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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHODS FOR THE DETERMINATION OF CEFPIMIZOLE IN PLASMA AND URINE

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SUMMARY

Quantitative analytical methods have been defined for the determination of cefpimizole, a new broad-spectrum cephalosporin antibiotic, in plasma and urine specimens. The methods employ ion-pair reversed-phase high-performace liquid chromatography with both ethylenediaminetetraacetic acid (EDTA) and tetrabutylammonium hydroxide as pairing agents for separation and ultraviolet detection at 254 nm. Sample preparation for plasma aliquots consisted of acetonitrile protein precipitation followed by phase separation; the aqueous phase was filtered and assayed. For urine, sample preparation consisted of diluting an aliquot with chromatographic eluent, filtering, and assaying. The methods had a linear range of 17—0.3 μg/ml for plasma and 800—15 μg/ml for urine and had sufficient precision and accuracy to provide quantitative data. Stability studies in plasma and urine indicated that cefpimizole degraded rapidly at room temperature. Addition of EDTA to the physiological fluid substantially increased the stability at room temperature, and little or no degradation was observed in plasma or urine stored at —30°C for over 100 days. Utility of the methods was demonstrated by assaying plasma and urine specimens obtained from a human volunteer receiving three dose levels. Estimates of various pharmacokinetic parameters are presented.

INTRODUCTION

Cefpimizole (I), 7-β-D-(—)-α-[4(5)-carboxyimidazole-5(4)-carboxiamido]-phenylacetamido-3-(4-β-sulfoethylpyridinium)-methyl-3-cephem-4-carboxylate, sodium salt (Fig. 1), is a third generation cephalosporin analogue and is being evaluated as a broad-spectrum antibiotic. This compound has reported potent activity against both Gram-positive and Gram-negative bacterial species including *Proteus vulgaris*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Streptococcus mitis*, and *Staphylococcus aureus* [1, 2].

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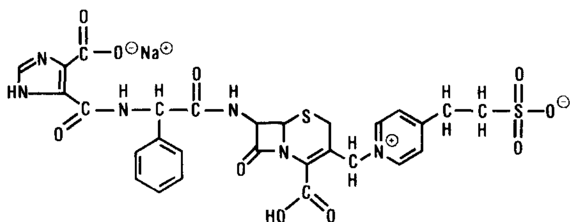

Fig. 1. Chemical structure of cefpimizole.

Pharmacokinetic and metabolism studies on I require a selective, quantitative assay. Previous studies on other cephalosporin analogues have employed high-performance liquid chromatography (HPLC) as the analytical technique for the determination of plasma and urine levels [3—7]. These methods employed reversed-phase HPLC or ion-pair HPLC to separate the cephalosporins from potentially interfering physiological fluid components. Previously reported pharmacokinetic evaluations on I in animals [8] and man [9] utilized microbiological techniques to determine serum and urinary levels of biological activity attributed to I. The results from the human studies indicated that the biological activity was rapidly excreted in the urine (80—90% of administered dose) and had a terminal serum half-life of 1.5—1.7 h. No definable metabolites of the antibiotic were observed and only minimal side effects were encountered in over 200 human patients. A bacteriological efficacy of 81.9% for a variety of Gram-negative strains was reported. While these data provide information of the disposition and efficacy of the antibiotic in humans, the methodology employed did not have adequate specificity to detect the parent compound or the precision and reproducibility for quantitative results.

This report describes quantitative HPLC methods for the determination of I in plasma and urine specimens. Applications of the methods are demonstrated by assaying for I in specimens obtained from a human volunteer and estimating various pharmacokinetic parameters from the data.

EXPERIMENTAL

Apparatus

A Laboratory Data Control (Riviera Beach, FL, U.S.A.) isocratic HPLC unit consisting of a Constametric III pump, UV Monitor III at 254 nm, and a Rheodyne (Cotati, CA, U.S.A.) Model 7125 variable-loop injector was employed. The HPLC column utilized was a Supelcosil LC-18, 5-μm particle size, 250 × 4.6 mm I.D. (Supelco, Bellefonte, PA, U.S.A.) with a Co:Pell ODS, 35-μm particle size, 50 × 2.1 mm I.D. guard column (Whatman, Clifton, NJ, U.S.A.). A Hamilton (Reno, NV, U.S.A.) Series 810RN 100-μl syringe was used to make injections.

Chemicals and reagents

Cefpimizole was supplied by the Pharmaceutical Research and Development Laboratories (Upjohn, Kalamazoo, MI, U.S.A.) and was used without further purification. Acetophenone, selected as the internal standard, and ethylenediaminetetraacetic acid (EDTA) were obtained from Aldrich (Milwaukee, WI,

U.S.A.). Tetrabutylammonium hydroxide (TBA) (Eastman-Kodak, Rochester, NY, U.S.A.) was 0.4 *M* titrant grade. Methanol and acetonitrile were UV, distilled-in-glass solvents (Burdick and Jackson Labs., Muskegon, MI, U.S.A.). All other chemicals used during these evaluations were of the highest purity available.

The final HPLC eluent was prepared by adding 40 ml of 0.1 *M* EDTA and 50 ml of 0.4 *M* TBA to 2510 ml distilled, deionized water. While stirring, 1400 ml methanol were added, and then the pH was adjusted to 6.0 with the dropwise addition of concentrated acetic acid. The prepared eluent was filtered through a 0.2-μm Nylon-66 (Rainin Instrument, Woburn, MA, U.S.A.) filter and helium-degassed for 45 min. The prepared eluent was stable for a minimum of five days; after that time, refiltration and degassing were performed to protect the analytical column. Prior to using the 0.1 *M* EDTA and 0.4 *M* titrant TBA solutions, they were filtered three times through 0.2-μm filters to remove particulate matter.

Sample preparation procedures

Plasma samples were prepared by adding 1.0 ml plasma to 1.0 ml of a solution containing 0.01 *M* EDTA and 0.05 *M* TBA, pH 5.0, and precipitating the plasma protein with the addition of 4.0 ml acetonitrile. After mixing, the plasma—acetonitrile mixture was allowed to stand a minimum of 2 h at 4°C to ensure complete protein precipitation. The sample was centrifuged and the supernatant transferred to a clean tube. The precipitate was washed with 2.0 ml (75:25, v/v) acetonitrile—0.01 *M* EDTA, 0.05 *M* TBA, pH 5.0; and after centrifugation, the wash was added to the original supernatant. Methylene chloride (200 μl) was added, and the solution was allowed to phase separate at —20°C. The upper organic layer was aspirated and discarded. The calculation internal standard (IS) (50 μl of a 200 μg/ml acetophenone in methanol solution) was added to the aqueous phase; the sample thoroughly mixed and filtered through a 0.45-μm filter (Acrodisc®-CR, Gelman, Ann Arbor, MI, U.S.A.). The prepared plasma sample was stored at 4°C until analysis by HPLC—UV (254 nm).

Urine samples were prepared for analysis by quantitatively pipetting a 100-μl urine aliquot into 4.0 ml HPLC eluent containing 10 μg/ml IS. The solution was filtered through a 0.45-μm filter and stored at 4°C until assay.

Plasma and urine specimens

Plasma and urine specimens were obtained from a normal volunteer who was administered three doses of I (1000, 2000, and 4000 mg) with a one-week wash-out period between doses. The drug was administered by intravenous infusion over a 20-min period. Blood specimens were obtained at 0 (predose), 0.33, 0.67, 1.0, 2.0, 4.0, 6.0, 8.0, 12.0, and 24.0 h using Vacutainers containing EDTA as anticoagulant and preservative. The blood specimens were centrifuged immediately after collection, and the plasma frozen and maintained at —30°C or lower until analysis. Urine specimens were obtained during the following collection periods: 0 (predose), 0—0.5, 0.5—0.75, 0.75—1.0, 1.0—2.0, 2.0—6.0, 6.0—12.0, 12—24, and 24—48 h after administration of the drug. During each collection period, urine was placed in a fask containing EDTA

as preservative and maintained at 4°C. After the collection period, the specimen was mixed, the urine volume measured, and an aliquot frozen and maintained at —30° C or lower until analysis.

Calculations

The level of I in plasma and urine specimens was calculated using the relative weight response (RWR, eqn. 1). Reference solutions of I in water for plasma and HPLC eluent for urine over a concentration range were analyzed with each sample set and the level of I in the sample aliquot determined by eqn. 2. Since some loss of I during the sample preparation was expected, fortified blank plasmas or urines over the concentration range were prepared with the sample set and the absolute recovery of I determined by eqn. 3. The level of I in the plasma and urine samples was corrected for sample preparation loss by eqn. 4. The method of calculation provided reference to a known quantity of I and served as a check on variations in the recovery of I between sample sets.

$$\text{RWR} = \frac{\text{Peak height I (standard)}}{\text{Peak height IS}} \times \frac{\mu\text{g IS}}{\mu\text{g I (standard)}} \quad (1)$$

$$\mu\text{g I/aliquot} = \frac{\text{Peak height I (sample)}}{\text{Peak height IS}} \times \frac{\mu\text{g IS}}{\text{average RWR}} \quad (2)$$

$$\text{Recovery I} = \frac{\mu\text{g I/aliquot found (fortified)}}{\mu\text{g I added}} \quad (3)$$

$$\text{Corrected } \mu\text{g/aliquot} = \frac{\mu\text{g I/aliquot}}{\text{average recovery}} \quad (4)$$

RESULTS AND DISCUSSION

HPLC parameter definition

Initial evaluations indicated that simple reversed-phase HPLC techniques employed for other cephalosporins did not provide acceptable chromatographic peak shape or retention for I. Likewise, single ion-pairing HPLC techniques reported for other cephalosporins either resulted in no retention (hexane-sulfonic acid) or complete retention (TBA) of I. A combination of acidic and basic ion-pairing reagents in a methanol—water eluent gave some retention of the compound, and further evaluations indicated that a combination of EDTA and TBA gave acceptable peak shape. Experiments were conducted to optimize the pH, EDTA, TBA, and methanol content of the eluent and the following characteristics were observed: (a) at EDTA and TBA levels below 0.0005 *M* and 0.0025 *M*, respectively, the retention volume of I decreased substantially, but the retention volume was not affected by higher concentrations; (b) the retention volume was fairly consistent between pH values of 5.5 to 6.8, however, below pH 5.5 the retention volume decreased as the pH was lowered; (c) a 32—38% methanol level gave a retention volume between 8 and 16 ml and a sharp, well defined peak; and (d) increased column temperatures did not improve the chromatographic characteristics.

After establishing the HPLC eluent, an IS was selected. Since a homologue of I was not available and the HPLC eluent was different from the eluents employed for other cephalosporins, acetophenone, which had good chromatographic characteristics in the defined analytical system, was selected as IS.

Linearity and precision of the analytical technique

The HPLC analytical technique was evaluated for linearity and reproducibility by preparing and analyzing concentration series of I from 0.5 to 20 μg/ml. Each of the six reference solution levels was prepared and assayed in duplicate on four separate days. Excellent precision was obtained at each concentration with a relative standard deviation (R.S.D.) range for the eight determinations of 2.9—5.5%. A least-squares linear regression evaluation of the peak height ratio versus concentration gave $Y = (0.197 \pm 0.012)X - (0.010 \pm 0.011)$ with a correlation coefficient of 0.999. The Y-intercept was statistically insignificant ($p > 0.05$ with 95% confidence). The slope of the linear-regression equation was directly proportional to the relative weight response of I since the intercept was statistically equal to zero.

Sample preparation procedure for plasma/serum samples

Techniques commonly employed to isolate a compound from plasma/serum protein, i.e. liquid—liquid extraction and protein precipitation, were evaluated for I. The ionic nature of the compound prevented extraction from aqueous solution even when the ionic groups of the antibiotic were ion-paired with EDTA and TBA. Protein precipitation with acetonitrile, perchloric acid, or trichloroacetic acid resulted in co-precipitation of I. When EDTA and TBA were added to the physiological fluid prior to protein precipitation, recovery of the compound in the supernatant was obtained with acetonitrile but not with the acids as the precipitating reagent. However, two volumes of acetonitrile for each aqueous volume were required for complete protein precipitation, and the increased volume of the final solution prevented quantification at the 1 μg/ml level. Increasing the injection volume resulted in poor chromatography of I, thus preventing this technique from providing the necessary sensitivity. Concentration of the supernatant at approximately 35°C under a stream of nitrogen gave acceptable chromatography and met the sensitivity requirement. This sample preparation technique was employed to optimize the conditions, i.e. EDTA, TBA level; pH; sample handling, which gave the highest recovery of the compound with minimal interference from plasma components. The addition of 1.0 ml of a 0.05 *M* TBA—0.01 *M* EDTA, pH 5.0 solution to 1.0 ml serum/plasma and protein precipitation with 4 ml acetonitrile gave approximately 70% recovery of I. At lower TBA, EDTA levels, lower pH values, or less than 1.0 ml added aqueous solution, the recovery was decreased. To improve the recovery, the protein precipitate was washed with a (75:25, v/v) acetonitrile—0.05 *M* TBA, 0.01 *M* EDTA, pH 5.0 solution and the wash added to the original supernatant prior to concentration. This procedure gave 75—85% recovery of the compound; however, the concentration step was time-

consuming. Earlier experiments [10] had shown that I had limited stability in aqueous solution at room temperature. Because of this limited stability, the concentration step was a potential source of error as well as requiring substantial time. Two reported techniques, ultrafiltration [6] and acetonitrile precipitation followed by methylene chloride extraction [7] were evaluated. Ultrafiltration of plasma or serum gave HPLC chromatograms which had interfering plasma components at the elution position of I and the IS. The plasma components prevented quantitation below the 2 μg/ml level. The precipitation—extraction technique was modified because a partial phase separation had been observed earlier (attributed to the high salt content and 4°C employed to ensure complete protein precipitation). To ensure maximum phase separation, 200 μl methylene chloride were added and the sample frozen. After thawing, the upper organic layer was aspired. The aqueous layer contained 75—85% of I, and the chromatogram was relatively free of plasma components. The acetonitrile—methylene chloride layer had no detectable I. Representative HPLC chromatograms of a plasma blank and sample are shown in Fig. 2.

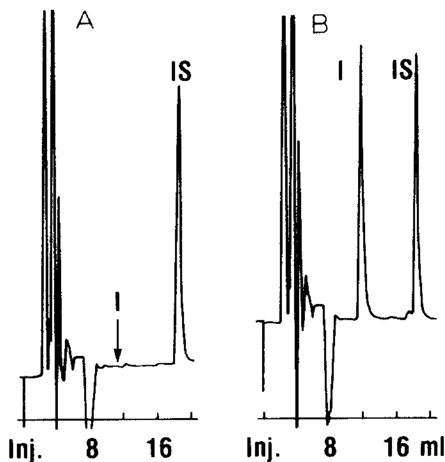

Fig. 2. HPLC—UV chromatograms of cefpimizole isolated from plasma; subject 1 given a 2000-mg dose. (A) Predose plasma; (B) 6-h plasma.

Sample preparation procedure for urine samples

The microbiology results [4] indicated that a relatively high urinary concentration of the parent compound (high μg/ml to mg/ml) could be expected. A simple procedure where a 100-μl aliquot of urine was diluted with 4.0 ml of HPLC eluent containing the IS and the solution filtered through a 0.45-μm filter provided an acceptable chromatogram with a quantifiable sensitivity of 15 μg/ml urine. Representative HPLC chromatograms of a urine blank and sample are shown in Fig. 3.

Linearity, precision and reproducibility of the sample preparation procedures

The procedures for the isolation of I in plasma and urine were validated for linearity, precision, and reproducibility by fortifying blank plasma and urine samples. For plasma samples, the concentration range was 17—0.33 μg/ml; and for urine was 800—17 μg/ml. The results of these validation studies are

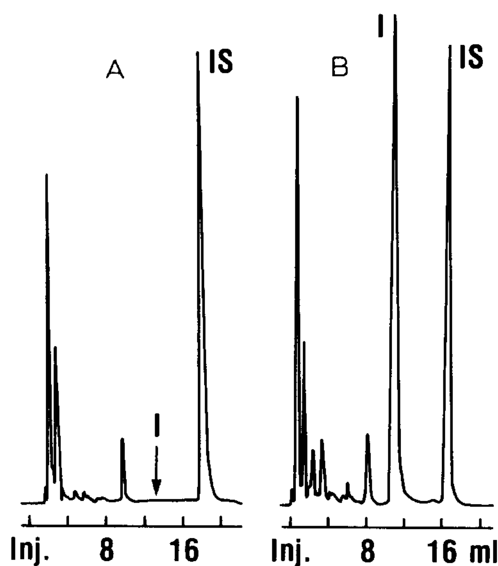

Fig. 3. HPLC—UV chromatograms of cefpimizole in urine; subject 1 given a 2000-mg dose. (A) Predose urine; (B) 6-h urine.

TABLE I

LINEARITY AND REPRODUCIBILITY OF PLASMA AND URINE DETERMINATION OF I

Plasma, 1.0-ml aliquots, n = 5, average recovery = 78.7 ± 1.3%. Urine, 100-μl aliquots, n = 4, average recovery = 99.3 ± 2.4%.

Plasma			Urine		
Concentration (μg/ml)	Average found (μg/ml)	Relative standard deviation	Concentration (μg/ml)	Average found (μg/ml)	Relative standard deviation
16.78	13.51	3.8	839	864	2.8
13.42	10.79	1.9	671	686	2.3
10.07	7.91	3.6	503	508	2.8
6.71	5.34	2.6	420	422	1.6
5.03	3.90	3.6	335	336	2.3
3.36	2.65	2.6	252	252	2.0
2.52	1.97	6.3	210	213	1.6
1.68	1.29	5.6	168	168	1.0
1.34	1.04	5.6	126	125	1.2
1.01	0.78	9.3	83.9	81.6	3.4
0.67	0.52	10.5	67.1	64.6	2.0
0.33	0.27	11.9	50.3	50.0	2.7
0	<0.05	—	33.6	32.7	2.8
			16.8	15.9	7.7
			0	<1.0	—

summarized in Table I. The least-squares linear-regression equations and correlation coefficients were $Y = (0.804 \pm 0.004)X - (0.044 \pm 0.031)$, r = 0.999 for plasma, and $Y = (1.028 \pm 0.005)X - (4.6 \pm 1.7)$, r = 0.999 for urine. The Y-intercept for the plasma and urine specimens was statistically

insignificant ($p > 0.05$). The minimal quantifiable level (defined as the lowest level assayed with an R.S.D. of less than 15% for multiple prepared samples on separate days) for plasma was 0.33 μg/ml and for urine was 16.8 μg/ml. The detection limit (based on signal-to-noise ratio of 5:1) was 0.05 μg/ml for plasma and 1.0 μg/ml for urine.

Stability in physiological fluids

The limited stability of I in aqueous solution indicated that stability studies in physiological fluids were necessary. The compound was added to plasma, serum, and urine and maintained at room temperature. At timed intervals, aliquots were taken and analyzed by the procedure described earlier. The results are shown graphically in Fig. 4 where the percent of the compound remaining (% T_0) is plotted versus time. Very rapid loss of I was observed with 80% of the original level being detected at 1 h for plasma, 4.5 h for serum, and 27 h for urine. The least-square linear-regression equations given in Fig. 4 showed that the loss of I in each matrix was linear ($r^2 > 0.96$) and the slope significant ($p < 0.05$). These data indicated that pharmacokinetic evaluations of I would be subject to substantial error due to the rapid loss of the compound in physiological samples.

When EDTA was added to plasma or urine containing I, a substantial increase in the room temperature stability was observed (Fig. 5). The 80% T_0 level was reached at 48 h for plasma containing EDTA and at 240 h for urine containing EDTA. Additional studies at −30°C for plasma—EDTA and urine—EDTA fortified with I indicated that collected specimens would be stable (greater than 95% of original level) for over 100 days when stored at −30°C.

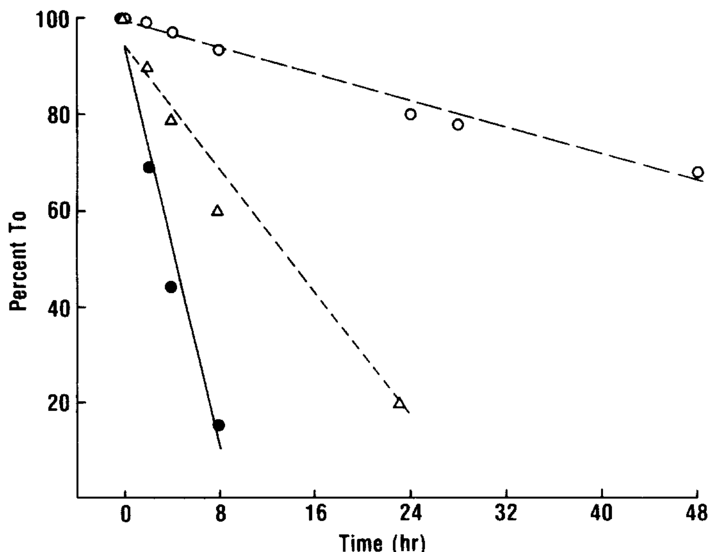

Fig. 4. Stability of cefpimizole in plasma, serum and urine samples maintained at room temperature. (•—•) Plasma; (△— —△) serum; (○— — ○) urine. Linear regression: plasma, $Y = (-10.4 \pm 0.8X) + (93.2 \pm 3.8)$, $r^2 = 0.96$; serum, $Y = (-3.17 \pm 0.22X) + (93.5 \pm 2.5)$, $r^2 = 0.96$; urine, $Y = (-0.68 \pm 0.04X) + (99.1 \pm 0.9)$, $r^2 = 0.96$.

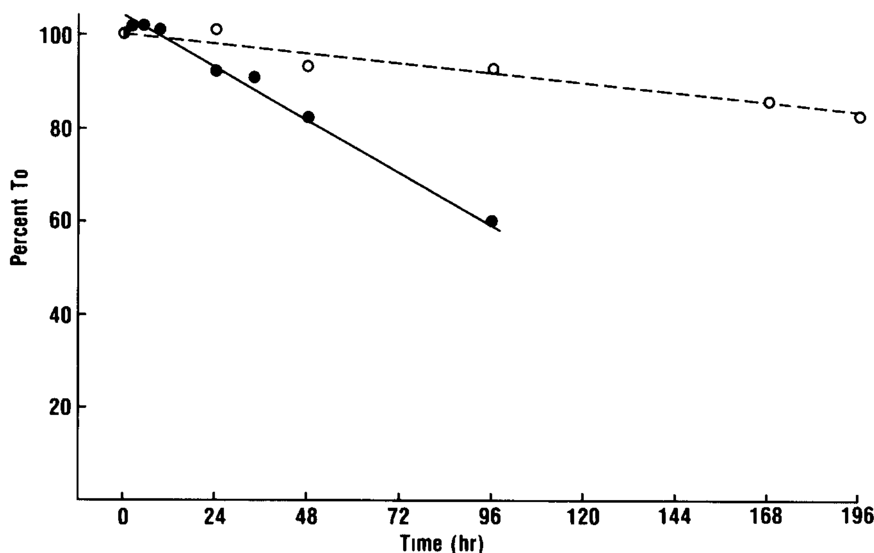

Fig. 5. Stability of cefpimizole in plasma—EDTA and urine—EDTA samples maintained at room temperature. (●—●) Plasma; (○— —○) urine. Linear regression: plasma, $Y = (-0.471 \pm 0.020X) + (104.1 \pm 0.8)$, $r^2 = 0.98$; urine, $Y = (-0.085 \pm 0.005X) + (100.0 \pm 1.0)$, $r^2 = 0.96$.

Plasma and urine specimen analysis

The utility of the developed HPLC methods for I in plasma and urine specimens was demonstrated by preparing and assaying samples obtained from a human volunteer receiving three intravenous doses of the drug.

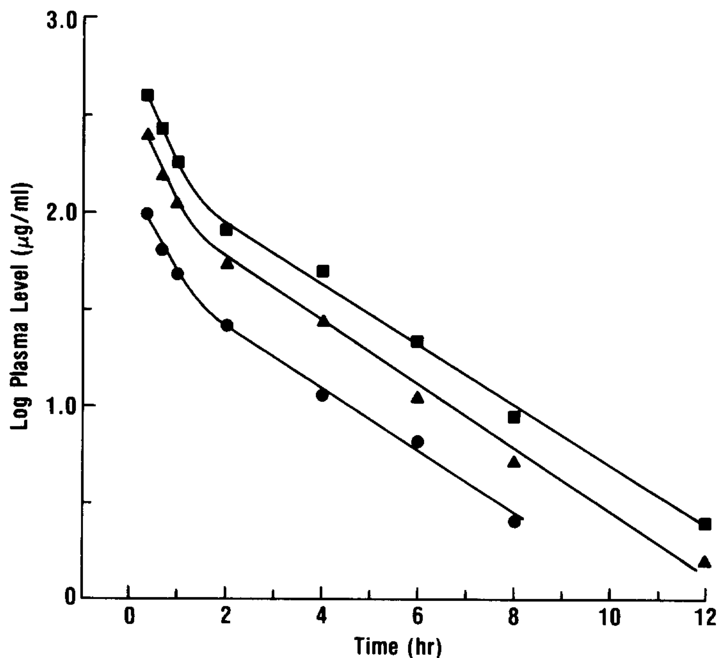

Fig. 6. Plasma concentration—time plot of cefpimizole from a human volunteer. Intravenous administration of three dose levels: (■—■) 4000-mg dose; (▲—▲) 2000-mg dose; (●—●) 1000-mg dose.

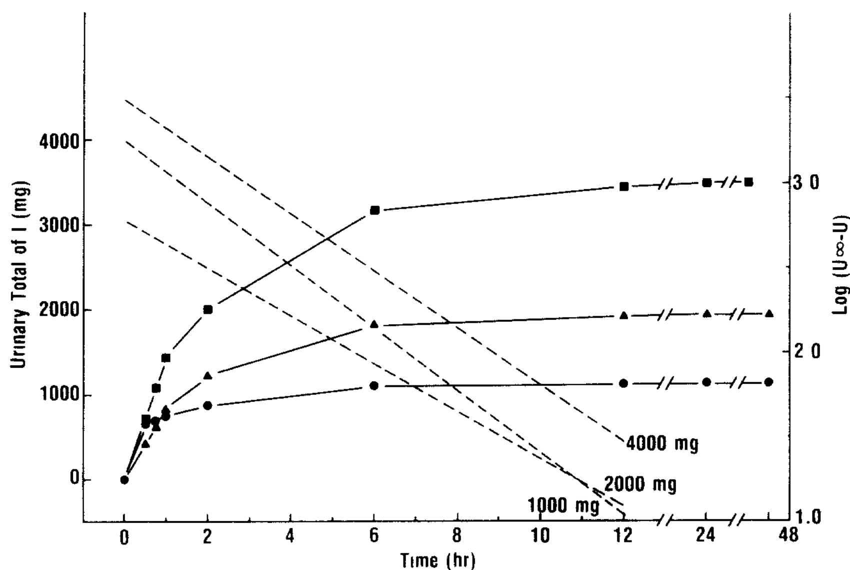

Fig. 7. Cumulative urinary excretion—time plot of cefpimizole from a human volunteer. Dashed lines represent log (U_T − U)—time plots for each dose level. (■—■) 4000-mg dose; (▲—▲) 2000 mg dose; (●—●) 1000-mg dose.

The results for the plasma assays of I are shown graphically in Fig. 6 where the log plasma concentration versus time is plotted for the three dose levels. The data indicate a two-compartment body model. Since the dose was administered by constant-rate infusion over a 20-min period and no samples were obtained during this time, the plasma level of the drug during the infusion process is not included. The urine assay results are shown in Fig. 7 where the cumulative amount excreted is plotted against time. The dashed lines represent least-squares linear-regression plots of the log (U_T − U), i.e., total amount excreted − amount excreted to time t, versus time and indicate a first-order elimination process for I. The average amount of the unchanged drug excreted in the urine was over 75% during the first 6 h after administration.

Estimates of various pharmacokinetic parameters were made by subjecting the plasma and urine data to computer fitting using a NONLIN [11] program for a two-compartment constant-rate infusion model. The estimated plasma pharmacokinetic parameters (Table II) were fairly uniform at the three dose levels indicating that the drug was behaving similarly independent of dose, i.e. dose-independent pharmacokinetics. The urine data was evaluated graphically

TABLE II

PHARMACOKINETIC PARAMETER ESTIMATES FOR I FROM A HUMAN VOLUNTEER

Dose (mg)	Distribution rate constant α (h^{-1})	Terminal rate constant β (h^{-1})	Volume of distribution V_d (l)	Area under curve AUC (μg h/ml)	Total body clearance Cl_T (ml/min)	Elimination rate constant K_e (h^{-1})	Percent dose excreted (%)
1000	2.31	0.366	16.1	169.8	98.2	0.322	114.4
2000	1.98	0.349	15.0	391.1	85.2	0.424	97.2
3000	2.32	0.358	17.2	648.9	102.7	0.352	87.5

by plotting the log ($U_T - U$) versus time to estimate the urinary elimination rate constant (K_e) (Table II). As with the plasma pharmacokinetic parameters, the K_e and percent dose excreted were similar at the three dose levels indicating that the excretion of I was independent of dose. Additional pharmacokinetic evaluations of I are planned to more fully define the physiological distribution and elimination process for this drug.

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