Pharmacokinetics of Mitomycin C in Dogs: Application of a High-Performance Liquid Chromatographic Assay

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Abstract □ A normal-phase high-performance liquid chromatographic (HPLC) assay was developed for the determination of mitomycin C in plasma and urine. The method involves extraction of mitomycin C from plasma or urine into ethyl acetate-2-propanol-chloroform (70:15:15) with UV detection at 365 nm. Quantitation was performed with an internal standard (porfiromycin) by the peak height ratio method. Excellent correlation was obtained between the HPLC assay and the established microbiological cup-plate bioassay. The pharmacokinetics of mitomycin C were investigated in beagle dogs following a 1-mg/kg iv (22-mg/m^2) bolus dose. The plasma mitomycin C concentration *versus* time data were analyzed by using an open three-compartment model. The average volume of distribution was 1.90 L or 17% of body weight for the central compartment and 7.7 L or 68% of body weight for the terminal elimination phase. The volumes of distribution at steady state, calculated by model-dependent and -independent methods, compared very well with each other and were 6.5 L or 58% of body weight. Total body clearance averaged 112 mL/min, and the mean terminal plasma half-life was 53 min. The 0-24-h urinary excretion of intact mitomycin C accounted for 19% of the dose. The terminal half-life and percent urinary recovery of mitomycin C in dogs is similar to that in humans. Based on these observations, the dog appears to be a good model for studying the disposition of mitomycin C.

Keyphrases □ Mitomycin C—pharmacokinetics in dogs, HPLC □ Pharmacokinetics—mitomycin C in dogs, HPLC

Mitomycin C, an anticancer antibiotic, was first introduced for clinical trials in the United States over two decades ago, but its use was limited due to bone marrow toxicity. The development of high-dosage, intermittent schedules and its use in combination with other anticancer agents has improved the therapeutic index of mitomycin C against a variety of tumors (1–3). The therapeutic index and toxicity are reported to be dependent on dose and schedule (4).

The pharmacokinetics of mitomycin C in animals and humans have not been fully described, mainly due to the lack of a sensitive and specific assay. Preliminary pharmacokinetics of mitomycin C have been reported in humans (5, 6) and rabbits (6). Recently, assay methodologies involving high-performance liquid chromatography (HPLC) (6, 7) and enzyme immunoassay (8) have been described for the determination of mitomycin C in serum.

The HPLC assays utilize reverse-phase columns with UV detection at 365 nm. One of the HPLC assays (7) has a sensitivity of 40 ng/mL in plasma, making it unsuitable for pharmacokinetic studies. In addition, the assay involves acidification of plasma prior to the extraction of mitomycin C into ethyl acetate. As mitomycin C is unstable in acidic and basic solutions (5, 9, 10), it may degrade before being extracted. A modified, stability-indicating HPLC assay involving extraction of mitomycin C at neutral pH in plasma has been reported (5). The disadvantage of this method relates to the fact that no internal standard is used in the assay. In a third HPLC assay (6), a nonionic exchange resin column is utilized for the extraction of mitomycin C and the internal standard. The assay has a sensitivity of ~5–10 ng/mL; however, it is expensive to run on a routine basis because of the cost of the resin columns. The enzyme immunoassay (8) may lack the necessary specificity for pharmacokinetic studies.

In the present study, a normal-phase HPLC assay for mitomycin C in plasma and urine was developed, compared with an established microbiological cup-plate assay (11), and applied to a study of the pharmacokinetics of mitomycin C after intravenous bolus administration in beagle dogs.

EXPERIMENTAL SECTION

Reagents—Mitomycin C[1], porfiromycin[2], tetrahydrofuran[3], dichloromethane[4] (glass distilled), ethyl acetate[3] (glass distilled), isooctane[5] (pesticide grade), 2-propanol[4] (glass distilled), methanol[5] (certified ACS), and chloroform[4] (glass distilled) were used as received from the suppliers.

Animals—Three healthy female beagle dogs (weight, 10.7–12 kg) were used in the study. Each dog was restrained in a sling; the urinary bladder was catheterized, and indwelling venous catheters were placed in the saphenous veins of both hind legs.

Dose Preparation and Administration—To each vial[6], containing 20 mg of mitomycin C and 40 mg of mannitol, was added 40 mL of sterile distilled water. The vials were gently shaken until a clear solution of mitomycin C was obtained. A 1-mg/kg dose was administered by a rapid bolus (1-min) injection through a saphenous vein catheter.

Sample Collection—Samples of venous blood (8 mL) were collected in heparinized tubes just before dosing and at 2, 5, 10, 15, 20, 30, and 45 min, and 1, 1.5, 2, 3, 4, 5, 6, and 7 h after drug administration. A venous catheter placed contralaterally to the one used for intravenous administration of the drug was used for all blood collections. Blood samples were centrifuged immediately after collection, and the plasma was separated and stored at −20°C. Urine samples were collected in a graduated cylinder and immersed in crushed ice before dosing and 1, 3, 5, 7, and 24 h after dosing. After each collection interval, the volume of urine was recorded, and 10 mL of urine was stored at −20°C.

Extraction of Mitomycin C from Plasma and Urine—The extraction solvent consisted of ethyl acetate-2-propanol-chloroform (70:15:15). The reconstitution solvent was chloroform-methanol (9:1). The internal standard was 1000-ng of porfiromycin/mL in methanol.

Plasma (2 mL) or urine (0.2 mL), 1.0 mL of distilled water, 7.0 mL of the extraction solvent, and 0.1 mL of the internal standard solution were transferred into a 16 × 125-mm screw-capped tube. The tube was capped with a polytef-lined screw cap and gently rotated[7] for 20 min. The tube was centrifuged[8] at 3000 rpm for 10 min and the upper organic phase was transferred into a clean 16 × 125-mm tube. The solvent was evaporated to dryness under nitrogen. The residue was dissolved in 50 μL of the reconstitution solvent, and 25 μL was injected on the liquid chromatograph.

Chromatography—The HPLC system was the same for mitomycin plasma and urine assays and consisted of a solvent pump[9], an injector[10], a 10-μm particle size normal-phase silica column[11], a fixed-wavelength UV detector[12]

[1] Lot no. 11210; Bristol Laboratories, Syracuse, N.Y.
[2] Lot no. 6891; Bristol Laboratories.
[3] MCB Manufacturing Chemist, Inc., Cincinnati, Ohio.
[4] Burdick and Jackson, Inc., Muskegon, Mich.
[5] Fisher Scientific Co., Fair Lawn, N.J.
[6] Mutamycin, 20 mg for injection, lot no. D1C01; Bristol Laboratories.
[7] Model 343, Roto-Rack; Fisher, Springfield, N.J.
[8] International Centrifuge, model UV; Scientific Glass, Bloomfield, N.J.
[9] Model M-45 solvent delivery system; Waters Associates, Milford, Mass.
[10] Model U6K injector; Waters Associates.
[11] μPorasil P/N 27477 N/SA no. 115735, Waters Associates.
[12] Model 441 UV absorbance detector; Waters Associates.

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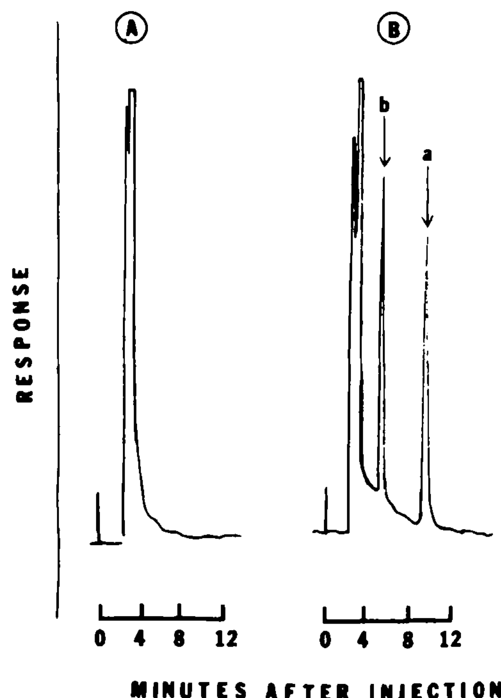


Figure 1—*Chromatograms from plasma containing no drugs (A) and plasma containing 116 ng of mitomycin C/mL (a in B) and 48 ng of porfiromycin/mL (b in B).*

with a 365-nm filter, and a guard column[13]. The mobile phase consisted of tetrahydrofuran-methanol-isooctane-dichloromethane (3:7:15:75). The solvent flow rate was maintained at 1.0 mL/min. HPLC analysis was performed at ambient temperature.

Data Processing—The peak heights were measured manually or with an automated laboratory data system[14], and the ratio of the mitomycin C to porfiromycin peaks for each chromatogram was calculated. After each series of injections, the regression of peak height ratio *versus* concentration of mitomycin C standard was calculated by least-squares analysis, and the concentration of mitomycin C in each plasma or urine sample was estimated by inverse prediction.

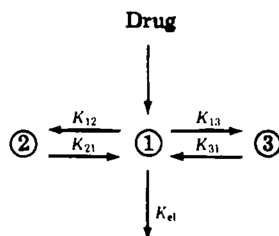
Comparison with Bioassay—Samples of plasma and urine from dogs have also been assayed by a cup-plate bioassay (11), with *Escherichia coli B* A22636 as the test organism. The assay was sensitive to 10 ng/mL of mitomycin C on plasma or urine.

Pharmacokinetic Calculations—After intravenous administration, unchanged mitomycin C concentration-time curves showed a triexponential decay on a semilogarithmic graph. Thus, an open three-compartment model, with elimination occurring from the central compartment, was proposed to describe mitomycin C kinetics (Scheme I).

The data were fitted according to:

$$C_p = Pe^{-\pi t} + Ae^{-\alpha t} + Be^{-\beta t} \quad \text{(Eq. 1)}$$

where C_p is the concentration of mitomycin C in plasma at time t; π, α, and β are the exponential coefficients during the three phases; and P, A, and B are the concentrations of the exponential terms at time zero. Estimates of these parameters were first obtained by standard graphical analysis and then by computer program P-AUTOAN which is a combination of the programs



Scheme I—*Linear three-compartment model with elimination from the central compartment.*

[13] 2.54 cm long packed with 37-50 μCorasil; Waters Associates.
[14] Model 3354; Hewlett Packard, Avondale, Pa.

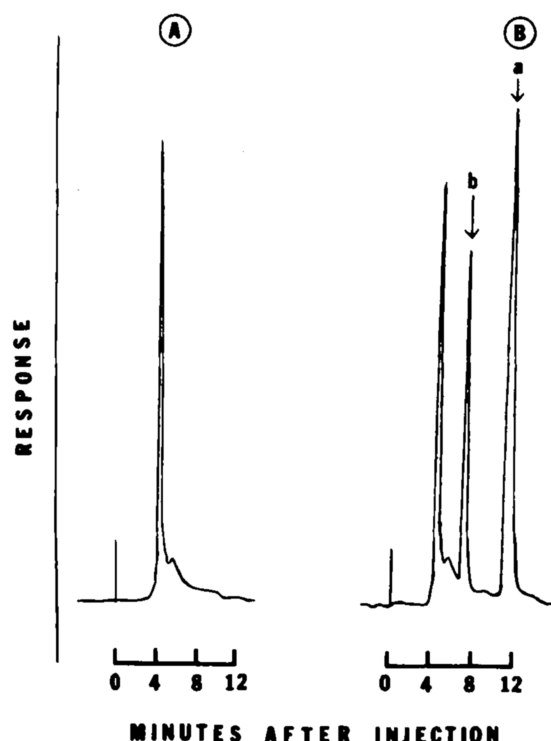


Figure 2—*Chromatograms from urine containing no drugs (A) and urine containing 878 ng of mitomycin C/mL (a in B) and 77 ng of porfiromycin/mL (b in B).*

CSTRIP (12) and GUESS (13). For computer analysis, all plasma level data were squared, weighted according to their reciprocals, and fitted to mono-, bi-, tri-, and tetraexponential equations. The model equation with Akaike's minimum information criterion (14) was chosen as the best representation for the time course data. The values obtained for each parameter were then used to calculate other kinetic parameters, such as intercompartment rate constants (k_{12}, k_{21}, k_{13}, and k_{31}), the overall elimination rate constant (k_{el}), terminal elimination phase and central compartment volumes of distribution (Vd_β and V_c, respectively), and total (CL_{tot}) and renal (CL_r) clearances, using conventional methods (15). The steady-state volume of distribution (Vd_{ss}) was calculated by model-dependent (16) and -independent (17) equations (Eqs. 2 and 3, respectively):

$$Vd_{ss} = (1 + k_{12}/k_{21} + k_{13}/k_{31})V_c \quad \text{(Eq. 2)}$$

$$Vd_{ss} = \frac{\text{Dose} \cdot \left(\int_0^\infty t\, C_p dt\right)}{\left(\int_0^\infty C_p dt\right)^2} \quad \text{(Eq. 3)}$$

The areas under the plasma concentration-time curves (AUC) were calculated by the trapezoidal rule with extrapolation to infinite time.

RESULTS AND DISCUSSION

Assay—Mitomycin C and the internal standard (porfiromycin) can be readily extracted into organic solvent from plasma and urine at neutral pH. As mitomycin C and porfiromycin are very stable at neutral pH and are subjected to rapid decomposition in acid and base (5, 9, 10), extraction was carried out at neutral pH. The extraction efficiency of mitomycin C and the internal standard in plasma and urine was not significantly affected between pH 7.0 and 7.9 and was >90% with a phase volume ratio of plasma extractant of at least 1:3.5. The normal-phase chromatographic procedure was developed to separate mitomycin C from endogenous plasma and urine components, the internal standard, and possible metabolite(s). A mobile phase consisting of ethyl acetate-2-propanol-chloroform (70:15:15) was found to be most suitable for the selective determination of mitomycin C in plasma and urine. Mitomycin C has maximum UV absorbance at 365 nm (5); detection at 254 nm resulted in decreased sensitivity and increased interference at or near the mitomycin C or porfiromycin peaks. A 365-nm UV filter was therefore selected for the optimum sensitivity and specificity.

Typical chromatograms obtained from blank plasma samples and from

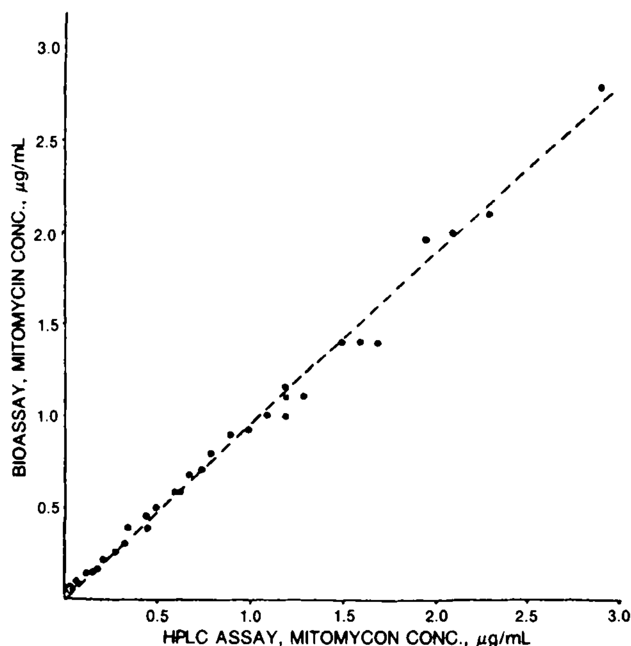


Figure 3—*Comparison of HPLC assay and bioassay of mitomycin C in plasma: slope 0.93; intercept, 0.008;* r *= 0.996.*

plasma samples containing mitomycin C and porfiromycin are shown in Fig. 1. Mitomycin C was completely separated from porfiromycin, and there was no interference from any endogenous substance in the plasma samples obtained from dogs. The retention times for mitomycin C and porfiromycin were 10.5 and 5.6 min, respectively, and the response (concentration *versus* peak height ratio) was linear in the range of 1-2000 ng of mitomycin C per mL. Standard curves obtained from repeated determinations of plasma standards were $y = 0.009x + 0.033$, $r = 0.999$. Typical chromatograms obtained from blank urine samples and from urine samples containing mitomycin C and porfiromycin are shown in Fig. 2. The retention times for mitomycin C and porfiromycin were the same as in the plasma assay. The urine assay for mitomycin C was linear in the range of 200-2000 ng/mL. Standard curves obtained from repeated determinations of urine standards were $y = 0.0008x - 0.027$, $r = 0.999$.

A set of 10 plasma samples was prepared to contain 5 ng of mitomycin C/mL and carried through the assay procedure for determining accuracy and precision of the assay. The estimated sample concentrations were then used to calculate the observed mean (5.3 ng/mL), *SD* (0.61), *RSD* (11.0%) and mean recovery (106%).

A set of 10 urine samples was prepared to contain 1000 ng of mitomycin C/mL and carried through the assay procedure for determining accuracy and precision of the assay. The estimated sample concentrations were then used to calculate the observed mean (1019 ng/mL), *SD* (35), *RSD* (3.39%), and mean recovery (102%).

Comparison of the Liquid Chromatographic Assay and the Bioassay—Since the lower limit of detection for the plasma bioassay was 10 ng/mL, values of <10 ng/mL found by the HPLC assay were excluded from the comparison of the two assays. The paired assay values of mitomycin C by HPLC assay and bioassay were highly correlated, with a correlation coefficient of 0.996 (Fig. 3). Excellent correlation between the HPLC assay and bioassay suggests that both assays are specific, and metabolite(s) of mitomycin C may not be microbiologically active. Pharmacokinetic calculations were performed by employing the values obtained from the plasma HPLC assay because of its greater sensitivity (1 ng/mL).

Pharmacokinetics of Mitomycin C in Dogs—Following the bolus intravenous administration of mitomycin C, a triexponential plasma level curve was observed in all three dogs (Fig. 4). The graphic and computer analyses of the data suggest that the pharmacokinetic evaluation would require a three-compartment open-model system (Scheme I), assuming that all processes are linear and that elimination occurs from the central compartment (plasma) which is connected to two peripheral compartments. The summary of pharmacokinetic parameters is shown in Table I.

The rate constants associated with the rapidly and relatively slowly equilibrating sites in the peripheral compartments ranged from 27.6 to 42.2 h^{-1} for π and from 3.73 to 7.86 h^{-1} for α, respectively. These values correspond to apparent half-life ranges of 1-1.5 and 5-11 min, respectively. The terminal elimination rate constant (β) ranged from 0.66 to 0.89 h^{-1}, corresponding to an apparent half-life range of 47-63 min. The magnitude of the three hybrid rate constants π, α, and β reflect an extremely rapid initial distribution of mitomycin C and a relatively slow elimination rate of the drug from plasma.

The individual rate constants k_{12}, k_{21}, k_{13}, and k_{31} reflect the rate of distribution into and out of the shallow and deep peripheral compartments. The mean ratio of k_{21}/k_{12} of 1.4, which reflects the return and entry of the drug within the shallow peripheral compartment, suggests rapid equilibration and free transfer of the drug between the central and shallow peripheral compartments. The mean ratio of k_{31}/k_{13} of 0.66, which reflects the return and entry of the drug within the deep peripheral compartment, suggests somewhat slower equilibration plus the possibility of drug binding to tissues or protein sites in this compartment, with a corresponding slower return of the drug to the central compartment. The mean ratio of β/k_{el} of 0.23 indicates that only 23% of the mitomycin C in the body is in the central compartment and available for elimination at any time (18).

The volume of distribution of mitomycin C in the central compartment (V_c) was calculated to be 16, 14, and 22% of body weight for dogs 17637, 17876, and 17516, respectively. The Vd_{ss} calculated by model-dependent (16) and -independent (17) methods were in agreement with each other and ranged from 35 to 62% of body weight in the three dogs. The values calculated for Vd_{β} were higher than Vd_{ss} and ranged from 58 to 78% of body weight.

The 24-h excretion of intact mitomycin C in urine ranged from 19.2 to 19.8% of the dose, most of which was excreted in the first 7 h. The total body clearance for mitomycin C ranged from 90 to 129 mL/min, and renal clear-

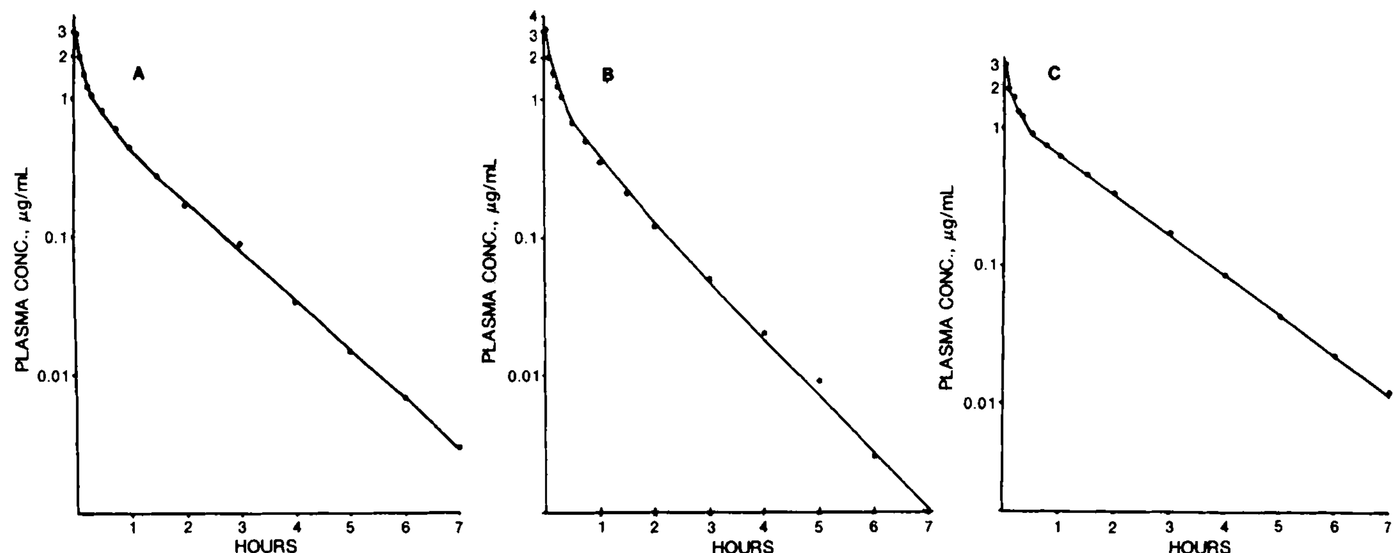


Figure 4—*Plasma levels of mitomycin C in three dogs after a single 1-mg/kg intravenous dose. Key: (●) experimental values; (—) computer-predicted curve; (A) dog 17516; (B) dog 17637; (C) dog 17874.*

Table I—Pharmacokinetic Profiles of Mitomycin C in Dogs after Single Intravenous Doses

Parameters	Dog 17637	Dog 17874	Dog 17516	Mean
Dose, mg	10.7	12.0	11.0	11.2
Dose, mg/kg	1	1	1	1
P, μg/mL	3.61	3.69	2.37	3.22
A, μg/mL	1.74	2.11	1.16	1.67
B, μg/mL	0.96	1.23	0.94	1.04
π, h^{-1}; $t_{1/2\pi}$, h	42; 0.016	38; 0.018	28; 0.025	36; 0.02
α, h^{-1}; $t_{1/2\alpha}$, h	4.8; 0.14	7.86; 0.09	3.73; 0.18	5.5; 0.14
β, h^{-1}; $t_{1/2\beta}$, h	0.89; 0.78	0.66; 1.05	0.82; 0.85	0.79; 0.89
k_{12}, h^{-1}	18.1	11.9	10.8	13.6
k_{21}, h^{-1}	20.0	21.1	14.3	18.5
k_{13}, h^{-1}	3.2	7.3	2.2	4.2
k_{31}, h^{-1}	2.2	3.0	2.0	2.4
k_{el}, h^{-1}	4.5	3.2	2.9	3.5
Ratio β/k_{el}	0.20	0.20	0.28	0.23
AUC, μg/mL/h	1.5267	2.2197	1.5477	1.7647
V_c, L; % of body weight	1.7; 16	1.7; 14	2.4; 22	1.9; 17
Vd_{ss}, L; % of body weight[a]	5.9; 55	4.2; 35	6.8; 62	5.6; 51
Vd_{ss}, L; % of body weight[b]	5.8; 54	6.8; 57	6.8; 62	6.5; 58
Vd_{β}, L; % of body weight	6.2; 58	8.2; 68	8.6; 78	7.7; 68
Percent of Dose Excreted in Urine (0-24 h)	19.2	19.2	19.8	19.4
Cl_{tot}, mL/min	129	90	118	112
Cl_r, mL/min	22	17	23	21

[a] Calculated by the model-independent method. [b] Calculated by the model-dependent method.

ance ranged from 17 to 23 mL/min in the three dogs. The large differences between renal and plasma clearances can be attributed to extensive biliary and/or metabolic clearances of mitomycin C. The terminal half-life and percent recovery of the mitomycin C dose in the urine of dogs is very similar to those values in humans (19).

The HPLC assay described for mitomycin C in plasma is sensitive to 1 ng/mL and should be suitable for elucidating the pharmacokinetics of mitomycin C in humans after therapeutic doses. There appears to be no interference from any metabolite in dogs. Drug-free human plasma and urine samples from different individuals do not have any interfering peak with mitomycin C or porfiromycin. However, possible interference from other drugs which may be coadministered with mitomycin C must be determined before use of this assay in humans. The pharmacokinetic data of mitomycin C in dogs are similar to preliminary pharmacokinetic data in humans (19). The dog may, therefore, be a good model for studying the disposition of mitomycin C.

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