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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF PIRLIMYCIN IN HUMAN SERUM AND URINE USING 9-FLUORENYLMETHYLCHLOROFORMATE

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SUMMARY

A reversed-phase high-performance liquid chromatographic (HPLC) assay method has been developed for determining pirlimycin in human serum and urine. The method involves chloroform extraction of pirlimycin free base followed by derivatization with 9-fluorenylmethylchloroformate to form a carbamate ester. The reaction is rapid, reproducible, and quantitative. 9-Fluorenylmethylchloroformate reacts with amines to form derivatives sensitive to both ultraviolet and fluorescence detection. Human serum and urine samples following 50-mg and 500-mg single oral doses of pirlimycin were analyzed. The samples were chromatographed on an RP-18 Spherisorb 5-μm, 250 × 4.6 mm I.D. reversed-phase HPLC column. The eluent for the serum assay was acetonitrile—water (58:42) containing 0.02% acetic acid, and for the urine assay was acetonitrile—methanol—tetrahydrofuran—water (48:2:1:49). Fluoranthene was used as an internal standard. The assay sensitivity by ultraviolet detection (λ_{max} = 264) was about 5 ng/ml and by fluorescence detection ($\lambda_{excitation}$ = 270 nm, $\lambda_{emission}$ = 300 nm) was 0.1 ng/ml. Statistical analysis indicates an average drug recovery of 101 ± 4.2% from serum and 102.0 ± 2.62% from urine.

INTRODUCTION

The development of pirlimycin (P), an analogue of clindamycin, required a sensitive analytical method for its measurement in biological fluids.

A major problem in developing a sensitive high-performance liquid chromatographic (HPLC) assay was the lack of useful ultraviolet absorption by the lincosaminide family of antibiotics of which pirlimycin is a member. Although some work has been reported [1] on an HPLC assay for pirlimycin using ultraviolet end-absorption at 214 nm, the procedure is not sensitive for measuring nanogram levels of drug in biological fluids.

Alkylchloroformate esters have been used for the gas chromatographic

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analysis [2—6] and for the HPLC analysis [7] of many amines. However, the HPLC procedure required heating at 100° C for 1 h which seemed excessive.

Recently, a new chloroformate reagent was developed as an amino-protective group in the solid phase synthesis of peptides [8—12]. This reagent, 9-fluorenylmethylchloroformate (9-FMClF), was found to react quantitatively and rapidly at ambient conditions with the amine group of the piperidine moiety of pirlimycin. This report describes the development of an HPLC assay procedure for pirlimycin in human serum and urine using pre-column derivatization of the drug with 9-FMClF.

EXPERIMENTAL

Reagents and materials

Pirlimycin · HCl and pirlimycin free base were synthesized within the Research Division of the Upjohn Company. Fluoranthene and 9-fluorenylmethylchloroformate were obtained from Eastman Kodak (Rochester, NY, U.S.A.) and from Aldrich (Milwaukee, WI, U.S.A.), respectively, and used as received. Acetonitrile, chloroform, dioxane, and methanol were distilled-in-glass UV grade from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.) All other chemicals were analytical reagent grade.

Synthesis of pirlimycin derivative

Method 1. Pirlimycin free base ($3.84 \cdot 10^{-3}$ mol) was dissolved in 25 ml dioxane and added to 10 ml of 10% (w/v) sodium carbonate. The solution was cooled in an ice-bath. An equimolar amount of 9-FMClF was dissolved in dioxane and then added drop-wise to the above solution with continuous stirring for about 20 min. The mixture was allowed to stir for an additional 45 min, and then poured into water; the formed derivative was extracted into chloroform. The extract was evaporated to dryness to yield an oily residue; the residue was dissolved in diethyl ether and crystalline material was obtained upon room temperature drying.

Method 2. Equimolar solutions of pirlimycin free base and 9-FMClF were prepared in 25 ml and 5 ml acetonitrile, respectively, then mixed. The mixture was allowed to stand at room temperature for about 45 min. The derivative was precipitated by adding 50 ml water to the reaction mixture and stirring for about 20 min. The filtered precipitate was dried under vacuum.

Chromatography instruments

An Altex Model 110A pump, a Rheodyne Model 7125 low-dead-volume injector, and an LDC Spectromonitor III variable-wavelength UV detector or Perkin-Elmer LS-5 variable-wavelength fluorescence detector were used. Data were recorded on a linear dual-channel strip-chart recorder. The column was a Spherisorb, 5-μm, 25 cm × 4.6 mm RP-18 column from Brownlee Labs. fitted with a Spherisorb, 5-μm, 3 cm × 4.6 mm RP-18 guard column, also from Brownlee Labs.

Operating parameters for serum and urine analysis

Serum samples were analyzed using acetonitrile—water (58:42) containing

0.02% acetic acid as the mobile phase at a flow-rate of 1.2 ml/min. For UV detection the absorbance was set at 264 nm, and for fluorescence detection excitation was set at 270 nm and emission at 300 nm. Under these conditions using UV detection, the retention times of pirlimycin derivative and fluoranthene (internal standard) were 14.5 and 37.5 min, respectively.

For the urine analysis, the mobile phase consisted of acetonitrile–methanol–tetrahydrofuran–water (48:2:1:49). With the exception of the flow-rate of 1.5 ml/min, all other conditions were identical to the conditions employed for serum analysis. Under these conditions using UV detection, retention times of pirlimycin derivative and internal standard were 28 and 60 min, respectively.

Thin-layer chromatographic (TLC) conditions

Thin-layer chromatography was performed on C_{18} reversed-phase TLC plates with a 200-μm layer (Whatman $MKC_{18}F$). The developing solution was acetonitrile–water (80:20) and detection was at 254 nm. Under these conditions the R_F for the pirlimycin carbamate derivative was 0.5.

Human study protocol

Pirlimycin · HCl capsules were administered orally at 50-, 125-, 250- and 500-mg dose levels. Blood and urine samples were taken at recorded time intervals. Blood samples were taken into non-heparinized tubes, allowed to clot, and then centrifuged at 1000 *g* for 10 min. The harvested serum was put into appropriately labelled vials and immediately frozen. The frozen samples were stored at −20° C until assayed.

Total urine volume was recorded for each interval before an aliquot was withdrawn, immediately frozen, and stored at −20° C until assayed.

ASSAY PROCEDURE

Preparation of serum samples

Stock solutions of pirlimycin · HCl (2.088 μg/ml) and fluoranthene internal standard (9.3 μg/ml) were prepared separately in methanol, while 9-FMClF derivatizing reagent, a stock solution (63.8 μg/ml), was prepared in acetonitrile. A series of standard serum samples of pirlimycin · HCl was prepared by pipetting pirlimycin · HCl stock solutions into 15-ml centrifuge tubes. The solvent was evaporated to dryness then reevaporated after adding 50 μl of internal standard solution; 1 ml of human serum and 0.3 ml of 0.1 *M* sodium hydroxide were added to each tube. The samples were extracted twice, each time with 4 ml of chloroform, then mixed and centrifuged; the chloroform extract was evaporated to dryness. Similarly, a blank serum sample was prepared from the same serum specimen. The 10 μl of $2.5 \cdot 10^{-4}$ *M* sodium hydroxide and 50 μl of 9-FMClF were added to the dry residue. The mixture was kept at room temperature for 2.5 h, and then 20–40 μl were injected for analysis into HPLC.

Test serum samples were prepared by pipetting 50 μl internal standard solution into a series of centrifuge tubes. After evaporating to dryness, 1 ml of serum and 0.3 ml of 0.1 *M* sodium hydroxide were added; subsequent steps

involving chloroform extraction and derivatization were carried out as described for the standard serum samples.

Preparation of urine samples

Stock solutions of pirlimycin · HCl (10.33 μg/ml) and internal standard (9.3 μg/ml) were prepared in methanol. A series of standard samples was prepared by pipetting pirlimycin · HCl stock solutions into 15-ml centrifuge tubes; 100 μl of internal standard were added to each tube, then evaporated to dryness. Blank human urine (1 ml), 0.5 ml of 0.1 *M* sodium hydroxide and 5 ml of chloroform were added; the samples were mixed and centrifuged and the choroform extract was evaporated to dryness. Similarly, a blank urine sample was prepared from the same human urine. The 10 μl of $2.5 \cdot 10^{-4}$ *M* sodium hydroxide and 80 μl of 150 μg/ml 9-FMClF were added to the dry residue. The mixture was kept at room temperature for about 2.5 h, then 20—70 μl were injected for analysis into HPLC.

Test urine samples were prepared by pipetting 100 μl of internal standard into series of centrifuge tubes. After evaporation to dryness, 1 ml urine for low-level doses and 0.1 ml urine for high-level doses were added to each centrifuge tube. Subsequent steps of chloroform extraction and derivatization were carried out as described for the standard urine samples.

Calculations

Peak height ratios were calculated by dividing the peak height of the pirlimycin derivative by the peak height of the internal standard. The peak height ratio was corrected by taking into account the corresponding blank serum samples. A calibration curve was constructed by plotting peak height ratios versus the concentrations of the pirlimycin · HCl standards. The concentration of pirlimycin · HCl in the unknowns was calculated from the peak height ratios using the slope and intercept obtained by linear-regression analysis of the calibration curve data. The amount of drug excreted in urine was calculated by multiplying the urine volumes by the analytical concentration.

RESULTS AND DISCUSSION

Synthesis and characterization of 9-FMClF derivative of pirlimycin

Pirlimycin free base reacts with 9-FMClF to form a pirlimycin carbamate derivative and an equimolar amount of hydrochloric acid (see Fig. 1). Hydrochloric acid can rapidly convert the unreacted pirlimycin free base into its hydrochloride salt. Since the salt does not react with 9-FMClF, only about 50% of the free base can be converted into its carbamate derivative. Sodium carbonate was used to neutralize the acid in the Method 1 synthesis. This method is similar to the one reported previously [8]. Since the Method 2 synthesis was carried out without the addition of a base, about 50% yield was obtained by this procedure. However, Method 2 is much simpler than Method 1.

TLC and HPLC analysis showed that the synthesized material was a single component. Elemental analysis, IR, and mass spectrometric analysis supported the proposed structure.

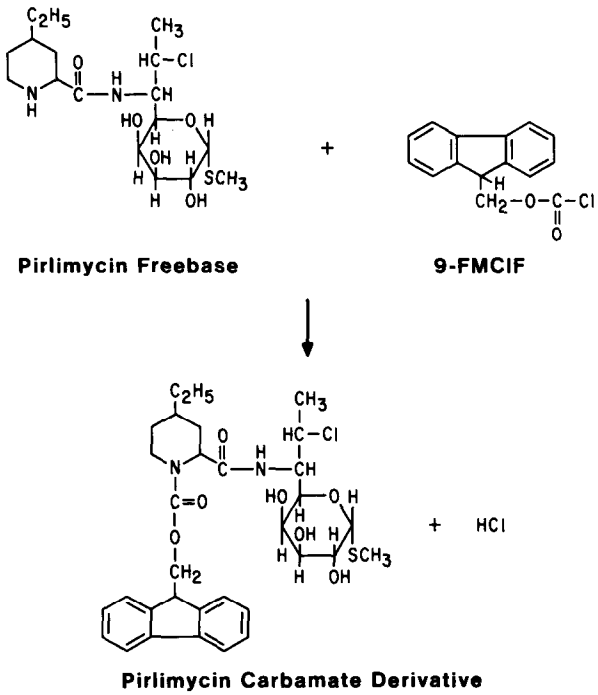

Fig. 1. Reaction scheme for formation of carbamate derivative of pirlimycin.

Chromatographic analysis

Serum and urine samples containing pirlimycin free base were extracted with chloroform, evaporated and reacted with 9-FMClF in an organic solvent (acetonitrile). In order to achieve complete derivatization of all the drug, the reaction had to be carried out in the presence of an inorganic base which could neutralize hydrochloric acid from the reaction mixture. The use of 18-Crown-6 ether is common for dissolving inorganic ions in organic solvents [13]. Several trials were made using potassium hydroxide and other inorganic bases in 18-Crown-6 ether to derivatize pirlimycin free base in acetonitrile. However, these efforts were not fully successful due to the interfering effects of 18-Crown-6 ether and the partial decomposition of the drug derivative. The use of a very small volume of sodium hydroxide solution in water yielded 100% derivatization of drug with reproducible results.

Chromatographic experiments were performed using C_8 and C_{18}, 5-μm and 10-μm particle size, reversed-phase columns, and various mobile-phase compositions consisting of acetonitrile—water, acetonitrile—tetrahydrofuran—methanol—water, acetonitrile—water—acetic acid, and methanol—water—acetic acid. Among these, a C_{18} reversed-phase column, having a 5-μm particle size and a mobile phase composed of acetonitrile—water (58:42) containing 0.02% acetic acid, provided optimum resolution for serum analysis. Fluoranthene was chosen as an internal standard because of its non-reactivity to the reagent, complete recovery from biological fluids and high UV-fluorescence detector response.

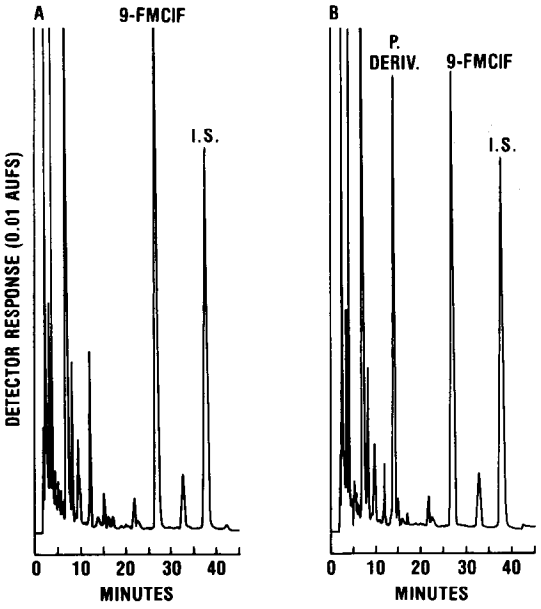

Fig. 2. Chromatogram of human serum extract of (A) blank serum containing internal standard (I.S.) and derivatizing reagent (9-FMClF); and (B) serum containing pirlimycin carbamate derivative (P. Deriv.), internal standard (I.S.) and derivatizing reagent (9-FMClF).

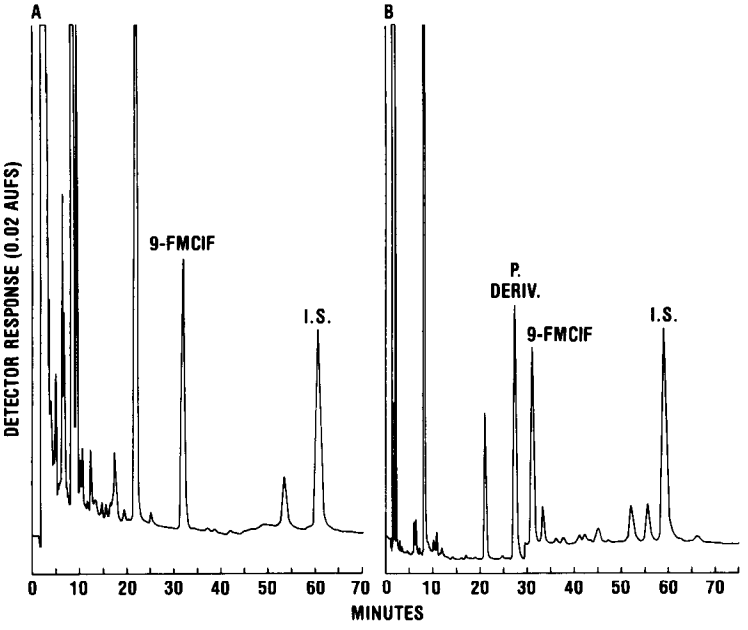

Fig. 3. Chromatogram of human urine extract of (A) blank urine containing internal standard (I.S.) and derivatizing reagent (9-FMClF); and (B) urine containing pirlimycin carbamate derivative (P. Deriv.), internal standard (I.S.) and derivatizing reagent (9-FMClF).

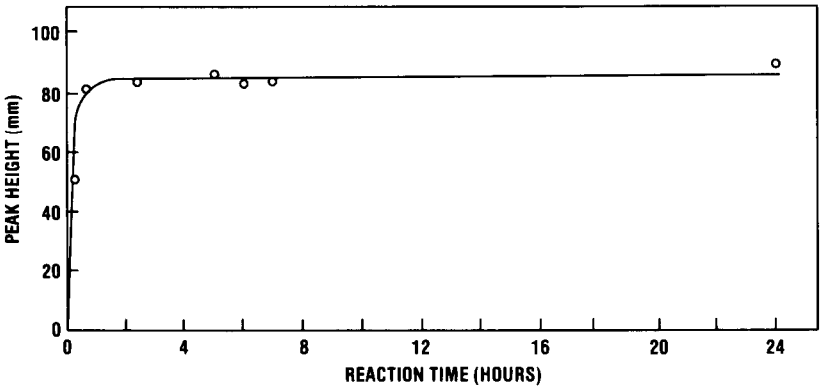

Fig. 4. Effect of reaction time on formation of pirlimycin derivative.

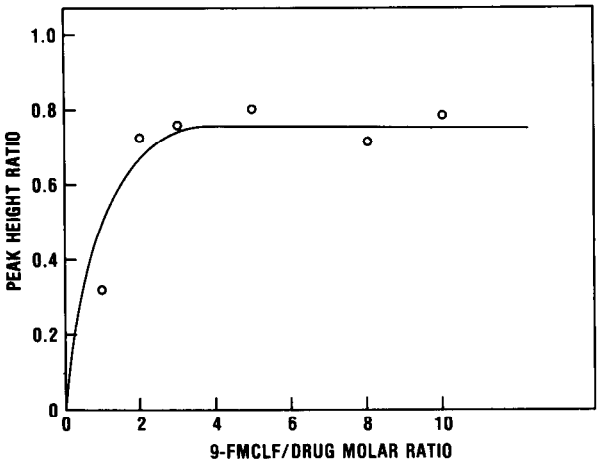

Fig. 5. Effect of 9-fluorenylmethylchoroformate on pirlimycin derivative formation. Reaction time = 2.30 h at room temperature.

Fig. 2A shows a typical HPLC chromatogram of an extract of blank human serum containing internal standard and derivatizing reagent (9-FMClF). Fig. 2B shows a chromatogram of serum containing pirlimycin carbamate derivative, internal standard, and 9-FMClF. Fig. 3A and Fig. 3B show similar chromatograms for blank urine and spiked urine extracts, respectively.

Fig. 4 shows the effect of reaction time on the formation of pirlimycin derivative at room temperature. The reaction is essentially completed within 1 h. The derivative is stable for at least 24 h. Fig. 5 shows the effect of derivatizing reagent concentration upon the extent of formation of pirlimycin derivative. A molar ratio of drug to reagent of 1:3 gives complete formation of the derivative.

Extraction efficiency

Serum or urine specimens (1 ml) were spiked with known concentrations of pirlimycin · HCl and the drug was extracted according to the procedure described in Experimental. These samples were assayed at 214 nm using chromatographic conditions reported by Asmus and Landis [1]. The extraction

TABLE I

EXTRACTION EFFICIENCY OF PIRLIMYCIN · HCl FROM HUMAN SERUM AND URINE

Trial	Serum extraction efficiency (%)	Urine extraction efficiency (%)
1	94.16	98.28
2	94.50	101.50
3	103.33	—
Mean (%)	97.33	99.89
Coefficient of variation (%)	5.3	2.3

efficiency of pirlimycin from serum and urine was calculated by comparing absorbance with the known standard drug solution at 214 nm. The results, listed in Table I, show an average extraction efficiency of 97.33% from serum an 99.89% from urine.

Reaction efficiency

Blank serum or urine (1 ml) was spiked with a known concentration of pirlimycin · HCl. The sample was extracted and reacted with the derivatizing reagent (9-FMClF) in acetonitrile in the presence of a small amount of base as described in Experimental. The sample was chromatographed along with a standard solution of the pure derivative in acetonitrile. The results confirmed stoichiometric conversion of drug into its carbamate derivative.

Assay precision and accuracy

Linear-regression analysis of the calibration curve data indicated no significant deviations from linearity for pirlimycin hydrochloride concentrations up to 2 μg/ml serum and up to 20 μg/ml urine. Correlation coefficients were better than 0.993 for the standard curves prepared at three different times.

Assay precision and accuracy were established by adding a known concentration (417.6 ng/ml serum) of pirlimycin hydrochloride to several 1-ml blank serum samples. These replicate samples were extracted, derivatized and assayed

TABLE II

ACCURACY AND PRECISION DATA FOR THE RECOVERY OF PIRLIMYCIN · HCl FROM HUMAN SERUM

Added (ng/ml)	Found (ng/ml)	Recovery (%)
417.6	439.11	105.15
	421.89	101.03
	407.95	97.69
	426.51	102.13
	436.51	104.53
	436.76	104.58
	386.81	92.63
	Average = 422.22	Mean = 101 ± 4.2%

Coefficient of variation = 4.5%

TABLE III

ACCURACY AND PRECISION DATA FOR THE RECOVERY OF PIRLIMYCIN·HCl FROM HUMAN URINE

Added (μg/ml)	Found (μg/ml)	Recovery (%)
5.165	5.346	103.51
	5.279	102.22
	5.071	98.18
	5.051	97.80
	5.439	105.31
	5.309	102.69
	5.373	104.02
	Average = 5.26	Mean = 102 ± 2.62%

Coefficient of variation = 2.8%

as described previously. The average interday recovery of pirlimycin · HCl obtained was 101 ± 4.2% (Table II).

Table III gives precision and accuracy data for the replicate analysis of urine samples spiked with a known concentration (5.165 μg/ml urine) of drug. An average interday drug recovery of 102.0 ± 2.62% was obtained.

Applicability of the methodology

The utility of the analytical method for pharmacokinetic studies was demonstrated by monitoring serum and urine levels of pirlimycin · HCl in humans. A typical serum profile is shown in Fig. 6 for a dose of 500 mg of pirlimycin · HCl. A typical cumulative urinary elimination profile is shown in Fig. 7 for a dose of 50 mg of pirlimycin · HCl. A summary of the pharmacokinetic parameters is given in Table IV. The area under the serum curve (AUC) data may indicate a non-linear dose response since the AUC for the 50-mg dose is much less than expected based on the 500-mg dose results. The values of U_∞

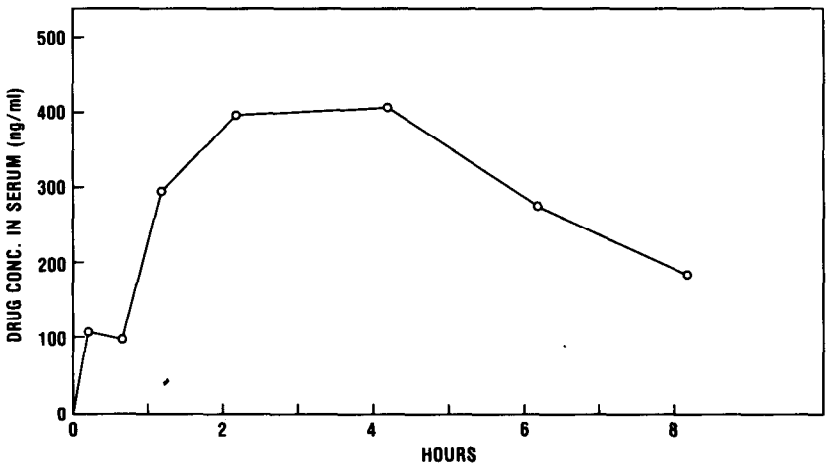

Fig. 6. Pirlimycin · HCl concentration in human serum following a 500-mg oral dose of pirlimycin · HCl to a human male.

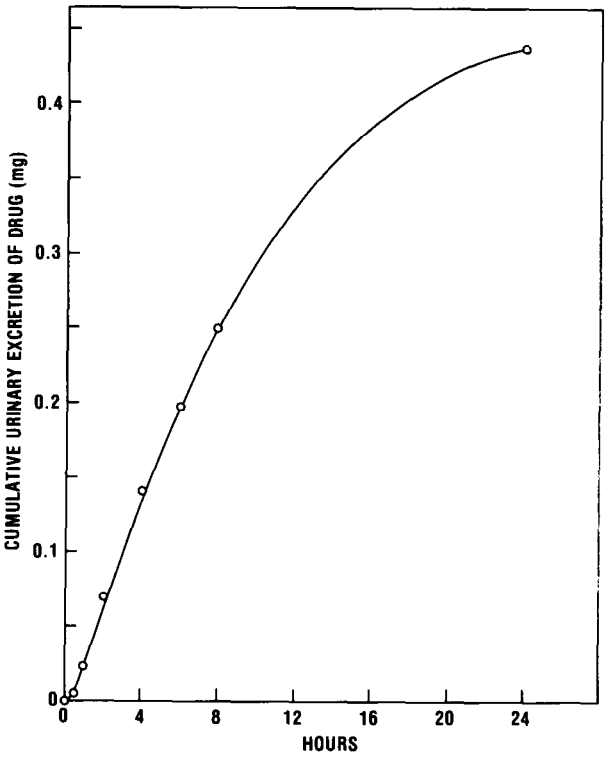

Fig. 7. Cumulative urinary elimination of pirlimycin ◦ HCl following an oral dose of 50 mg pirlimycin ◦ HCl to a human male.

TABLE IV

PHARMACOKINETIC PARAMETERS IN MALE HUMANS FOLLOWING ORAL DOSES OF PIRLIMYCIN · HCl

	500-mg Dose		50-mg Dose
	Subject 1	Subject 2	Subject 1
AUC_0 8 h (ng h ml^{-1})	2721	2462	44.5
k_e* (h^{-1})	0.097		0.101

* The value of U_∞ used to calculate these rate constants were estimated assuming first-order kinetics.

were estimated assuming first-order kinetics for the calculation of the renal elimination rate constants. Therefore, these rate constants should be considered only as approximations.

Fluoresence detection

Derivatives prepared from 9-FMClF are highly fluorescent and may be useful in increasing the assay sensitivity to sub-nanogram levels. A preliminary evaluation of the fluorescence sensitivity using blank serum samples spiked with the 9-FMClF derivative of pirlimycin indicated an analytical sensitivity of 0.1 ng/ml.

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