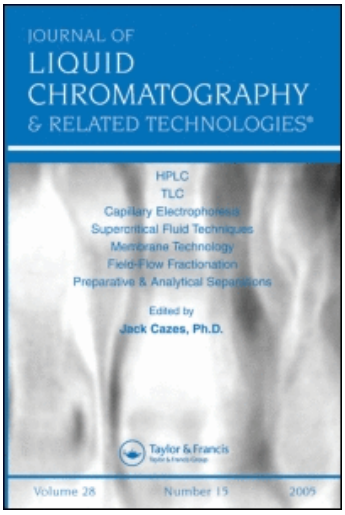

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An Application of a Statistical Approach to the Calibration and Validation of Analytical Assays for Investigations Drugs: Quantitation of CGP19984D, A Thiazolidinedione Derivative with Antitumor Activity, and its Two Diastereomers in Biological Fluids by High Performance Liquid Chromatography

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AN APPLICATION OF A STATISTICAL APPROACH TO THE CALIBRATION AND VALIDATION OF ANALYTICAL ASSAYS FOR INVESTIGATIONAL DRUGS: QUANTITATION OF CGP19984D, A THIAZOLIDINEDIONE DERIVATIVE WITH ANTITUMOR ACTIVITY, AND ITS TWO DIASTEREOMERS IN BIOLOGICAL FLUIDS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A set of statistical procedures has been developed for: 1) the construction of linear standard curves for cases in which the sensitivity of the assay spans a 1000-fold range; 2) the estimation of unknown compound concentrations with associated fiducial limits; 3) the validation of the assay; and 4) the generation of appropriate quality control charts. This statistical methodology was applied to a new investigational drug, CGP19984D. CGP19984D is a thiazolidinedione derivative that suppresses gonadal function and inhibits tumor growth in both hormone-dependent and hormone-independent mammary and prostatic adenocarcinoma in the rat. This compound has

undergone extensive pharmacologic and toxicologic evaluation in preparation for initial clinical trial in man. This paper describes the analytical procedure that has been developed to quantitate the two diastereomers of CGP19984D in plasma and urine and the statistical evaluation of this procedure.

INTRODUCTION

In the development and validation of analytical assays for new investigational agents, several complications routinely arise: (1) when the sensitivity of the anaytical assay spans a 1000-fold range, a proper construction of linear standard curves for data with nonhomogeneous variance is required; (2) estimates of unknown drug concentrations in biological fluids must be computed along with a good measure of the uncertainty of the estimate; (3) both the analytical and calibration procedures for the assay must be validated; (4) since the stability of a new drug in seeded control samples is unknown, such samples may not be useful for quality control purposes, and alternate quality control procedures must be devised. This paper describes a general approach to the solution of these problems and the application of this approach to the calibration and validation of an analytical assay for a new chemotherapeutic agent, CGP19984D.

CGP19984D (Figure 1) is a thiazolidiendione derivative (phosphoric acid, monomethyl mono [3-methyl-2-[[5-methyl-3-(2-methyl-2-propenyl) thiazolidinylidine]hydrazono]-4-oxo-5-thiazolidinyl]ester, compound with 2-aminoethanol) that has been shown to have antitumor activity against hormone-independent tumors (Walker 256, Colon 26, R3230 AC mammary tumor, MTW-9B mammary tumor) and hormone-dependent tumors (DMBA induced rat mammary tumor, androgen-dependent R3327 prostate tumor) [1,2]. Studies performed in the androgen dependent Dunning 3327 rat prostate

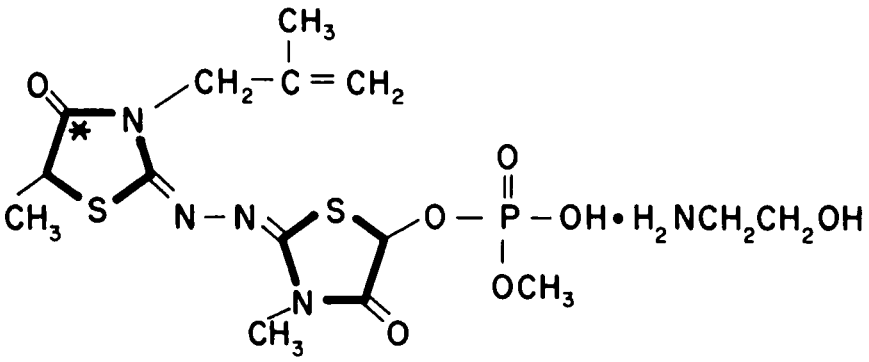

Figure 1 Structure of CGP19984D. The "*" corresponds to the position of ^{14}C in the labeled compound used for animal studies.

adenocarcinoma demonstrate that concomitant with tumor inhibition, a significant decrease in circulating luteinizing hormone (LH) and testosterone is observed with no change in serum prolactin or corticosterone levels. These data suggest that the antitumor effects of drug treatment resulted primarily from the inhibition of LH release and subsequent decline in testosterone synthesis [2]. Mechanistic studies performed in Sprague Dawley rats suggest that CGP19984D inhibits LH secretion through a hypothalamic mechanism [3]; however, it is not yet clear whether the underlying lesion is in the secretion of luteinizing hormone releasing factor (LHRH) or in its interaction with receptors in the pituitary gland, or both. Further studies in the estrogen-independent MTW-9B rat mammary tumor demonstrate that drug treatment reduces the growth of the tumor, tumor progesterone receptors, and uterine weight [2]. The ability of CGP19984D to suppress gonadal function and to inhibit tumor growth implies that this drug may be useful in the treatment of both hormone-dependent and hormone-indendent prostate and breast carcinoma.

In preparation for the initial clinical trial in man, CGP19984D has undergone extensive toxicological and pharmacological investigations. The purpose of this paper is to describe an analytical procedure that has been developed to quantitate the two diastereomers of CGP19984D in plasma and urine and the statistical evaluation of the reliability of the assay procedure.

MATERIALS AND METHODS

CGP19984D

CGP19984D was supplied by the Pharma Research Division of CIBA-GEIGY Ltd. (Basle, Switzerland).

Mobile Phase

All aqueous solutions were made with HPLC-grade water prepared with a Milli-Q Water System (Millipore Company, Bedford, MA). Tetrabutyl ammonium phosphate was purchased from Waters Chromatography Division (Milford, MA). Methanol (HPLC grade) was purchased from J.T. Baker (Phillipsburg, NJ).

Sample Preparation

Stock solutions of CGP19984D in distilled water were prepared at a concentration of 5 mg/ml; the actual concentration was confirmed by measuring the uv absorbance of an aliquot of a solution at 287 nm. Standards of CGP19984D in plasma were prepared by adding 20 µl of the stock solution to 980 µl of plasma to form a solution of concentration 100 µg/ml; this solution was then serially diluted with plasma to obtain solutions of concentration 10, 1, 0.25 and 0.1 µg/ml. Standard solutions of CGP19984D in urine were prepared similarly, except that the lowest standard concentration was 0.5 µg/ml.

Five hundred µl aliquots of the standard solutions were transferred to 50 ml plastic conical tubes (Corning, Wexford, PA) to which 2 ml of acetonitrile (Burdick and Jackson HPLC grade) were added. After vortexing, the tubes were centrifuged for 30 minutes at 2000 g at room temperature. The supernatants were carefully decanted into scintillation vials (VWR, Rochester, NY), evaporated under dry N_2 for 60 minutes and lyophilized for 2 hours. Five hundred µl of mobile phase was added to each vial, the solution was filtered through a 0.23 µ GS Millipore filter (Millipore Co., Bedford, MA) and injected into the HPLC system.

Analytical Procedure for the Separation of Diastereomers

The HPLC instrument was manufactured by Waters Chromatography Division (Milford, MA) and consisted of a model 710 WISP, a model 590 Solvent delivery system and a model 481 Variable wavelength u.v. detector. The chromatography data system consisted of an HP9816 microcomputer interfaced to the HPLC via a Nelson Analytical Model 962SB interface box and running Nelson Analytical Software (XTRACHROM, revision 7.02). The HPLC conditions used were: column-IBM C18 (5µ d_p; 4.6 mm x 50 mm); mobile phase-58% 0.005M Tetrabutylammonium phosphate in water: 42% methanol; flow rate 1 ml/min; injection volume 10 - 20 µl; detector wavelength: 287 nm and data rate - 2 points/second.

Procedure for Preparing Pure Diastereomers

The HPLC instrument was manufactured by Waters Chromatography Division and consisted of a Model 590 Solvent delivery system with extended flow heads, a PREP-PAK 500 radial compression module containing a 5.7 x 30 cm radial compression cartridge packed with VYDAC C18 (15-20 µ), and a 441 UV absorbance

detector. The chromatograph data system was the same as that described above. The HPLC conditions used were mobile phase - 60% 0.005M ammonium formate in water: 40% methanol; flow rate - 45 ml/min; detector wavelength - 254 nm. Heart cuts of each eluting peak were collected and lyophilized.

The lyophilized powders were then converted to the free base with 1N HCl; the free base was extracted sequentially with methylene chloride:propanol (3:1) and methylene chloride. The organic phases were combined, dried with $MgSO_4$, filtered and evaporated to dryness with a stream of N_2 during which the material crystallized. The white solid was further dried under vacuum; the resulting material showed a single peak in the analytical HPLC procedure at a retention time that corresponded to that of the expected diastereomer.

Routine Construction of Standard Curves and Estimation of the Concentration of CGP19984D in Plasma and Urine

Guidelines and mathematical formulas for the routine construction of standard curves, for the estimation of the concentrations of CGP19984D in plasma and urine, and for the quantitation of the uncertainty in the estimation were adpated from Draper [4] and Sharaf [5]. Standard concentrations of CGP19984D in plasma and urine were prepared in duplicate and analyzed as described above. From the area measurements, three different standard curves are routinely constructed: one for each diastereomer and one for the mixture. The standard concentration of isomer 1 is calculated as 44% of the mixture concentration, and the concentration of isomer 2 is calculated as 56% of the mixture concentration. These calculations are justified in the Results section.

Since both the sample concentration and peak areas range over several orders of magnitude (e.g., the isomer mixture in plasma, Figure 4), and since the standard deviation of peak

areas for each concentration was found to be roughly proportional to its mean, the areas and the concentrations for all six standard curves are logarithmically transformed (base 10 logarithm) when the standard curves are constructed. Since the peak area should be proportional to sample concentration [area = (K)(concentration); where K is a proportionality factor], the logarithmic transformation results in an easily interpretable relationship:

$$[\log_{10}(\text{area}) = \log_{10}(K) + \log_{10}(\text{concentration})]$$

The y-intercept of the transformed standard curve is equal to the logarithm (base 10) of the proportionality factor, and the slope of the transformed curve should be equal to 1. The standard curve is fitted with weighted linear regression; the weights are the same for each particular type of standard curve from day to day. The calculation of appropriate weights, which are normalized to sum to the number of data points, is a side product of the assay validation procedure and is explained in the Results section.

The drug concentration of unknown samples is calculated as:

$$X_0 = \text{antilog}_{10}[(Y-b_0)/b_1]$$

where X_0 is the point estimate of the drug concentration, Y is the mean of the $\log_{10}$ of the measured peak areas for replicates of a sample, b_0 is the y-intercept of the standard curve, and b_1 is the slope of the standard curve. The 95% fiducial (confidence) limits for these estimates are calculated by taking the antilog_{10} of the upper and lower confidence limits computed with equation 1.7.6 from Draper and Smith [4] as adapted for means of individual observations (page 50). A typical example of a set of confidence envelopes for individual observations in singlet is provided in Figure 4 (isomer mixture in plasma). The appropriate statistics for use in Equation 1.7.6 are taken from the weighted linear regression of the standard curve.

A computer program was written in HP Basic to run on the HP9816 microcomputer to quantitate the amount of total CGP19984D, diastereomer 1, and diastereomer 2 in plasma and urine from animals treated with CGP19984D. The area counts for the standards and the unknowns, each of which is determined in duplicate, are entered into a formatted video screen. The program uses the formulas described above for the fitting of the standard curve, for the estimation of unknown drug concentrations and for the calculation of the 95% confidence limits. The printout includes summary tables and the graph (Figure 4) of the standard curve.

The entire assay procedure, including sample preparation, analytical procedures, standard curve construction, the estimation of unknown drug concentrations, and the use of the computer program was rigorously validated as described in the Results section.

RESULTS

Analytical Procedure

CGP19984D contains two assymetric centers; the material supplied for development to clinical trial consisted of a mixture of the diastereomers. Since it is possible that the diastereomers may be metabolized at different rates, it was the goal from the outset to develop a procedure that would allow quantitation of the diastereomers independently. Figures 2 and 3 show an HPLC tracing of standard samples of CGP19984D in plasma and urine that have been extracted and analyzed as described in the Materials and Methods; the drug is separated from the other components of plasma and urine and the diastereomers are resolved. In order to use the procedure to quantitate the individual diastereomers, it was necessary to demonstrate that they have the same extinction coefficients.

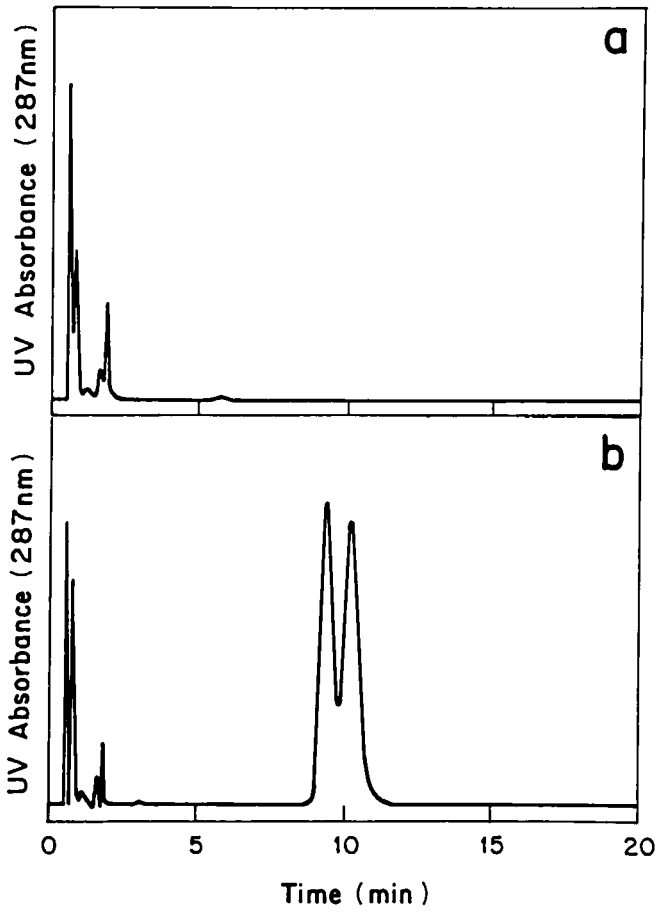

Figure 2 Chromatogram of plasma blank (a) and plasma spiked with CGP19984D (1 μg/ml) (b).

Therefore, the individual diastereomers were isolated by the procedure described in the Materials and Methods and a UV spectrum was obtained on a 20 mg/L solution of each in 0.1 N NaOH. The spectra were superimposable with a λmax of 285 nm; the ε were 14,800 ($M^{-1}cm^{-1}$) (first diastereomer) and 14,700 ($M^{-1}cm^{-1}$) (second diastereomer).

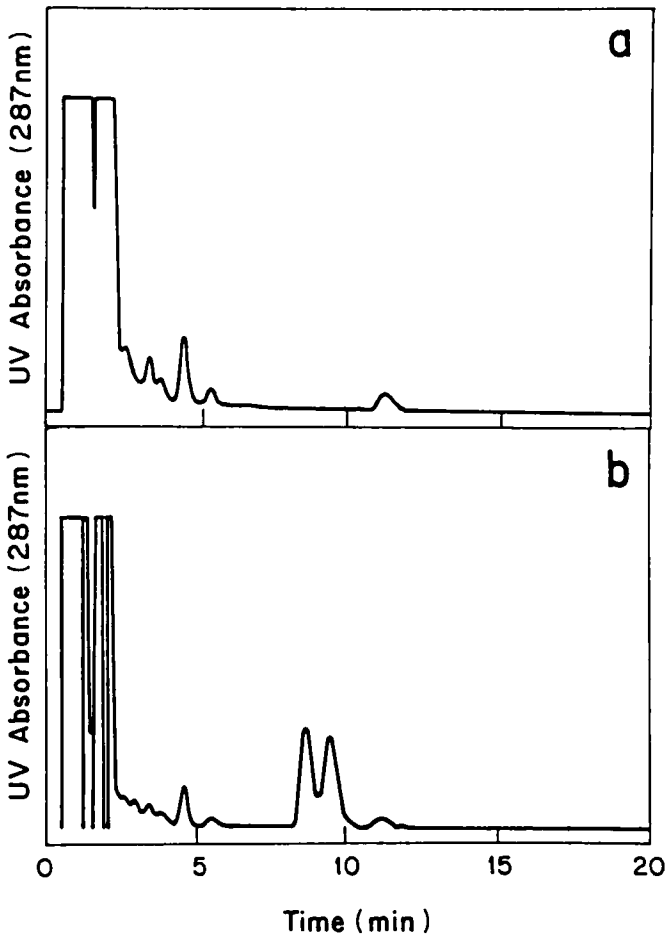

Figure 3 Chromatogram of urine blank (a) and urine spiked with CGP19984D (1 μg/ml) (b).

Validation of the CGP19984D Assay for Plasma Samples

In order to determine the reliability of the HPLC system, a sample of CGP19984D in mobile phase (10 μg/ml) was prepared on three different days and analyzed 10 times each day with the HPLC procedure. The relative standard deviations (=100 X S.D./mean) for the measured peak areas for the three days were

2.24, 1.4 and 1.5. Therefore, there is little variability in the instrumental procedure.

In order to test the reliability of the entire procedure for quantitating CGP19984D in plasma, 5 standard concentrations of CGP19984D in plasma were prepared on 3 different days in triplicate from the freshly prepared stock solution and extracted. The lyophilized samples from each day were assigned random positions in the autosampler and analyzed by the HPLC procedure described above. In Table 1, the raw data are

TABLE 1

Calibration Standards for Mixture of Diastereomers in Plasma

Concentration (μg/ml)	0.1	0.25	1.0	10.0	100
Day 1	2601[a]	7340	28,831	283,964	2,793,174
	2843	8298	29,272	271,567	2,854,346
	2384	7217	26,305	276,710	2,798,204
Day 2	2535	6241	26,357	280,502	2,745,850
	3515	7200	26,023	290,554	2,703,568
	2582	8184	32,150	284,540	2,779,841
Day 3	3870	8732	27,839	262,034	2,667,801
	2650	8126	26,966	277,133	3,268,148
	2984	7500	28,767	268,566	2,844,877
Mean	2885	7649	28,057	277,285	2,828,421
Standard Deviation	497	758	1,958	8,841	175,721
Relative S.D., %	17.2%	9.91%	6.98%	3.19%	6.21%

[a]Entries in the upper part of the table are measured peak areas. Summary statistics are included in the lower part of the table.

arranged according to the sample concentration and day for the mixture of diastereomers. Summary statistics for the peak areas of each standard concentration are listed on the lower part of the table: mean, standard deviation, and relative standard deviations.

For the data in Table 1, a one way analysis of variance (ANOVA) of log (AREA) by DAY was then performed for each concentration and the resulting error mean squares were used as measures of the variance of the data at each concentration. Weights for each data point, which are to be included in future routine use of the assay as described in the Experimental section, were estimated by calculating the reciprocal of each variance estimate. A weighted linear regression was performed of Log (AREA) versus Log (CONCENTRATION) and DAY for all 45 data points. A plot of the weighted residuals was visually examined and the weights were adjusted empirically to result in a uniform variance of the weighted residuals across all concentrations. A weighted regression was rerun to check the effects of the new weights. Finally, the weights were normalized such that the sum of the weights equaled the number of data points. For the 5 standard concentrations, from smallest to largest, the final weights were 0.385, 0.769, 1.28, 1.28, 1.28 for isomer 1; 0.328, 0.574, 0.820, 1.64, 1.64 for isomer 2; and 0.328, 0.656, 0.938, 1.56, 1.56 for the mixture.

The relationship of log (AREA) to log (CONCENTRATION) was tested for curvature by regressing log (AREA) vs. log (CONCENTRATION), $\log^2$ (CONCENTRATION), and Day for all 45 data points. By a partial F-test [4] with a type I error rate of 0.05, it was determined that the $\log^2$ (CONCENTRATION) term was not a significant explanatory variable. Thus, there is no evidence to suggest curvature in the standard curve and one can conclude that:

$$\log (\text{AREA}) = b_0 + b_1 \log (\text{CONCENTRATION})$$

is an appropriate model for this data.

The data from Table 1 was also used to determine how well the assay procedure was able to predict the known standard concentration of subsets of the data. For each day, the data from Table 1 was divided into 3 groups: the first line of entries (one measured peak area for each standard concentration), was designated as group 1, the second line was designed as group 2 and the third line as group 3. For each day separately, the data from groups 1 and 2 (10 points), 2 and 3, and 1 and 3 were used to construct standard curves (9 total curves) (Figure 4). For each of these curves, the 5 data points not used in its construction were used to simulate unknown samples to compare the computed (back-calculated) vs. known standard concentrations (Table 2). There is good agreement between the actual and predicted concentrations; only 4 of 45 samples (8.89%) had 95% confidence limits that did not include the true concentration. The confidence limits tended to be quite narrow. For example, the 95% limits for the back-calculated concentrations in the first row of Table 2 were respectively: 0.0764 to 0.1097, 0.2182 to 0.3076, 0.8679 to 1.20, 8.64 to 11.9 and 84.7 to 119. It should be noted that in order to construct Table 2, each data point in Table 1 is used 3 times, twice for a standard curve, and once as an unknown sample. Therefore, the 45 back-calculated concentrations in Table 2 are not truly independent; the analyses described in the next paragraph should be regarded as only approximate and empirical, but adequate for describing the accuracy and precision of the assay.

For each of the five standard concentrations, a oneway ANOVA was performed of back-calculated concentrations by day; since P was greater than 0.05, there was no significant effect of day. Table 2 includes summary statistics of the back-calculated concentrations for each of the standard concentrations: mean, bias (mean - true standard concentration), % bias (bias X 100/true standard concentration), standard deviation and relative

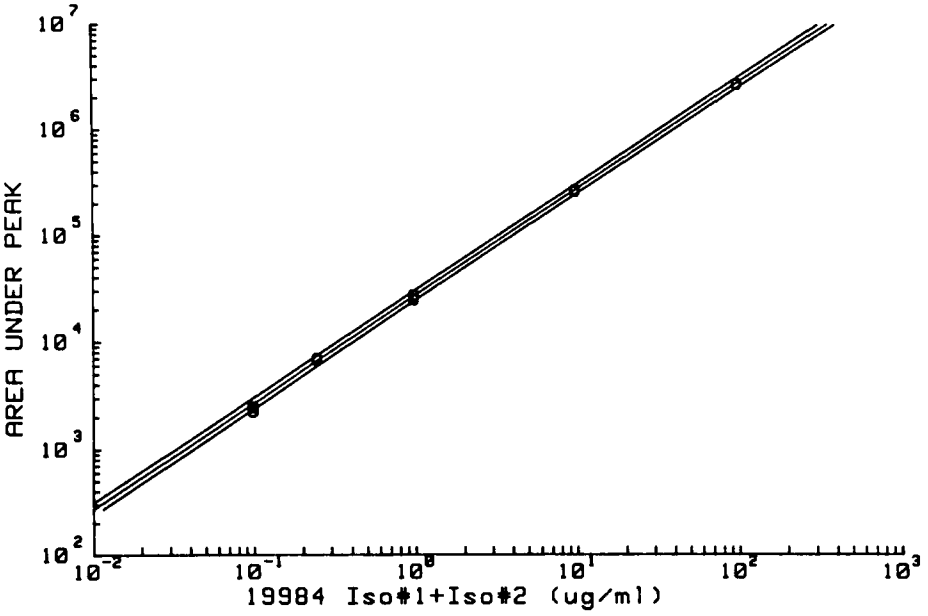

Figure 4 Typical standard curve for CGP19984D in plasma. The best fit line and confidence envelopes for the standard curves for the mixture of isomers was determined as described in the text. The 95% confidence envelopes were constructed for the case of an unknown sample in singlet. Data includes 5 standard concentrations in duplicate.

standard deviation. The 95% confidence intervals for the mean back-calculated concentrations encompass the true standard concentration for all five standards. Although the magnitude of the bias tends to increase from 0.1 to 100 μg/ml, there is no obvious pattern to the % bias. The standard deviations for the back-calculated concentrations tend to increase with increasing standard concentration. The relative standard deviation is highest for the 0.1 μg/ml standard but follows no obvious pattern from 0.25 to 100 μg/ml.

TABLE 2

Back-Calculated Concentrations of Standards for Mixture of Diastereomers in Plasma

	Standard Concentrations (μg/ml)				
	0.1	0.25	1.0	10.0	100.0
Day 1	0.0917[a]	0.2594	1.022	10.13	100.2
	0.1036	0.3013	1.058	9.739	101.5
	0.0807	0.2466	0.9081	9.732	100.2
Day 2	0.0828	0.2068	0.8926	9.847	99.79
	0.1244	0.2551	0.9239	10.36	96.70
	0.0940	0.2977	1.168	10.33	100.8
Day 3	0.1365	0.3067	0.9723	9.053	91.14
	0.0843	0.2658	0.9090	9.906	124.3
	0.0985	0.2501	0.9742	9.329	101.5
Mean	0.0996	0.2655	0.9809	9.825	101.8
Bias	-0.0004	0.0155	-0.0191	0.175	1.80
% Bias	0.40%	6.20%	-1.91%	-1.75%	1.80%
Standard Deviation	0.0192	0.0320	0.0896	0.433	9.06
Relative S.D., %	19.3%	12.1%	9.13%	4.41%	8.90%

[a]Entries in the upper part of the table are back-calculated concentrations in units of μg/ml. Summary statistics are included in the lower part of the table.

Routine Quality Control

From the validation data and analysis, four quality control charts were started, one each for the slope, y-intercept, error mean square and g (where g is an additional precision statistic, defined in [4], that will tend to be large, indicating poor precision, when the magnitude of the slope is small and/or if the slope is poorly determined). For the slope and y-intercept, X control charts were started [6], where each slope and y-inter-

cept is individually recorded. For the error mean square and g, S control charts [6] were started. Means and standard deviations of these four statistics to be used in the quality control charts will be updated with new data from each day's routine run. Extreme values of either the slope or intercept (>3 standard deviations from the mean) or drifts over time in any of the 4 quality control charts will suggest that the analytical procedure be examined.

Validation of the CGP19984D Assay for Urine Unknown Samples

Four standard concentrations of the mixture of diastereomers in urine were prepared on three different days in triplicate from freshly prepared stock solutions, extracted, and analyzed as described for plasma. In Table 3, the raw data are arranged according to sample concentration and day. This data was analyzed as discussed above for the plasma standards. Back-calculated values for the standards are presented in Table 4; only 2 of 36 samples (5.56%) had 95% confidence limits that did not include the true concentration.

Analyses were performed with the back-calculated concentrations for urine samples in Table 4 in the same way as that described for plasma samples in Table 2. Results were similar. There were no significant biases, and there was not a significant effect of day on the back-calculated concentrations.

Quantitation of Individual Diastereomers in Plasma and Urine

Since analytical standards for the individual diastereomers are not available for the construction of standard curves, it was necessary to estimate the amount of each diastereomer in the standards by multiplying the total CGP19984D by the percent for each diastereomer. In order for this approach to give satisfactory results, the extinction coefficients for the two diastereomers must be the same and the ratio of the two

TABLE 3

Calibration Standards for Mixture of Diastereomers in Urine

Concentration (µg/ml)	0.5	1	10	100
Day 1	11831[a]	23195	277520	2943356
	10331	24415	255813	2534402
	12207	25646	284020	2947701
Day 2	13305	25208	281939	2923691
	12807	22461	274706	2717555
	14633	26297	331561	2720516
Day 3	14731	28005	302889	2900503
	13370	26122	291114	2763472
	13365	28405	294998	2782705
Mean	12953	25528	288284	2803763
Standard Deviation	1377	1986	21072	138261
Relative S.D., %	10.63%	7.78%	7.31%	4.93%

[a]Entries in the upper part of the table are measured peak areas.

diastereomers as determined by the HPLC procedure must not vary with concentration. Five standard concentrations (0.1 - 100 µg/ml) of the diastereomer mixture were prepared in quintuplicate from a freshly prepared stock solution by the procedure described above, and the individual concentrations of the two diastereomers were determined by the HPLC technique. The ratio (I1%) of the concentration (by HPLC) of diastereomer 1 divided by the sum of the concentration (by HPLC) of diastereomer 1 + diastereomer 2 was calculated. The ratio, I1%, was used as a dependent variable with the base 10

TABLE 4

Back-Calculated Concentrations of Standards for Mixture of Diastereomers in Urine

	0.5	1	10	100
Day 1	.4956[a]	.9534	10.64	105.6
	.4317	.9866	9.433	85.47
	.5295	1.084	11.05	105.7
Day 2	.5086	.9550	10.32	103.6
	.4726	.8229	9.744	93.58
	.5889	1.042	12.30	95.57
Day 3	.5385	1.019	10.85	102.4
	.4701	.9167	10.14	95.62
	.4815	1.018	10.40	96.59
Mean	0.5019	0.9775	10.54	98.24
Bias	0.0019	-0.0225	0.54	-1.76
% Bias	0.38%	-2.25%	5.40%	-1.76%
Standard Deviation	0.0461	0.0772	0.833	6.68
Relative S.D., %	9.19%	7.90%	7.90%	6.80%

[a]Entries in the upper part of the table are back-calculated concentrations in units of μg/ml.

logarithm of the standard concentration of the diastereomer mixture as the independent variable for unweighted, simple linear regression (the variance of I1% was uniform across the range of standard concentrations of the mixture, 0.1 - 100 μg/ml). The y-intercept was 0.444 ± 0.0026 (S.E.) and the slope was 0.00228 ± 0.0023. Since the 95% confidence interval for the slope encompassed zero, there is no evidence to suggest that the proportion of either isomer is dependent upon the standard concentration of the mixture of diastereomers. Also, the

percent of diastereomer 1 in the mixture was concluded to be 44%, and that for diastereomer 2 to be 56% for both plasma and urine samples. For both individual diastereomers, analyses were carried out on back-calculated standard concentrations in the same way as that described for plasma and urine samples of the mixture of isomers in Tables 2 and 4. Results were similar. There was only one case of a significant bias, and for the same case a significant effect of day on the back-calculated concentrations was found. The specific case was for isomer 1, 4.4 μg/ml, in plasma. However, since 27 total individual bias (and ANOVA) tests were made with a type I error rate of 0.05 for the 2 isomers and the mixture in plasma and urine, 1/27 or about 4% of biased cases is consistent with an overall unbiased assay.

Example of the Use of the CGP19984D Assay: Pharmacokinetics of CGP19984D in the Dog (500 mg/kg i.v.)

A beagle dog was given a dose of CGP19984D (500 mg/kg) intravenously over 2 minutes. Blood samples were drawn into heparinized syringes at timed intervals after drug administration, the RBC were removed by centrifugation, and the plasma was frozen for later analysis; urine samples were collected via a catheter for 24 hours and frozen for analysis. Pretreatment plasma and urine from the same animal were used to construct standard curves; the analytical procedure described above was used to quantitate the concentration of total drug, diastereomers 1 and 2 in plasma and urine. The plasma data (Figure 5) for all 3 species of parent drug were fit individually by a biexponential model with nonlinear regression with the PC NONLIN [7] software package running on an IBM PC/AT microcomputer. The estimated alpha and beta phase half lives (in minutes ± S.E.) for diastereomer 1, diastereomer 2, and the mixture were respectively: 24.2 ± 4.4, 61.1 ± 12; 24.1 ± 5.2,

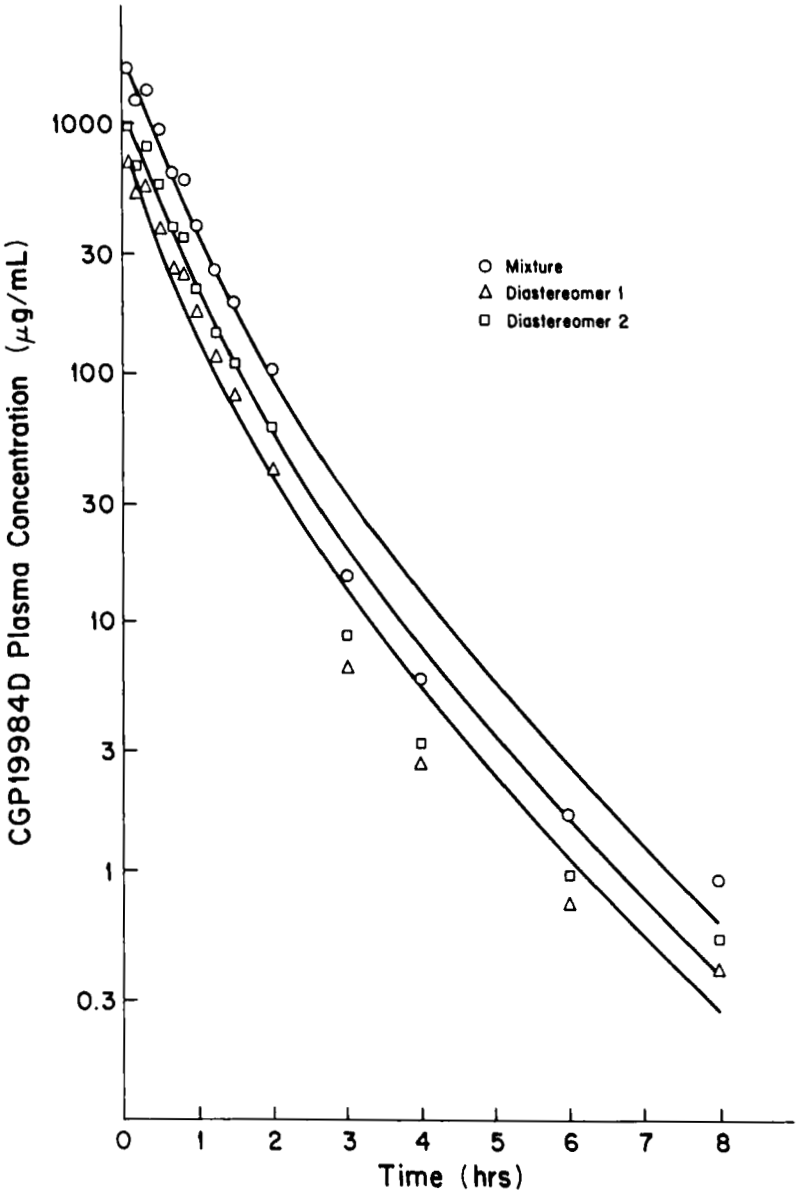

Figure 5 Concentration of CGP19984D in the plasma of a beagle dog after an i.v. dose of 500 mg/kg of CGP19984D.

60.1 ± 13; and 24.4 ± 5.0; 60.2 ± 13. Because the half lives for the two isomers were found to be the same and because the percent of isomer 1 and isomer 2 remained constant at about 44% and 56%, respectively from 5 to 480 minutes after drug administration, it is concluded that the two isomers are cleared from plasma at the same rate. Forty-three percent of the dose was eliminated in the urine as unchanged drug. Further studies with ^{14}C-labeled CGP19984D will quantitate the fraction of the clearance due to metabolism.

DISCUSSION

CGP19984D has been developed to clinical trial in man since it represents a new chemical class of antitumor agents, has antitumor activity against two types of solid tumors that are important in man, and has both direct cytotoxic and hormone-modulatory effects in experimental systems. The goal of this study was to develop a method to quantitate CGP19984D in biological fluids that would form the basis for a detailed pharmacological study in the rat and dog. The structure of CGP19984D contains two assymetric centers, one in each heterocyclic ring, so that the compound exists as two sets of enantiomeric species that form a pair of diastereomers. Since diastereomers differ in their chemical properties, they can be separated on stationary phases that do not contain optically active ligands. Since this compound is a strong acid, a separation using ion pairing with the tetrabutylammonium cation was developed on a C18 column; our results demonstrate that this technique resolves the drug from the other components of plasma and urine and separates the two sets of diastereomers from one another.

The goal in developing a new antitumor agent is to demonstrate that the drug is of therapeutic benefit to patients when administered as a single agent or in combination chemotherapy,

so that a New Drug Application (NDA) can be filed with the Food and Drug Administration (FDA) for the introduction of the drug into standard medical practice. Therefore, the pharmacological studies performed in animals and in patients during phase I, II, III trials should be carried out in such a way that the data obtained is acceptable to the FDA in support of an NDA. A basic concern of the FDA is the precision and accuracy of the methods used to quantitate the drug in biological fluids. Since no particular approach to this problem is mandated by the FDA, a secondary goal of this paper was to outline a statistical approach to the evaluation of the precision and accuracy of the assay for CGP19984D that may serve as a paradigm for the evaluation of other analytical assays.

The approach to ensure good precision and accuracy includes: (a) the design of the standard curve methodology to guard against potential biases and large variability in the prediction of drug concentrations in test samples; (b) the provision of diagnostic measures of precision for a routine run of the assay; (c) a formal short-term statistical validation of the complete assay; and (d) a long-term continuous assessment of the assay with quality control procedures.

The general need for standard curves to span many orders of magnitude has been generated by the combination of a need to study unknown samples spanning a wide range of concentration, and the availability of sensitive analytical instrumentation. The methodology used in this paper, which includes logarithmic transformations of the data and empirical weighting factors, seeks to improve upon the practice of routinely constructing two standard curves (one for the low concentration range and one for the high range) for one set of unknowns and to eliminate potential biases and unacceptible variability. There was no evidence found to suggest that the standard curve for CGP19984 does not follow a straight line over a thousand-fold range of concentration.

Routine construction of a standard curve and analysis of a set of unknown samples include several related diagnostic measures of precision: the mean square error, the statistic g, the 95% confidence envelope surrounding the graph of the standard curve, and the 95% confidence limits around the estimate of unknown sample concentrations. If poor precision is detected in the analysis of a set of unknowns, the analytical procedure can be examined for errors and the samples can be re-analyzed.

The routine method for estimating CGP19984D in plasma and urine was validated for each diastereomer individually and for the mixture. The accuracy of the method was tested by back-calculating the concentration of the standards (Tables 2,4). There were no important biases or patterns to the biases seen over the range of standard concentrations. Precision was examined by comparing the variability in back-calculated concentrations between days to the variability within days. There was no evidence to suggest that day-to-day variability was large or important. Except for the 0.1 μg/ml plasma standard for the mixture (and individual isomers), the relative standard deviations for the back-calculated concentrations were always less than 13%.

Four quality control charts have been started for the monitoring of the assay on a routine basis. The four recorded statistics are the slope and y-intercept of the standard curve, the mean square error, and g. It is not, however, a common nor well established practice to monitor these four statistics. Therefore, the four quality control charts will be used on an empirical basis to alert the analyst to changes in the assay and to establish criteria for the acceptability of an analysis of a set of unknowns. As experience accumulates with these diagnostic statistics, more rigorous guidelines for their use will be established.

A more standard approach to the construction of quality control charts is to make a large batch of seeded control samples and to assay a set of these with each routine standard curve; means and standard deviations are calculated for the estimated concentrations of seeded controls, and are plotted on traditional X and S quality control charts [9]. The difficulties with this practice, especially for investigational agents, is that seeded control samples may not be stable over time, and that even if seeded control samples would be stable it would require a prohibitive amount of time to confirm the stability. Thus, the investigation of the above 4 statistics for use in quality control may have general applicability for investigational compounds.

The pharmacokinetic results for a dog treated with 500 mg/kg i.v. of CGP19984D are presented as an illustration of the application of the method. The results of this experiment demonstrate that the disposition of CGP19984D from the plasma in this dog is adequately fit by a biexponential model, that the pair of diastereomers are cleared from the plasma at the same rate, and that 43% of the dose is eliminated in the urine as unchanged drug. The method is being used in an extensive study of the pharmacology of CGP19984D administered by the i.v. route to experimental animals (rat and dog) and to patients entered into a Phase I trial.

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