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Note

High-performance liquid chromatographic determination of mitomycin C in rat and human plasma and urine

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Mitomycin C (MMC) is an antitumor agent produced by the actinomycete *Streptomyces caespitosus* It is used clinically by intravenous administration to treat systemic disease [1,2] and by intravesical administration to treat superficial bladder cancer [3,4] There are limited plasma pharmacokinetic data [3] and there have been no studies elaborating the concentration–time profile of MMC in urine during intravesical administration This is partly due to a lack of sensitive assays of MMC to measure the low drug concentrations after this mode of administration The present report describes an analytical method to determine the concentration of MMC in the plasma and urine of patients and rats that receive intravesical MMC

MMC has been analyzed by microbiological [2,5], immunological [6], and high-performance liquid chromatographic (HPLC) [7–9] assays Both normal- and reversed-phase HPLC have been used In most methods, MMC is separated from the biological matrix using a solid–liquid extraction where the sample is passed through an adsorption resin and MMC is subsequently eluted from the resin [3,10] Choi et al [9] have described a sample pre-treatment using a loop-column procedure which requires modification of a Rheodyne injector Barbhaiya et al [5] used a liquid–liquid extraction followed by normal-phase HPLC The detection limits of these previous methods are reported to range from 1 to 25 ng/ml We report here a method to analyze MMC using a liquid–liquid extraction and reversed-phase HPLC with an improved detection

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limit of 0 5 ng/ml in human plasma This method does not require specialized equipment, avoids the additional expense of the pre-treatment resin columns, and avoids the potential complications of normal-phase HPLC Preliminary disposition data obtained from a patient and a rat are also reported here to demonstrate its applicability to the analysis of the low plasma MMC concentrations following intravesical treatment

EXPERIMENTAL

Chemicals

All HPLC solvents and reagents were of analytical grade and were purchased from Fisher Scientific (Fair Lawn, NJ, U S A) MMC was a gift from Bristol-Myers (Wallingford, CT, U S A) Porfiromycin (PFM) was a gift from American Cyanamid (Pearl River, NY, U S A) 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 three weeks with negligible degradation (<4%) as determined by chromatographic analysis

Apparatus

Similar HPLC techniques were used for plasma and urine samples The HPLC system consisted of a solvent delivery system (Spectroflow 400, Kratos Analytical Instruments, Ramsey, NJ, U S A), an automated injector (WISP 710B, Waters Assoc , Milford, MA, U S A) or a manual injector (Model 7125, Rheodyne, Cotati, CA, U S A), a fixed-wavelength UV detector with 365- and 254-nm filters (Model 441, Waters Assoc), and an HP 3390A integrator (Hewlett-Packard, Menlo Park, CA, U S A) The stationary phase was a reversed-phase C_{18} column (Pecosphere™, 83 mm×4 6 mm I D , 3 μm, Perkin-Elmer, Norwalk, CT, U S A) The aqueous mobile phase consisted of 12 5% acetonitrile and 2 5 m*M* phosphate buffer adjusted to pH 6 9 with dilute phosphoric acid The solvent flow-rate was maintained at 1 5 ml/min All analyses were performed at ambient temperature

Animal study

A six-month-old female Fischer rat (Charles River Breeding Lab , Kingston, NJ, U S A) weighing 170 g was used for the intravesical study One day prior to the experiment, a permanent catheter was inserted into the right external jugular vein of the rat under light ether anesthesia The animal was housed in a metabolic cage with access to food and water ad libitum overnight On the day of the experiment, the animal was anesthesized and the bladder was catheterized with a 15-cm piece of polyethylene tubing (PE-10, I D 0 38 mm and O D 1 09 mm, Becton Dickinson, Parsippany, NJ, U S A) The polyethylene tubing was covered with 4 cm of flexible tubing (Silastic®, I D 0 63 mm and O D 1 19 mm, Dow Corning, Midland, MI, U S A) to prevent leakage of the

instilled MMC around the urethral catheter. The bladder was emptied by gentle suction through the catheter and 1 mg MMC in 0 1 ml of a 50 50 mixture of propylene glycol–water was placed in the bladder The urethral catheter was then flushed with a small amount of air to insure complete instillation of the drug The urethral catheter and MMC remained in the bladder for a period of 2 h. Serial blood samples (150–200 μl each) were withdrawn through the venous catheter and collected for a period of 2.5 h Blood samples were placed in heparinized tubes and kept on ice to prevent clotting and/or MMC decomposition After each sampling, the lost blood volume was replaced with an equal volume of saline. Blood samples were centrifuged at 1100 *g* for 10 min at 0°C. The plasma fraction was stored at −20°C until assay No urine samples were obtained from the intravesical study in the rat due to the limited volume of the bladder instillate in this experiment At the completion of the experiment, exploratory surgery and macroscopic examination were performed to ascertain that the bladder mucosa stayed intact and that the catheterization procedure did not produce any physical damage to the bladder.

Patient study

A patient with superficial bladder cancer was treated by transurethral tumor resection Intravesical MMC was administered one day after surgery. Intravesical therapy consisted of 20 mg MMC in 40 ml saline placed in the bladder through an in-dwelling Foley catheter and retained for 2 h Afterwards, the bladder MMC instillate and the Foley catheter were removed. Serial venous blood samples (8 ml each) were taken through a heparin lock and urine samples (0.1 ml each) were taken from the urethral catheter Blood and urine samples were taken prior to, during, and after the 2-h instillation period for up to 6 h Blood and urine samples were processed and stored as described above.

Sample extraction

Plasma and urine samples obtained from patients and rats were processed as follows PFM (1.07 mg in 50 ml of 0.5 *M* phosphate buffer, pH 7) was used as an internal standard The patient urine samples were diluted 100-fold with water prior to extraction. For the patient urine samples and rat plasma samples, either 50 μl of diluted urine or 50 μl of rat plasma was mixed with 10 μl of PFM and then extracted with 1.2 ml of ethyl acetate–chloroform–2-propanol (70 15 15) The sample was vortex-mixed for 10 s and then centrifuged at 1100 *g* for 10 min at ambient temperature The organic layer was transferred to an Eppendorf tube and evaporated to dryness in a vortex vacuum evaporator (Buchler, Saddle Brook, NJ, U.S A.). For human plasma, 1 ml of plasma was mixed with 10 μl of PFM and 90 μl of 2 *M* phosphate buffer (pH 7) and then extracted with 10 ml of ethyl acetate The sample was vortex-mixed for 10 s, frozen rapidly in a mixture of dry ice–acetone after settling, allowed to thaw for 1 min, and then vortex-mixed again for 10 s. Following centrifugation at

1100 *g* for 10 min at ambient temperature, the organic layer was transferred to a 10-ml disposable glass tube and evaporated to dryness under a stream of nitrogen at ambient temperature The glass tubes were then rinsed with two separate 100-μl aliquots of methanol. The methanol fraction was transferred to an Eppendorf tube and evaporated to dryness in the vortex vacuum evaporator Standard curves were prepared for MMC in patient and rat plasma and urine.

Comparison of extraction solvents

Blank plasma samples from rats were spiked with various concentrations of MMC Triplicate sets of spiked plasma were carried through the extraction procedure as outlined above for rat plasma. Peak-height ratios of MMC to PFM were determined for plasma samples extracted utilizing PFM as both an internal and an external standard. Extraction yields were calculated as peak-height ratios obtained with an external standard divided by peak-height ratios obtained with an internal standard

RESULTS AND DISCUSSION

HPLC analysis

The elution volumes of MMC and PFM were 5 8 and 9 0 ml, respectively Representative chromatograms of the HPLC analysis of human plasma samples extracted with ethyl acetate are shown in Fig. 1. Fig. 1A is a blank plasma sample and Fig 1B is a plasma sample containing 2 ng/ml MMC

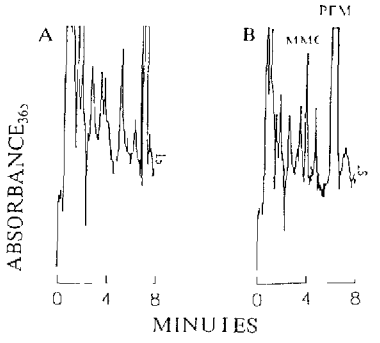

Fig 1 Representative chromatograms of human plasma samples extracted with ethyl acetate The stationary phase was a C_{18} Pecoshere, 83 mm×4 6 mm I D , 3 μm column The mobile phase consisted of 12 5% acetonitrile and 2 5 m*M* phosphate buffer adjusted to pH 6 9 (A) Blank (injected 70 μl of 100-μl sample), (B) plasma spiked with 2 ng/ml MMC (injected 50 μl of 100-μl sample)

Comparison of extraction solvents

Patient urine samples and rat plasma samples were extracted with the ethyl acetate–chloroform–2-propanol (70 15 15) mixture as described. For patient plasma samples, it was necessary to compare different extraction solvents because of the low MMC concentrations and the interfering endogenous substances. Three solvents were compared in regards to their percentage extraction yield, sample clean-up, and convenience of extraction. The extraction yields and standard deviations were 88 7 ± 2 0% for ethyl acetate–chloroform–2-propanol (70 15 15), 85.4 ± 8.9% for chloroform–2-propanol (50 50), and 93 1 ± 4.7% for ethyl acetate (100%) The extraction yields of the three solvents were not significantly different at a 5% level of significance The mixture of chloroform–2-propanol (50 50) was found to be inconvenient for extraction, due to the higher density of the organic phase relative to the aqueous plasma layer. This resulted in a more difficult transfer of the organic phase A considerable loss in the volume of the aqueous plasma layer also occurred upon extraction with this mixture. The mixture of ethyl acetate–chloroform–2-propanol (70 15 15) did not exhibit a higher density than the aqueous plasma layer at room temperature. However, it was serendipitously discovered that the density of this mixture varies significantly enough with temperature to undergo inversion of the two phases at temperatures near 10°C This mixture also produced a considerable loss in the volume of the aqueous plasma layer In both cases, the loss of aqueous plasma layer may be attributed to the uptake of water by 2-propanol present in the organic phase In addition, the hydrophilic nature of 2-propanol led to the extraction of the more hydrophilic endogenous compounds in plasma This resulted in additional peaks in the chromatogram which either interfered with MMC elution or significantly prolonged the analysis time. Ethyl acetate alone has a lower density than the aqueous plasma layer. Hence, the transfer of the organic phase was easier. Further, the endogenous interferences which were extracted with the mixtures of ethyl acetate–chloroform–2-propanol and chloroform–2-propanol were not extracted by ethyl acetate alone. For these reasons, ethyl acetate was chosen as the extraction solvent for human plasma

Following the extraction of human plasma, the organic layer was dried under a stream of nitrogen at ambient temperature. Use of the vacuum vortex evaporator for drying purposes provided an incomplete and variable dissolution of MMC from the glass tubes An apparent tight binding of MMC to the glass tubes occurred under vacuum conditions Attempts to re-dissolve and transfer MMC using mobile phase, methanol, and siliconized glass tubes did not improve the completeness and/or reproducibility of the extraction and transfer yield. A similar problem in transferring MMC was noted by Den Hartigh et al [7]. We found that this tight binding could be avoided by the evaporation of the organic phase under nitrogen coupled with the dissolution in two 100-μl aliquots of methanol.

Standard curves

The standard curve obtained from the extraction of spiked rat plasma was linear over the concentration range 0 1–10 μg/ml The regression equation for this line was $y=0\ 658x-0\ 010$ with a correlation coefficient of 0 999 The intra-day coefficients of variation ranged from 1 3 to 5 6%

The standard curve obtained from the extraction of spiked human plasma was linear over the concentration range 0 5–50 ng/ml The regression equation for this line was $y=0\ 0107x+0\ 0015$ with a correlation coefficient of 0 999 The intra-day coefficients of variation ranged from 2 4 to 7 5%. The lower detection limit of this assay was 0 5 ng/ml The enhanced detectability of this assay, as compared to those previously reported, is due to the higher efficiency of the stationary phase and the lack of interference from endogenous substances under the assay conditions The small particle size (3 μm) gives a greater number of theoretical plates and thus a larger peak height-to-width ratio and improved resolution

The standard curve obtained from the extraction of spiked human urine was linear over the concentration range 100–1000 μg/ml The regression equation for this line was $y=0\ 00308x-0\ 00740$ with a correlation coefficient of 0.999 The intra-day coefficients of variation ranged from 0 7 to 5.4% for this curve. The detection limit of the urine assay could be increased by using 1 ml of the diluted urine sample instead of the 50-μl sample assay described

Application to human and rat samples

Fig 2 shows the plasma concentration–time profile of MMC in a rat given an intravesical dose of 1 mg No MMC was recovered from the bladder at the end of the 2 5-h experiment, suggesting that the entire dose was absorbed MMC was not detected in 5- and 10-min plasma samples The first plasma sample which showed an appreciable MMC concentration was the 15-min sample, this indicating a lag time for MMC absorption The post-administration plasma concentration declined exponentially

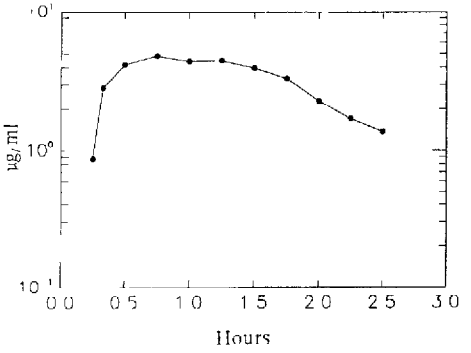

Fig 2 Plasma MMC concentration–time profile in a rat given an intravesical dose of 1 mg MMC

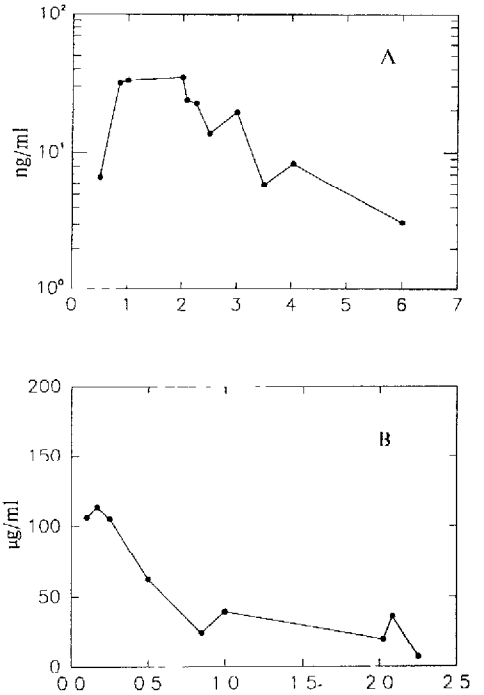

Fig 3 Concentration–time profiles in a patient given an intravesical dose of 20 mg MMC (A) Plasma profile, (B) urine profile The MMC concentration in the dosing solution was 351 µg/ml

The plasma and urine concentration–time profiles in a patient given an intravesical dose of 20 mg MMC per 40 ml are shown in Fig 3A and B The urine MMC concentration declines from the original concentration of the dosing solution of 351 1 to 19.8 µg/ml at the end of the 2-h instillation period This decline in concentration is due partly to the absorption of MMC from the bladder and partly to dilution by the production of 112 ml of urine MMC was not detected in the 5-, 10-, and 15-min samples. The first plasma sample which showed a measurable MMC concentration was the 30-min sample This again indicates a lag time for absorption of MMC as in the rat. The post-administration plasma concentrations declined exponentially.

CONCLUSIONS

We have described here an analytical method for MMC utilizing a liquid–liquid extraction and reversed-phase chromatography This assay has an improved lower limit of detection (0 5 ng/ml) than previously published assays for human plasma. As shown in this report, this assay is useful for the phar-

macokinetic studies of MMC in the plasma and urine during and after intravesical administration in rats and patients

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