03

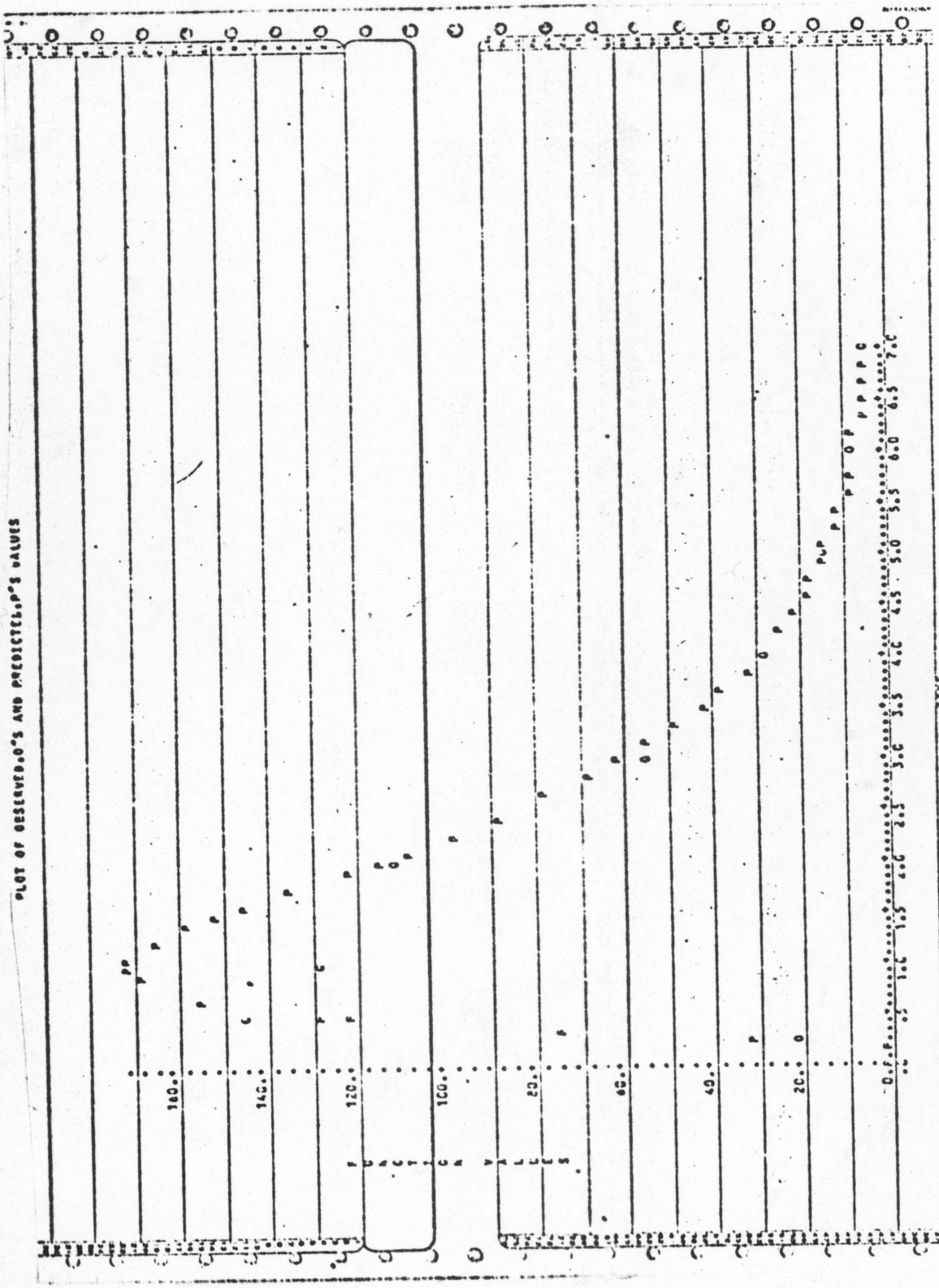
F I N A L F U N C T I O N V A L U E S A N D R E S I D U A L S

WEIGHTED LEAST MEAN SQUARE RESIDUAL = 2.2745137+01 NO. DEGREES OF FREEDOM = 7
THIS IS THE SCALE UNIT IN THE GRAPH OF THE WEIGHTED RESIDUALS.

EXPT. NO.	VAR. NO.	PREDICTION	OBSERVATION	RESIDUAL	WEIGHTED RES.	GRAPH OF WEIGHTED RESIDUAL (-2 -1 0 +1 +2)
1	1	2.8515[illegible]+01	[illegible]	-8.5190381+00	-5.5190381+00	
2	1	1.1915[illegible]+02	1.44[illegible]+02	2.4848[illegible]+01	2.4848[illegible]+01	
3	1	1.6[illegible]+02	1.27[illegible]+02	-4.1218559+01	-4.1218[illegible]+01	
4	1	1.4[illegible]+02	1.[illegible]+02	3.4378[illegible]+01	3.4378[illegible]+01	
5	1	1.1[illegible]+02	1.[illegible]+02	-5.4018[illegible]+00	-5.4018[illegible]+00	
6	1	5.[illegible]+01	[illegible]	-4.3103762+00	-4.3103762+00	
7	1	2.7[illegible]+01	2.7[illegible]+01	-6.5[illegible]-01	-6.5[illegible]-01	
8	1	1.2[illegible]+01	1.2[illegible]+01	-8.2169926-01	-8.2169926-01	
9	1	5.[illegible]+00	7.[illegible]+00	1.[illegible]+00	1.[illegible]+00	
10	1	2.7[illegible]+00	2.[illegible]+00	-7.[illegible]-01	-7.[illegible]-01	

TIME SINCE END OF THE LAST ITERATION = .113 SECONDS TOTAL TIME = .289 SECONDS

R-SQUARED FOR FUNCTION = .[illegible]
COR FOR FUNCTION = .[illegible]


PLOT OF OBSERVED,O'S AND PREDICTED,P'S VALUES
TIME

Appendix 10 - A Method to Obtain Separate Estimates of First Pass Effect (f) and Fraction of Dose Absorbed (F) From Overall Bioavailability (ω).

From Gibaldi (81):

fraction of dose, f, surviving the first-pass effect assuming fraction of dose absorbed, F, is 1, is either

$$f = \frac{Q}{Q + \frac{D_o}{A_o}} \qquad \text{or} \qquad f = 1 - \frac{D_I}{A_I Q}$$

where Q is blood or plasma flow rate through the liver, D is the oral (O) or intravenous (I) dose, and A is oral (O) or intravenous (I) area under the curve.

Let $\omega = \frac{A_o}{A_I}$ represent overall bioavailability.

An estimate of F can be obtained as follows. By Gibaldi's three-compartment model,

$$\omega = \frac{A_o}{A_I} = \frac{\frac{FDk_{21}}{V_1 k_{12} k_2}}{\frac{D(k_{21} + k_2)}{V_1 k_{12} k_2}} = \frac{Fk_{21}}{k_{21} + k_2}$$

Multiply by $\frac{V_2}{V_2}$: $\omega = \frac{FV_2 k_{21}}{V_2 k_{21} + V_2 k_2}$

and assume $k_{21}V_2 = k_{12}V_1 \cong Q$ for liver blood flow rate-limited transport,

$$\omega = \frac{FQ}{Q + V_2 k_2} \qquad \text{But,} \quad A_o = \frac{FD_o}{V_2 k_2}$$

↑ EQUATION A ↑

Therefore,

$$\omega = \frac{FQ}{Q + \frac{FD_o}{A_o}} \qquad \underline{\text{Equation B}}$$

This is essentially Gibaldi's Equation 9.

To obtain ω in terms of intravenous data, rewrite Equation A as:

$$\omega = \frac{FQ + FV_2k_2 - FV_2k_2}{Q + V_2k_2} = \frac{F(Q + V_2k_2 - V_2k_2)}{Q + V_2k_2}$$

or

$$\omega = F\left\{1 - \frac{V_2k_2}{Q + V_2k_2}\right\} \qquad \underline{\text{Equation C}}$$

From the model, $$A_I = \frac{D_I(k_{21} + k_2)}{V_1k_{12}k_2}$$

Multiply by $\frac{V_2}{V_2}$: $$A_I = \frac{D_I(V_2k_{21} + V_2k_2)}{V_2k_2V_1k_{12}}$$

Since $V_1k_{12} = V_2k_{21} \cong Q$ $$A_I = \frac{D_I(Q + V_2k_2)}{QV_2k_2}$$

Thus, from Equation C,

$$\omega = F\left\{1 - \frac{D_I}{A_IQ}\right\} \qquad \underline{\text{Equation D}}$$

This is similar to Gibaldi's Equation 11, but now includes the F term.

Now set the two expressions for ω equal to each other (Eqn. B for oral administration and Eqn. D for i.v. administration), and solve for F,

$$\frac{FQ}{Q + \frac{FD_o}{A_o}} = F\left\{1 - \frac{D_I}{A_I Q}\right\}$$

$$\frac{A_o Q}{A_o Q + FD_o} = \frac{A_i Q - D_I}{A_I Q}$$

$$A_o A_I Q^2 = (A_o Q + FD_o)(A_I Q - D_I)$$

$$F(D_o A_I Q - D_o D_I) = A_o Q D_I$$

$$F = \frac{A_o Q D_I}{D_o(A_I Q - D_I)} \qquad \underline{\text{Equation E}}$$

Thus, knowledge of oral and intravenous doses and areas under the curve for a drug allows the calculation of ω as $(A_o D_I)/(A_I D_o)$. The fraction of drug absorbed, F, can then be obtained using Equation E. Fraction of the drug surviving the first pass through the liver, f, can then be calculated from $\omega = fF$ using the values obtained for F and ω.