Plasma mitomycin C concentrations determined by HPLC coupled to solid-phase extraction

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The aim of this study was to set up a method for quantification of plasma mitomycin C (MMC) concentrations during intravesical chemotherapy delivered in the presence of local bladder hyperthermia (HT). In comparison with existing methods, this assay, characterized by relative simplicity and efficiency, resulted in the facilitation of performance with nondedicated instrumentation or nonspecialized staff. Purification from plasma matrix was carried out by solid-phase extraction under vacuum. The purified drug was then collected directly into the vials of the HPLC autosampler. Chromatographic analysis was performed on a reversed-phase C18 column with water:acetonitrile (85:15 by vol) as the mobile phase and the UV detector set at 365 nm. The use of porfiromycin as internal standard provided a method with good within-day precision (CV 6.0% at 5 \( \mu \)g/L, \( n = 6 \)), linearity (0.5–50 \( \mu \)g/L), and specificity. The lower limit of detection (\( \leq 0.5 \mu \)g/L) proved to be suitable for plasma pharmacokinetics monitoring in two tested patients treated with MMC+HT for superficial bladder cancer.

INDEXING TERMS: plasma assay • bladder hyperthermia • intravesical chemotherapy • porfiromycin • pharmacokinetics

Mitomycin C [aziridine(2',3',3,4) pyrrole (1, 2-\( \alpha \)) indol-4, 7-dione-6-amino-1,1a,2,8a,8b-exahydro-8-(hydroxymethyl) 8a-metoxy-5-methylcarbamate] (MMC) is an antimicrobial and anticancer agent that was isolated in 1958 from the fermentation fluid culture medium of *Streptomyces caespitosus* [1].2 Since then, several clinical trials involving MMC as a single agent or in combination with other chemotherapeutic drugs and with different dosage schedules have been reported [2]. The most significant MMC toxicity in humans is myelosuppression, widely confirmed to be a delayed and dose-related effect [3]. Anemia, hepatotoxicity, and renal failure, although unusual, have also been well documented. One of the most peculiar MMC administration routes is intravesical instillation as a preventive therapy for the recurrence and stage progression of superficial bladder cancer after transurethral resection. Intravesical administration has resulted in extremely low serum concentrations, thus with virtually no systemic side effects [4, 5]. Several investigations [6] have clearly evidenced a synergistic interaction between local increase of tumor temperature and anticancer agents' tumoral cell killing capacity. In our Institute, a new system (SBTS-101®) designed to simultaneously deliver local bladder hyperthermia (HT) and intravesical chemotherapy (ICT) has been recently developed and clinically tested on recurrent superficial transitional cell carcinoma of the bladder (STCCB) [7, 8]. Preliminary results [9] were suggestive of a better effectiveness of microwave-induced HT combined with local chemotherapy in respect to chemotherapy alone. However, the question is whether temperature enhancement of bladder mucosa could potentially affect the systemic absorption of the cytostatic drug, rendering important the monitoring of plasma concentration–time profile during ICT+HT treatment.

To quantify MMC in biological fluids, a wide number of different analytical methods have been applied on the basis of microbiological [10], immunological [11], and chromatographic (HPLC) procedures [12–17]. None of the aforementioned methods, however, completely presented the characteristics of sensitivity (<0.5 \( \mu \)g/L), precision, rapidity, and simplicity ideally required. Our aim was to develop a low-cost HPLC assay for MMC determination in serum that could be easily transferred to the routine laboratory and, therefore, also carried out by nonspecialized personnel and without dedicated or unusual instrumentation.
**Materials and Methods**

**CHEMICALS**
The organic solvents (HPLC grade) were obtained from J.T. Baker (Deventer, The Netherlands). Bidistilled water was used throughout the study.

**CALIBRATION SOLUTIONS AND CALIBRATORS**
As calibrator MMC, Mitomycin-C® (injectable powder at 4% purity in NaCl) from Kyowa Hakko Kogyo (Tokyo, Japan) was used. As internal standard, porfiromycin (PFM) was synthesized in Japan (>99% pure) and kindly donated by Kyowa Hakko Kogyo. MMC and PFM stock solutions were prepared at 1.72 mmol/L (0.5 g/L) and 2.87 mmol/L (1 g/L) in methanol, respectively. These solutions were stored at −20 °C for up to 1 month without notable degradation. MMC working solution was 3.44 and 1.72 µmol/L (1 and 0.5 mg/L) in water, whereas PFM was 2.87 µmol/L (1 mg/L) in 2 mol/L PBS (potassium phosphate buffer), pH 7.4. New working solutions were freshly prepared for dilution of the stocks at each analytical series. For unknown sample measurement, calibrators in plasma were prepared at three different MMC concentrations (1, 5, 20 µg/L) and treated as described below in the sample extraction procedure.

**APPARATUS**
Chromatographic analysis was carried out on a C18 column (UltraspHERE 150 × 4.6 mm, 5 µm; Beckman, Palo Alto, CA) connected to a Kontron chromatographic system (Kontron, Zurich, Switzerland) equipped with two pumps (Model 420), an autosampler (Model 460), a double-beam UV detector (Model 430) set at 365 nm, and a Data System 450. The mobile phase of water:acetonitrile (85:15 by vol) was delivered at 1.0 mL/min.

**SAMPLE PREPARATION AND STORAGE**
As a preliminary study, we evaluated MMC systemic absorption in two patients with STCCB in which MMC instillation was associated with microwave-induced local HT (homogeneous heating of bladder mucosa within a medium temperature of 42.5 °C–46.5 °C). A total amount of 40 mg of MMC was administered in 60 min but, to avoid the drug degradation throughout the entire procedure, the dose was delivered with two 30-min instillations of 20 mg/50 mL bidistilled water. The procedure followed in this study was in accordance with the ethical standards of our Institution’s responsible committee.

Blood samples were taken into heparinized tubes before and every 15 min during the 60-min instillation. As soon as possible, plasma was obtained and stored at −20 °C until analysis took place.

**SAMPLE EXTRACTION PROCEDURE**
After thawing and avoiding any exposure to light, plasma samples (1 mL) were added to the internal standard PFM (50 µL = 50 ng) and diluted 1:1 (by vol) with water. For solid-phase extraction, 1-mL Supelclean extraction tubes filled with 100 mg of LC-18 end-capped sorbent were used (Supelco, Bellefonte, PA). The disposable tubes were connected to the Visiprep Solid Phase Extraction Vacuum Manifolds (Supelco) and, after vacuum application with a water pump, preactivated by washing with 2 mL of methanol followed by 2 mL of water. The vacuum was then disconnected to prevent the columns from drying, the diluted plasma samples were loaded, and the vacuum was applied again, not exceeding the flow rate of ~1 mL/min. The C18 columns were washed with water (2 × 1-mL aliquots), followed by water:methanol 90:10 (1 mL) and water:methanol 70:30 (0.2 mL) mixtures. Columns were completely dried by flushing air for 1–3 min; then the vacuum was disconnected and the 12-mL polypropylene tubes used to collect washings were discharged and replaced with the 0.5-mL polypropylene microvials of the HPLC autosampler. MMC was eluted under vacuum with 2 × 200-µL fractions of water:methanol 50:50. The vials were directly positioned into the sample tray of the autosampler and 100–200 µL of the samples injected into the HPLC column.

**Results**

**CHROMATOGRAPHIC ANALYSIS**
Under the conditions described in Materials and Methods, the MMC and PFM showed a Rf of 4.9 and 7.2 min, respectively, with a total analysis time of 10 min. In Fig. 1A, the chromatogram of a blank plasma sample enriched with MMC (20 µg/L) and PFM (50 µg/L) before solid-phase extraction is shown, whereas in Fig. 1B and C the same control plasma was supplemented with 2.5 and 0.5

![Fig. 1. Chromatographic separation of plasma samples purified by solid-phase extraction as described in Materials and Methods.](image-url)
µg/L MMC, respectively. In Fig. 1D, the analysis of a serum sample taken from a patient after a 60-min instillation with MMC and homogeneous heating of the bladder walls at 46.5 °C is shown.

We found that the length of the chromatographic separation could be properly reduced to only 3 min (1.9 and 2.7 min $R_t$ of MMC and PFM, respectively) simply by replacing the 15-cm $C_{18}$ column with a 3 cm $\times$ 3 mm $\times$ 3 µm $C_{18}$ column (Supelco) (data not shown). Because of the rapidity of the preanalytical phase, however, we considered the analysis length of 10 min acceptable for our purpose, and we preferred the use of the 15-cm column, thus reducing the possibility of chromatographic interferences and improving the specificity of the separation.

ANALYTICAL VARIABLES

The use of PFM as the internal standard ensured good reproducibility of the entire procedure. The extraction efficiency and reproducibility of MMC and PFM from the $C_{18}$ columns were assessed in triplicate on plasma samples supplemented with 50 µg/L of the internal standard PFM and with MMC (50, 20, and 5 µg/L). MMC recovery was 98% ± 4%, 92% ± 6%, and 89% ± 7% at the three concentrations tested, respectively, whereas PFM showed a mean recovery of 85.0% ± 6.1% ($n = 9$). The interference study was carried out with caffeine, theophylline, amphotericin B, epirubicin, ampicillin, amikacin, cefazolin, and ceftriaxone. None of these commonly administered drugs was detected under the analytical conditions used for MMC assay. The intraday precision of the method (CV) calculated on the MMC/PFM area ratio gave 5.8% ($n = 6$) and 6.0% ($n = 6$) at 10 and 5 µg/L, respectively. The reproducibility of the method calculated within 1 year showed a CV of 10.7% and 9.5% for the 10 and 5 µg/L concentrations ($n = 10$), respectively. Linearity of the assay was assessed by supplementing human control plasma in the 0.5–50 µg/L concentration range. Linear regression of the area ratio ($y$) vs MMC concentration expressed in µg/L ($x$) gave the equation $y = 0.0108(±0.0001)x + 0.005(±0.003)$, $r = 0.999$, $S_{y/x} = 0.0072$. By injecting 0.5 µg/L directly after solid-phase extraction, the MMC signal was still evaluable with a peak area of 0.011 mVmin. To achieve a better response at this concentration, avoiding larger injection volumes, we reduced the methanol fraction of the sample by subjecting it to nitrogen evaporation for few minutes before HPLC injection, thus potentially improving the detection limit (Fig. 1C). Control plasma showed no peaks at the $R_t$ of the two drugs.

MITOMYCIN C PHARMACOKINETICS

The plasma pharmacokinetics concentration profiles recorded in two patients when an intravesical dose of MMC (20 mg/50 mL replaced after a 30-min instillation) was given in the presence of adjuvant local HT are shown in Fig. 2. In both patients, the curves reached the maximum peak of MMC plasma concentration between 45 and 60 min of instillation, with concentrations from 1.2 to 5.9 µg/L in the first patient and a slightly more increased systemic absorption in the second one (plasma concentration 8.1–13.3 µg/L).

Discussion

The method set up for MMC quantification was suitable for monitoring the kinetics of absorption in plasma during ICT associated with local HT.

The chosen chromatographic conditions were the same as those described by Tjaden et al. [16], but without buffering the mobile phase because we were interested in MMC determination only. Those authors obtained a fully automated analysis with a continuous-flow system and a valve-switching technique coupled to a 100 $\times$ 3 mm $C_{8}$ HPLC column. Although feasible for a routine working load, this assay procedure needed dedicated instrumentation with nonstandard chromatographic apparatus and >2 mL of plasma to reach a MMC sensitivity of <1 µg/L.

Den Hartigh et al. [12] used a liquid–liquid extraction with chloroform:2-propanol (1:1 by vol), whereas Dalton et al. [17] used ethyl acetate alone. Despite the very high solvent:sample ratio used by these authors (1:10 by vol), and the very critical step of the solvent drying up under reduced pressure or nitrogen and thus causing MMC decomposition and affecting the recovery rate, this kind of procedure appeared unsuitable for a clinical chemistry laboratory where air exhausters and (or) equipment for solvent evaporation are not always easily available. Moreover, the chromatographic separation of the ethyl acetate extract shown by Dalton et al. [17], although referring to only 1 ng of MMC injected, appears contaminated and full of potentially interfering peaks. The use of solid-phase extraction for MMC purification from serum has already been reported [13, 14]. However, Tjaden et al. [13] used homemade Amberlite XAD-2 columns requiring high
washing and elution volumes, with a drying step of the purified methanol extract (6 mL) before HPLC analysis.

The main feature of our proposed assay consists of the solid-phase extraction under vacuum of 1 mL of sample with commercially available 100-mg disposable columns, which assures a reproducible performance, avoids the use of conspicuous extraction volumes, and allows the direct injection of the sample eluted in the last purification step. The chromatographic analysis does not require a dedicated apparatus [16] or an unusual detector [13], so MMC analysis can be easily scheduled on the same HPLC apparatus used for other routine assays (drugs or metabolites). In addition, the manual preanalytical purification can be fully automated with ASPEC (Gilson, Villiers Le Bel, France) equipment for automated column extraction directly linked to the HPLC system. The manual handling of the purification procedure allows the simultaneous cleanup of 12 samples every 15 min, with the use of <3 mL of solvent and a quite low cost analysis (<$1 including the column and the extraction eluents).

The pharmacokinetics curves reported here are only preliminary results of a larger trial in which the effects of local HT when associated with ICT will be thoroughly investigated. Moreover, any eventual relation between temperature delivered during the hyperthermic treatment and the systemic absorption of the drug is presently under careful examination.

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References