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Research Article

A Method to Study Drug Concentration–Depth Profiles in Tissues: Mitomycin C in Dog Bladder Wall

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Determination of the depth of penetration of locally applied drug therapy and evaluation of possible mechanisms of drug transport require knowledge of drug concentration-versus-tissues depth profiles. A method to determine the drug concentration–depth profile is needed. We have devised such a method and used it to determine the penetration of mitomycin C (MMC) in the dog bladder wall after intravesical drug instillation. This method is based on sectioning of frozen tissue into 40-μm segments, followed by drug extraction and high-pressure liquid chromatography analysis. Tissue concentrations could be detected with a sensitivity of 1 ng/sample, or 20 ng/g for tissue samples of approximately 2 × 2 cm. This sensitivity was sufficient to describe the penetration of MMC in the bladder wall of dogs, using an identical instillation technique, dwell time, and MMC concentration as in human patients. Tissue concentrations were expressed relative to tissue weight or tissue protein contents. For MMC, standardization to tissue weight yielded a better mathematical fit of the concentration-versus-depth profiles than standardization to protein content. The time interval between tissue harvesting and freezing was critical. The MMC concentration at the urothelial side of dog bladders was 2- to 10-fold higher in samples processed immediately after harvesting, compared to samples processed after 1 hr or longer. This significant decrease was not due to drug metabolism *in situ*. In separate *in vitro* experiments, we found that the degradation of MMC in 8% tissue homogenate was relatively slow, with only a 30% decline in concentration over 24 hr. We speculate that the decrease in concentration was due to passive diffusion of MMC, away from the urothelial side. In summary, the present study demonstrates that determination of drug penetration into tissues *in vivo* is feasible.

KEY WORDS: tissue concentration profile; bladder wall; dog; mitomycin C.

INTRODUCTION

In various therapeutic situations, drugs are applied to biological membranes to achieve therapeutic concentrations in the underlying tissue or as a means to deliver drug to the systemic circulation. Examples include the intravesical administration of anticancer agents for the treatment of superficial bladder cancer, administration of ophthalmic drugs, transdermal administration for local or systemic therapy, and intraperitoneal instillation of drugs for the treatment of ovarian and colorectal tumors (1). The characterization of drug concentrations as a function of depth in tissue is critical to the understanding of the process of drug absorption, and pharmacodynamics of local therapy. Various models of drug penetration through biological barriers have been proposed. Simple membrane models assuming a linear concentration decline with distance (2), combined models assuming aqueous diffusion through pores and cellular membrane partitioning (3), and a distributed model including diffusion and capillary absorption (4,5) have all been applied to studies of drug penetration. Experimental verification of these models has been limited because of a lack of effective methods for the determination of *in vivo* tissue concentrations, and the proposed models have been verified by measurement of overall tissue permeability and breakthrough times rather than actual tissue concentrations (6–8).

Flessner *et al.* used quantitative autoradiography to determine the gradient of radiolabeled substances in adjacent tissues following peritoneal dialysis (9). This procedure provides detailed tissue concentration data but requires the availability of a radiolabeled form of the compound of interest and analysis by computerized microdensitometry. Aeikens *et al.* obtained random biopsy specimens from bladders of patients treated with intravesical chemotherapy with mitomycin C (MMC) (10). Drug concentrations at various tissue depths were analyzed. The data showed large variability and were not suitable to verify mathematical models of drug transfer. Detailed methodology and concentration–depth profiles were not reported. Schaefer *et al.* determined a concentration–depth profile of retinoic acid in skin (11). The

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large variability of their results was possibly the result of degradation of the retinoid on the skin surface (12).

As a part of our studies to determine the efficacy of MMC after intravesical instillation, the depth of penetration of MMC in the bladder wall is of importance. The present study describes a method for the determination of tissue concentrations and gradients in the bladder wall, based on sectioning of frozen tissue in thin segments parallel to the epithelial surface, tissue extraction, and drug analysis in these tissue layers.

MATERIALS AND METHOD

Chemicals

All high-pressure liquid chromatographic (HPLC) solvents and reagents were of analytical grade and were purchased from Fisher Scientific Co. (Fair Lawn, NJ). MMC was a gift from Bristol-Myers Co. (Wallingford, CT). The internal standard, porfiromycin (PFM), was a gift from American Cyanamide (Pearl River, NY). HPLC analysis showed that MMC and PFM were >99% pure. Both chemicals were used as received. MMC and PFM solutions can be stored at −20°C for up to 3 weeks with negligible degradation (<4%) as determined by chromatographic analysis.

Apparatus

Frozen tissues were sectioned by cryotome (Reichert/American Optical, Buffalo, NY). The HPLC system consisted of a solvent delivery system (Spectroflow 400, Kratos Analytical Instruments, Ramsey, NJ), an automated injector (WISP 710B, Waters Associates, Milford, MA) or a manual injector (Model 7125, Rheodyne, Cotati, CA), a fixed-wavelength UV detector with 365- and 254-nm filters (Model 441, Waters Associates, Milford, MA), and an HP 3390A integrator (Hewlett-Packard, Menlo Park, CA). Tissue samples were homogenized at room temperature with a Biohomogenizer (Biospec Products, Inc., Bartlesville, OK). Tissue weights were determined with a precision of 0.1 mg on a Mettler balance (Mettler AE-100, Mettler Instruments Corp., Hightstown, NJ). Protein determination was performed using a spectrophotometer (Beckman DU-40, Beckman Instruments, Inc., Arlington Heights, IL).

Harvesting and Freezing of Tissue Specimens

Tissue concentrations in the bladder wall of dogs were determined after intravesical instillation of MMC. Drug was instilled through a 10–14 french Foley catheter and left in the bladder for periods of 5 to 120 min. At the end of the instillation period, the dog was anesthetized with pentobarbital. The bladder was exposed by a midline abdominal incision, and the left and right lateral sides of the bladder were marked with superficial stitches of surgical silk. The following steps were executed in rapid succession. (a) The bladder contents were removed via the indwelling Foley catheter. (b) The blood supply to the bladder was clamped off by placement of a clamp around the urethra and adjacent major blood vessels. (c) The bladder was excised. (d) The bladder was separated in tissue segments of approximately 2 × 2-cm surface area as follows. The bladder was opened with scissors, starting from the urethra, and separated into the right and left lateral sides. The incision was ended approximately 1 cm before the tip of the dome. Then the dome area was cut out as a circle of 1-cm radius around its tip. The remaining bladder tissue was separated in the left and right lateral sides. The tissue of the trigone area and tissue in excess of the desired 2 × 2-cm surface area were trimmed. (e) The tissue segments were blotted with absorbent paper to remove unabsorbed drug solution. (f) All tissue segments were rapidly frozen by placement of the urothelial side on a flat stainless-steel plate, cooled on dry ice. The middle part of the tissue segment was gently pressured against the cold surface first, and subsequently the remaining tissue areas were brought in contact with the steel surface. Care was exercised not to distend the tissue during the freezing process. Liquid nitrogen was poured on top of the tissues for rapid freezing. The time interval between clamping of the blood flow and freezing of the tissues was less than 2 min.

Cryotome Sectioning of Tissue

The frozen tissue segments were trimmed at −20°C with a No. 22 scalpel blade to remove tissue at the perimeter. This step eliminated the risk of contamination of the tissue edges by the intravesical dosing solution. Also, parts of the tissue that did not have a flat urothelial surface were removed. The frozen tissue was glued onto the cryotome object holder with Tissue-tek cryoadhesive (Miles Laboratories, Inc., Naperville, IL), such that the outer surface of the serosa was anchored on the cryotome object holder, and the urothelial surface was exposed for section. The cryoadhesive material was applied in moderation so that it was present only between the bladder specimen and the object holder. This was to avoid contaminating the sectioned tissue segments with cryoadhesive material, which would interfere with the tissue weighing and analysis. The position of the tissue in the cryotome was carefully adjusted to bring the tissue urothelial surface in exact alignment with the cutting blade. Tissues were sectioned into 40-μm thicknesses using the cryotome. Tissue segments were placed in preweighed nalgene microcentrifuge tubes (Sarstedt, Princeton, NJ), and stored frozen. For tissues between 0 and 400 μm, each 40-μm segment was analyzed individually. For tissues between 400 and 1200 μm, two segments were pooled for analysis. For tissues beyond 1200 μm, four segments were pooled. Weights of collected tissue samples (~5 to 50 mg) were determined by the difference of the empty and tissue-containing tubes. A maximal error of 0.6 mg is expected in this measurement, based on differences of ≤0.6 mg between repeated measurements of the same tubes (n = 10). Collection of tissue segments in 50-ml centrifuge tubes would be attractive, as it eliminates one transfer step in the procedure. However, the weight of these tubes (~10 g) precluded accurate determination of the tissue weights by the differential measurement technique. At selected tissue depths (400, 1200, 2800 μm), a 5-μm frozen section was taken and stained with hematoxylin and eosin to correlate the histological tissue type with depth. Further, a transverse frozen tissue section was taken from each tissue specimen prior to sectioning, in order to estimate the actual thickness of the bladder wall and its histological structures.

Tissue Concentration Analysis

Tissue concentration analysis was developed based on the plasma and urine assay described previously (13). The procedure is outlined in Fig. 1. To the weighed tissue samples collected in nalgene microcentrifuge tubes, 1 ml of a 200 m*M* phosphate buffer, pH 7.0, was added. The samples were vortexed until all tissue floated freely in the buffer. The samples were then transferred to a 50-ml nalgene culture tube. Porfiromycin (PFM; 25 μl of a 10-μg/ml solution in water) was added as the internal standard, and 10 ml of ethyl acetate as the extraction solvent. The total sample was homogenized for 60 sec with a rotor/stator type homogenizer, centrifuged (2000*g* × 10 min), and stored frozen (−20°C) overnight. The organic phase was decanted in 16 × 100-mm glass culture tubes while the aqueous phase remained frozen. The organic phase was then evaporated to approximately 0.5 ml under a flow of nitrogen gas. The concentrated extracts were transferred to microcentrifuge tubes, followed by an ethyl acetate rinse (0.2 ml). At the time of HPLC injection, the remaining organic phase was evaporated under nitrogen. The samples were reconstituted in 50 μl of mobile phase, and 20 μl was injected. Sample evaporation and transfer were a critical step in the extraction procedure (13). The duration of sample evaporation was, therefore, kept to a minimum.

HPLC Analysis

MMC concentrations in the tissue extracts were determined by HPLC using a reversed-phase C_{18} column (Pecosphere, 83 × 4.6 mm, 3 μm, Perkin–Elmer, Norwalk, CT). The aqueous mobile phase consisted of 12.5% acetonitrile in a 5 m*M* phosphate buffer adjusted to pH 6.9 with NaOH. The solvent flow was maintained at 1.5 ml/min. All analyses were performed at ambient temperature. The elution volumes for MMC and PFM were 7.5 and 11.8 ml, respectively. Blank tissue shows no detectable interference at the elution time of MMC. The limit of detection was 20 ng/g for a 50-mg tissue sample.

MMC Degradation in Tissue

The rate of degradation of MMC in whole bladder tissue and in tissue homogenates was determined. To determine the *in situ* MMC degradation in whole tissue, dogs received intravesical instillation of MMC. At the time of tissue harvesting, adjacent segments of bladder wall were either directly frozen or maintained at room temperature for 1 or 2 hr. Tissue concentrations in specimens with or without immediate freezing were compared. The *in vitro* degradation of MMC in tissue homogenates was determined using 8% homogenates of bladder tissue from untreated dogs in phosphate-buffered saline, pH 7.4. The initial MMC concentration was approximately 2 μg/ml, or 30 μg/g wet tissue. Separate tubes were prepared for time intervals of 0- to 1440-min incubation at room temperature.

2 cm × 2 cm frozen tissue block
↓
mount on cryotome
↓
cut in 40 micron segments
↓
place 1-4 segments in pre-weighed microcentrifuge tubes
↓
weigh, transfer to 50 ml tubes, add internal standard
↓
homogenize with 10 ml ethyl acetate
↓
evaporate, reconstitute, inject on HPLC

Fig. 1. Schematic outline of bladder tissue analysis.

Protein Determination

The protein contents of bladder tissue specimens of some animals were determined after extraction of MMC. The aqueous phase remaining after extraction was heated in a 100°C water bath for 15 min to remove residual ethyl acetate. The sample was diluted to exactly 2.5 ml with saline and made alkaline with 1 ml NaOH, 5 *N*. Samples were incubated overnight at 37°C and heated in a 100°C water bath for 30 min to ensure digestion of the tissue protein. The protein concentration was determined in an aliquot of the clear solution by the Coomassie blue method (Sigma kit 610, St. Louis, MO) (14). Bovine albumin was used as protein standard. Standard samples were treated as tissue samples, except for the incubation and heating steps. These steps were omitted as the bovine albumin formed a precipitate during the digestion procedure.

Regression and Statistical Evaluations

Best-fit curves of tissue concentration-versus-depth profiles and MMC decomposition over time were determined by nonlinear least squares regression using the Gauss–Newton method and the NLIN procedure of SAS (Cary, NC) (15). The following equation was used to fit the tissue concentration-versus-depth profile (5).

$$C(\text{depth}) = C(u) \cdot e^{-0.693/w_{1/2} \cdot \text{depth}}$$

where depth was the tissue depth from the urothelial surface, $C(u)$ was the extrapolated tissue concentration at zero depth, $0.693/w_{1/2}$ was the logarithmic slope of the concentration–depth relationship, and $w_{1/2}$ was the half-width or distance over which the tissue concentration fell to one-half of its original value.

Standardizations of tissue concentrations to tissue weight or to protein content were compared by the coefficients of determination (r^2) and the unpaired Student's *t* test. For the Student's *t*-test comparison, the percentage deviation of each data point from the best-fit line was determined for the two standardization methods. The null (H_0) hypothesis was that the distributions of the percentile deviations from the best-fit lines were equal for the two standardizations.

RESULTS

Evaluation of Methods of Tissue Homogenization

Bladder tissue segments were cut as previously described. Five methods were evaluated to achieve optimal conditions for tissue extraction of MMC. Tissue extraction was attempted after (a) no homogenization, (b) homogenization by a Potter–Elvehjem tissue grinder with Teflon piston,

(c) ultrasonic dismembrator, (d) rotor/stator-type homogenizer in aqueous solution, and (e) rotor/stator-type homogenizer in aqueous/organic two-layer system. Direct extraction of these segments without homogenization resulted in erratic concentration–depth profiles. The tissue segments often formed tight rolls that may have hampered effective extraction. Homogenization with a Potter–Elvehjem-type tissue grinder tended to flatten, but not homogenize the bladder wall tissue. The ultrasonic dismembrator disrupted the tissue, but complete homogenization could not be achieved in 5 min. Homogenization of bladder tissue in 1 ml of phosphate buffer with the rotor/stator-type homogenizer was essentially complete within 60 sec except for some membranous tissue elements. Some tissue remained attached to the rotor of the homogenizer and was difficult to recover quantitatively. Sample homogenization in the presence of the aqueous and the organic phases achieved sample homogenization and extraction simultaneously and reduced the amount of tissue remaining in the homogenizer assembly. This method was selected for sample preparation.

Determination of MMC Penetration in Dog Bladder Tissue

Figure 2 shows the MMC concentration-versus-depth profiles of the bladders of two dogs. The concentrations apparently declined as an exponential function of tissue depth, although some data points deviated from the best-fit concentration–depth relationship. This may be due to the heterogeneous nature of the tissue, as observed by histological examination. Histologic sections at 400-μm tissue depth showed that most of the surface area consisted of connective tissue (Fig. 3). Occasionally, an area was visible where the urothelium had recessed by more than 400 μm. These areas appeared as a narrow band without cells, surrounded by a dense layer of urothelial cells. Sections at 1200- and 2800-μm tissue depth showed muscle bands, alternating with areas of loosely packed connective tissue.

MMC Degradation in Bladder Tissue

MMC undergoes enzymatic and nonenzymatic degradation. The effect of tissue processing on the drug concentration–depth profile was examined. Bladder tissue concentrations were analyzed after immediate freezing of the tissue specimens. The effect of a time delay between tissue harvesting and freezing is shown for two dogs in Fig. 2. A decrease in the logarithmic slope of the concentration–depth curve was apparent in both animals. The computer-fitted half-width (distance corresponding to a 50% decline in tissue concentrations) was approximately two to three times larger in the tissues that were not immediately frozen subsequent to harvesting when compared to tissues which were immediately processed. The tissue concentrations at the urothelial side declined approximately 10-fold or more.

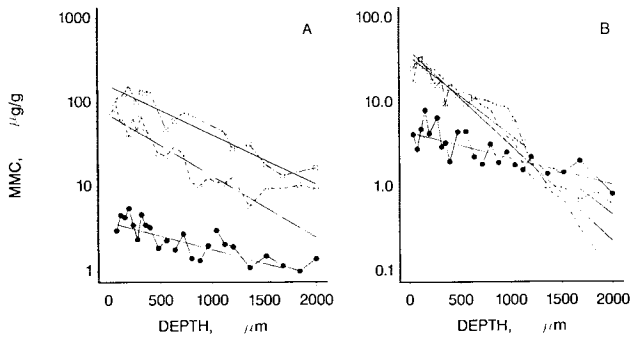

Fig. 2. Bladder wall concentration versus depth profiles of MMC in two dogs after instillation of MMC (20 mg/40 ml water) for 120 (A) or 7 min (B). Samples were from different sites of the bladder, i.e., dome (open circles) and right lateral side (open squares). The effect of a time delay between tissue harvest and freezing is also shown. Filled circles: frozen after 60 min (A) or 130 min (B) at room temperature. Open symbols: tissue segments from the same bladder, processed immediately.

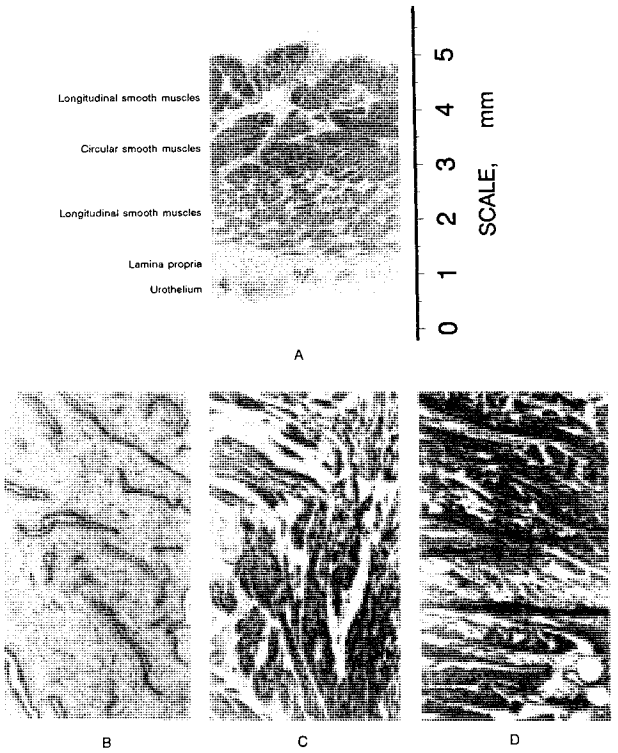

Fig. 3. (A) Transverse section of the dog bladder wall, showing, from bottom to top, the urothelium (three to five cell layers thick), the lamina propria, and three muscle layers. (B, C, D) Cryotome sections, cut in parallel with the urothelium at distances of 400, 1200, and 2800 μm from the urothelial surface, respectively. All photomicrographs were taken at the same magnification.

In phosphate-buffered saline, MMC disappeared with a half-life of approximately 90 hr. In comparison, the half-life of MMC in an 8% bladder tissue homogenate was 45 hr. The shorter half-life of MMC in tissue homogenate indicates that MMC was removed by bladder tissue through metabolism and/or tissue binding.

Standardization of Bladder Wall Concentrations Relative to Tissue Weight or Protein Contents

Bladder tissue is heterogeneous, as it is composed of various types of cells, matrix materials, extracellular space, and capillary space. MMC may have a preferential distribution in the extracellular space or be associated with any of the tissue components. Expression of tissue concentrations

relative to tissue weight is a conventional method of standardization. Another method of standardization, more reflective of cellular density in a tissue sample, is expression of concentrations relative to tissue protein. These methods were compared (Fig. 4). The standardization of concentrations relative to protein contents show more variability in the concentration–depth profile than standardization relative to tissue weight. The coefficient of determination (r^2) for the logarithm of concentration versus depth was greater than 0.9 for the weight-standardized concentrations. The corresponding r^2 for the protein-standardized concentrations was less than 0.8. When variability was expressed as the deviation (percentage) of the observed data from the best-fitting line, the variability in the protein standardized data (78.5 ± 98.6 and 41.9 ± 41.6%; Figs. 4A and B, respectively; filled symbols) was larger than that in the data standardized to tissue weight (28.7 ± 16.5 and 28.0 ± 25.2%; open symbols). In one of the two samples (Fig. 4A), the variability in the weight-standardized concentrations was significantly lower than in the protein-standardized concentrations ($P < 0.05$).

The protein contents per milligram of wet tissue weight is shown in Figs. 4C and D and shows a greater than 10-fold range of values for this ratio throughout the tissue. The average protein contents of the bladder tissue was approximately 1%, lower than the 10% reported by others for rat bladder (16). The reason for this difference is unclear. The variable protein contents at different tissue depths supports the heterogeneous nature of the bladder wall. Another possible cause of variation is a poor assay precision for the protein determination, or incomplete tissue digestion. Samples were included in the data evaluation only if tissue digestion resulted in visually clear solutions, suggesting that tissue digestion was complete. The precision of the protein assay was determined in samples taken from a well-mixed batch of minced tissue and was 24%. This value was considerably smaller than the coefficient of variability in the protein contents per milligram wet tissue weight (70 and 121% for Figs. 4C and D, respectively), suggesting that the precision in the protein assay could not account for the variability in the protein data.

DISCUSSION

We have described a method to study the *in vivo* penetration of drugs into tissues, using MMC and the dog bladder as a model. Our data indicate a logarithmic decline in drug

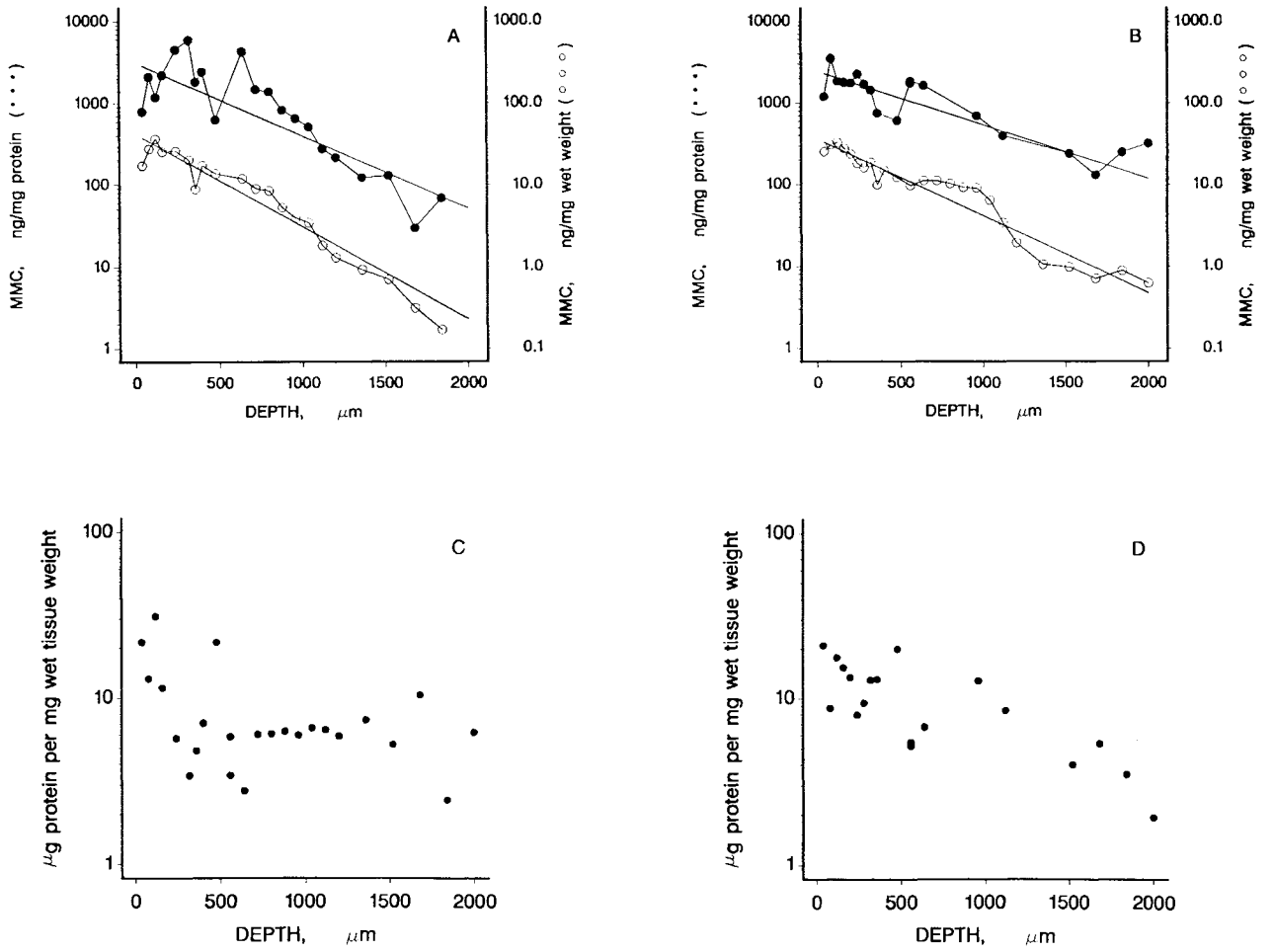

Fig. 4. Comparison of MMC concentrations relative to tissue weight (open circles) or tissue protein contents (filled circles) in two different animals. (A) Dog 1; (B) dog 2. The tissue protein contents per milligram of wet tissue weight as a function of tissue depth in the bladders of the same dogs are shown in C (dog 1) and D (dog 2).

concentration with respect to tissue depth, consistent with the distributed model of drug penetration (4,5). In the distributed model of drug penetration, the concentration–depth profile is determined by passive drug diffusion and drug removal by the capillary blood flow. After the tissue is excised from the host, the tissue perfusion has ceased, while passive diffusion continues. Therefore, the time interval between harvesting of bladder tissue and freezing of the tissue is an important factor for the accurate determination of the *in vivo* concentration–depth profile. A more shallow concentration–depth profile was found in tissue specimens maintained at room temperature for 1 hr or longer before processing, as compared to immediately processed specimens (frozen in <2 min after clamping of the blood supply). This indicates that diffusion of MMC from the urothelial to the serosal side has indeed affected the tissue concentration profile. Further studies are necessary to determine whether significant drug diffusion occurs within the 2-min tissue handling time.

The bladder wall is a heterogeneous tissue and is composed of an epithelial tissue layer, basement membrane, lamina propria, and three intertwined muscle layers. Loosely packed connective tissue is found between muscle bundles, with blood vessels interspersed throughout the tissue (17). The unbound or reversibly bound MMC that is analyzed by our method could be associated mainly with extracellular water or mainly with cellular material. Expression of concentrations relative to tissue weight was selected as a convenient and reproducible means of standardization. This method allows direct comparison of concentrations in tissue with concentrations in urine and plasma and appears appropriate for MMC. Other parameters, such as protein and/or DNA measurements, may be useful to standardize drug concentrations for compounds which have a predominant association with these tissue components. Similarly, standardization relative to a marker for extracellular volume is potentially useful.

The described method to prepare bladder tissue for determination of concentration–tissue depth profiles was developed to study the extent of penetration of MMC into the bladder wall. This method can be used for the *in vivo* study of the mechanisms of drug transport in bladder tissue, as well as for other tissue sites. The acceptability of drug candidates is limited only by assay sensitivity, since small samples sizes (6 to 100 mg in our study) are used. This limitation can be overcome in some cases by the use of radiolabeled compounds.

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