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**A Validated High Performance Liquid Chromatography – Diode Array Detector
Method for the Quantification of Mitomycin C in Plasma, Peritoneal Fluid and Urine**

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Abstract

Mitomycin C (MMC) is a quinone-containing alkylating agent, that has been extensively studied in preclinical and clinical work due to its antitumor activity. A thoroughly validated high performance liquid chromatography-diode array detector method is provided to quantify MMC in plasma, peritoneal fluid and urine. Porfiromycin served as internal standard. The mobile phase for the plasma, peritoneal fluid and urine analysis consisted of 27% MeOH and 73% 20mM ammoniumacetate buffer (pH 6.5) and 9% ACN and 91% 20mM phosphate buffer (pH 6.5) respectively. The residue from 100 μ L ACN deproteinated plasma was dissolved in 250 μ L mobile phase. Peritoneal fluid and urine were diluted 10-fold in their respective mobile phases. UV detection was performed at 365nm. Quantification of MMC was achieved over a linear range of 0.05-5 μ g/mL and 5-50 μ g/mL in plasma; 0.1-5 μ g/mL and 5-100 μ g/mL in peritoneal fluid; 0.25-5 μ g/mL and 5-100 μ g/mL in urine. The limit of quantification was 0.05 μ g/mL in plasma, 0.1 μ g/mL in peritoneal fluid and 0.25 μ g/mL in urine. The method was further validated for selectivity, specificity, inter- and intraday precision and accuracy ($\leq 15\%$), extraction recovery and stability. MMC remains stable in the different biofluids for 20 days (short-term stability) at 4°C, -27°C and -80°C and for 80 days (long-term stability) at -80°C.

Keywords

Mitomycin C; HPLC-DAD; Plasma; Urine; Peritoneal Fluid

Introduction

Mitomycin C (MMC) is a quinone-containing alkylating agent, isolated from the actinobacteria species, *Streptomyces caespitosus* [1]. The antibiotic has been extensively studied in preclinical and clinical work due to its antitumor activity. Its most important mechanism of action is through DNA cross-linking. Although MMC is not regarded as a prodrug, it is not active against cancerous tissue as unchanged molecule. The drug is modified as it enters the cell into its active state [2]. It is inactivated by microsomal enzymes in the liver and metabolized in the spleen and kidneys. Today, MMC is widely used for the intraperitoneal (IP) treatment of peritoneal carcinomatosis (PC) from colorectal, appendiceal, ovarian, gastric cancer and diffuse malignant peritoneal mesothelioma [3-8]. Treatment involves a combination therapy of cytoreductive surgery (CRS) and hyperthermic intraperitoneal perioperative chemotherapy (HIPEC). During HIPEC, a heated chemotherapy solution (41-43°C) is instilled in the peritoneal cavity for a predefined time, depending on the chemotherapeutic agent [9]. At present, however, no standardized IP chemotherapy treatment modalities exist, due to variations in choice of drug, dosage, duration, etc. Therefore, there is a pressing need for conducting pharmacologic research towards standardization amongst the myriad of IP treatment protocols currently applied.

Throughout literature, various methods have been published regarding the determination and quantification of MMC [10-11]. The most widely used analytical technique to detect MMC in biofluids is reversed phase high performance liquid chromatography (HPLC) combined with UV detection [12-17]. Dalton *et al.* reported a HPLC method to detect mitomycin C in both human and rat plasma and urine [12]. Using liquid-liquid extraction as sample preparation, a detection limit of 0.5 ng/mL in human plasma could be achieved. Schmid *et al.* reported the use of a fast and sensitive HPLC-UV method with an online sample clean-up to determine MMC in plasma but data of the development of this analytical method were not published [16]. Eksborg *et al.* presented a method, using solid phase extraction (SPE) (Sep-Pak C18) as sample

preparation, to determine MMC in human plasma and urine within the range of 2-1000 ng/mL and 0.5 – 4.4 µg/mL, respectively [17]. All the above-mentioned methods have the limitation of either having time-consuming processing procedures or being reported for only a limited number of matrices. Moreover, most publications lack a thorough validation of the described method. Our aim was to develop a validated HPLC-diode array detector (DAD) method to allow quantification of MMC in plasma, peritoneal fluid and urine. The described method can easily be used in a routine setting and results are presented of a case study of pharmacologic research regarding the IP administration of MMC in a cancer patient.

Materials and methods

Safety considerations

When working with chemotherapy, standard safety precautions were applied. These include wearing personal protective equipment (eye protection, protective gloves and protecting clothing) and using standardized handling procedures, including the use of BD PhaSeal™ closed system transfer devices (Dublin, Ireland), to minimize chemotherapy associated risks. All chemotherapy associated materials and patient samples were disposed in WIVA medical waste containers.

Chemicals and reagents

MMC, 2 mg, was purchased from Kyowa (Takeda Belgium, Brussels, Belgium) (Figure 1). The internal standard, porfiromycin, was kindly synthesized by the Laboratory for Organic and Bio-organic Synthesis of Ghent University (Ghent, Belgium) (Figure 1). HPLC-grade acetonitrile (ACN) and methanol (MeOH) were purchased from VWR International (Fontenay-sous-Bois, France). Potassium dihydrogen phosphate (KH₂PO₄) was purchased from Merck Millipore (Darmstadt, Germany). LC-MS-grade ammonium acetate (C₂H₇NO₂) and syringe filters, 25 mm with 0.45 µm nylon membrane, were purchased from VWR International (Leuven,

Belgium). At all times, ultrapure water of $18.2 \text{ M}\Omega\text{cm}^{-1}$ was used in the preparations (Advantage A10 Water Purification System, Merck Millipore, Overijse, Belgium). Nitrogen for evaporation was purchased from Messer (Messer Group GmbH, Krefeld, Germany). Drug-free human heparin plasma, urine and peritoneal fluid were kindly provided by the Department of Laboratory Medicine at the Ziekenhuis Oost-Limburg (Genk, Belgium).

Calibration standards and validation standards

A standard stock solution of MMC was prepared in ultrapure water at a concentration of 0.4 mg/mL and was stored at room temperature in the dark for up to 1 week. The internal standard solution, porfiromycin, was prepared in MeOH at a concentration of 51.5 mg/mL. Internal standard working solutions were prepared by serially diluting the stock solution in MeOH to reach final concentrations of 10, 100 and 1000 $\mu\text{g/mL}$. The internal standard stock and working solutions were stored in the dark at -80°C and -27°C , respectively.

Plasma

MMC calibration standards in human plasma were prepared by serially diluting the MMC standard stock solution in pooled drug-free plasma to reach final concentrations of 0.05, 0.1, 0.5, 1, 5, 10, 20 and 50 $\mu\text{g/mL}$. Quality control samples were prepared by diluting the MMC standard stock solution with pooled drug-free plasma to reach final concentrations of 0.15, 2, 5, 16 and 25 $\mu\text{g/mL}$. Calibration standards and quality control samples were freshly prepared each day.

Peritoneal fluid and Urine

Calibration standards in peritoneal fluid and urine were prepared by serially diluting the MMC standard stock solution in drug-free peritoneal fluid and urine to reach final concentrations of 0.1, 0.25, 0.5, 1, 5, 10, 50 and 100 $\mu\text{g/mL}$. Quality control samples were prepared by diluting the MMC standard stock solution with drug-free peritoneal fluid and urine to reach final

concentrations of 2, 5, 20 and 80 µg/mL. Calibration standards and quality control samples were freshly prepared each day.

Sample preparation

Plasma

Human heparin plasma was obtained by centrifuging blood at 3000 x g for 15 min at 21°C. To 95 µL plasma sample volume, 5 µL of the internal standard working solution containing 10 µg/mL porfiromycin was added. Protein precipitation was performed with 1 mL ACN. After thoroughly mixing on a vortex-mixer for 10 seconds, the mixture was centrifuged at 3000 x g for 10 minutes at 21°C. Subsequently, the supernatant was transferred to a clean glass tube and evaporated to dryness under a steady stream of N₂ at 39°C. The residue was dissolved in 250 µL mobile phase and transferred to dark HPLC vials for injection.

Peritoneal fluid and Urine

Peritoneal fluid and urine were centrifuged at 3000 x g for 10 min at 21°C. To 270 µL peritoneal fluid and 95 µL urine sample volume, 30 µL and 5 µL of the internal standard working solutions containing 100 µg/mL and 10 µg/mL porfiromycin respectively, was added. The matrices were transferred to a clean polypropylene tube, diluted 10-fold in mobile phase, subsequently filtered through 0.45 µm nylon syringe filter and transferred to dark HPLC vials before injection.

Instrumentation and chromatographic conditions

The HPLC system consisted of a Hitachi LaChrom Elite (VWR) system equipped with a Hitachi L-2200 autosampler, a Hitachi L-2100/2130 pump, a Hitachi L-2300 column oven and a Hitachi L-2455 DAD (VWR, Leuven, Belgium). The analytical column was a reversed phase Polaris 3 C18-A, 150 x 3.0 mm, 3 µm particle size (Agilent Technologies, Brussels, Belgium). The mobile phase for the plasma and peritoneal fluid analysis consisted of 27% MeOH in 73% 20 mM ammoniumacetate buffer (pH 6.5). The mobile phase for the urine analysis consisted

of 9% ACN in 91% 20 mM phosphate buffer (pH 6.5). Both mobile phases were run isocratically at a flow rate of 0.4 mL/min, to ensure back pressures below the threshold of 300 bar. The injection volume was set at 50 μ L, the plasma and peritoneal fluid analysis were performed at 25°C and the urine analysis at 30°C. The DAD detector was set to measure absorbance at 365 nm. Analytical column care was performed each day by rinsing the column with ultrapure water for 10 column volumes, followed by ACN for approximately 20 column volumes.

Method validation

The described HPLC-DAD method was validated for the following parameters according to the generally accepted guidelines for method validation [18].

Linearity

Linearity was assessed using the previously described calibration standards in plasma, peritoneal fluid and urine. The ratio of the areas of MMC and the internal standard, porfiromycin, was plotted against the analyte concentration and fitted in a linear manner.

Limit of Detection

Limit of detection (LOD) was defined as the concentration of MMC in the different biofluids at which the signal-to-noise ratio corresponded to at least 3.

Limit of Quantification

Limit of quantification (LOQ) was defined as the concentration of MMC at which the signal-to-noise ratio corresponded to at least 10, that could be measured with an acceptable precision and accuracy. Precision was expressed as the relative standard deviation (RSD%); calculated as $((SD/mean) \times 100)$. The accuracy was expressed as the relative error (RE%); calculated as $((\text{found concentration} - \text{theoretical concentration})/\text{theoretical concentration}) \times 100$.

169 Acceptable precision and accuracy (n=10) was defined as RSD and RE $\leq 15\%$ or $\leq 20\%$ at the
170 lowest level, i.e. the LOQ [19].

171 *Specificity and Selectivity*

172 The possible interference of endogenous matrix components with the HPLC-DAD assay was
173 evaluated. Resulting chromatograms of plasma, peritoneal fluid and urine samples containing
174 neither MMC nor internal standard were compared with the analysis of the biofluids containing
175 their respective LOQ. Analysis were performed with biofluids from 5 different donors,
176 including two HIPEC treated patients.

177 *Precision and Accuracy*

178 Interday (n=10) and intraday (n=10) precision and accuracy were evaluated at different
179 validation levels; 0.15, 2, 5, 16 and 25 $\mu\text{g/mL}$ MMC in plasma; 2, 5, 20 and 80 $\mu\text{g/mL}$ MMC
180 in peritoneal fluid and urine. Acceptable precision and accuracy was defined as RSD and RE
181 $< 15\%$.

182 *Extraction recovery*

183 Extraction recovery of MMC in plasma, peritoneal fluid and urine was evaluated at three
184 different validation levels by comparing the areas of the MMC signal in the respective biofluids
185 with the areas of the MMC signal measured in the corresponding mobile phases. Quality control
186 samples of MMC in matrix were prepared by diluting the MMC standard stock solution in drug-
187 free biofluids to reach final concentrations of 1, 10 and 50 $\mu\text{g/mL}$ MMC in plasma; 1, 50 and
188 100 $\mu\text{g/mL}$ MMC in peritoneal fluid and urine. For each concentration, three independent
189 samples were analyzed.

190 *Stability*

191 Stability of MMC in the biofluids was assessed at three different validation levels; 2, 16 and 25
192 $\mu\text{g/mL}$ MMC in plasma; 2, 20 and 80 $\mu\text{g/mL}$ MMC in peritoneal fluid and urine and expressed

in terms of recovery as compared to freshly prepared samples. Short-term and long-term stability were evaluated by preserving the different samples at 4°C, -27°C and -80°C for 20 days and 80 days respectively. For each concentration, three independent samples were analyzed.

Case study

The described HPLC-DAD method was employed in a clinical pharmacokinetic case study. A patient diagnosed with PC from colorectal origin was treated with the CRS and HIPEC procedure. During the 90-minute HIPEC, a total dose of 35 mg/m² MMC was administered using the ‘Dutch High Dose Mitomycin C Regimen, Triple Dosing Regimen’ [20]. At each 15-minute time interval, plasma, peritoneal fluid and urine were sampled and processed as previously described. Samples were stored at -80°C until day of analysis.

Results

Chromatography

The total runtime for the plasma (Figure 2a) and peritoneal fluid (Figure 2b) analysis was 12 min. MMC eluted with a retention time of 6.9 min and the internal standard eluted with a retention time of 9.4 min. The total runtime for the urine (Figure 2c) analysis was 30 min, with a retention time of 14.9 min and 23.0 min for MMC and porfiromycin respectively. All peaks exhibit a symmetric shape with only limited tailing. MMC displays two signals at 3.97 min and 6.83 min when measured in the plasma and peritoneal fluid mobile phase (Figure 2d) and at 6.21 min and 15.34 min when measured in the mobile phase for the urine analysis (Figure 2e).

Validation

Linearity

Calibration curves of MMC in plasma, peritoneal fluid and urine were divided into two sections to cover a large linear range. The HPLC-DAD method covered a linearity range of 0.05 – 5 µg/mL and 5 – 50 µg/mL in plasma with typical standard curves of $y = 2.66x - 0.041$ with a coefficient of determination (r^2) of 0.9998 and $y = 2.76x - 0.818$, $r^2 = 0.9997$ respectively. Linearity in peritoneal fluid ranged from 0.1 – 5 µg/mL and 5 – 100 µg/mL with typical standard curves of $y = 0.11x - 0.0017$, $r^2 = 0.9999$ and $y = 0.11x - 0.0021$, $r^2 = 0.9997$ respectively. Linearity in urine ranged from 0.25 – 5 µg/mL and 5 – 100 µg/mL with typical standard curves of $y = 0.31x - 0.0331$, $r^2 = 0.9995$ and $y = 0.34x - 0.2699$, $r^2 = 0.996$ respectively.

Limit of detection

The LOD was 5 ng/mL MMC in plasma and 20 ng/mL MMC in peritoneal fluid and urine.

Limit of Quantification

The LOQ was 0.05 µg/mL MMC in plasma (n=10; RSD: 5.53%, RE: -15.07%), 0.1 µg/mL MMC in peritoneal fluid (n=10; RSD: 2.09%, RE: 8.30%) and 0.25 µg/mL MMC in urine (n = 10; RSD: 6.45%, RE: 14.97%).

Specificity and Selectivity

Representative chromatograms of drug-free plasma, peritoneal fluid and urine analysis together with chromatograms of the respective biofluids spiked with their LOQ, evaluating specificity and selectivity are depicted in Figure 2. All samples are from a HIPEC treated patient. We confirm that there was no interference of endogenous matrix components with the HPLC-DAD assay.

Precision and Accuracy

The inter- and intraday precision and accuracy were investigated at different MMC levels; 2, 16 and 25 µg/mL MMC in plasma; 2, 20 and 80 µg/mL MMC in peritoneal fluid and urine (Table I). Both the RSD and RE are less than 8.94% for the HPLC-DAD analysis of all biofluids. As MMC concentrations in plasma are very low in a cancer patient treated with MMC-based HIPEC (see section case study), an additional level of 0.15 µg/mL MMC in plasma was evaluated for accuracy and precision. RSD and RE were less than 9.72%. Inter- and intraday precision and accuracy were also validated at a fourth level, 5 µg/mL MMC, i.e. at the intersection of the calibration curves in the respective biofluids, using both calibration curves (Table II). Both the RSD and RE are less than 9.58% for all biofluids.

Extraction recovery

The extraction recovery from plasma, peritoneal fluid and urine using the sample preparation methods described in the materials and methods section is presented in Table III. In summary, the recovery was assessed at three different MMC levels; 1, 10 and 50 µg/mL in plasma; 1, 50 and 100 µg/mL in peritoneal fluid and urine. The reported recoveries range from 82.13 – 99.44%.

Stability

The short-term and long-term stability at 4°C, -27°C and -80°C of MMC in plasma, peritoneal fluid and urine is summarized in Table IV. Stability was assessed at three different MMC levels; 2, 16 and 25 µg/mL MMC in plasma; 2, 20 and 80 µg/mL MMC in peritoneal fluid and urine, and expressed in terms of recovery. For all biofluids, recovery ranged from 76.61 – 112% after 20 days of storage at all defined storage conditions. As to long-term stability; when stored at 4°C and -27°C, recoveries ranged from 26.52 – 93.25% with high SDs. When stored at -80°C, recoveries of all biofluids ranged from 76.74 – 103%. Freeze-thaw stability was not investigated as part of this validation study. For the application of conducting pharmacologic research

regarding the intraperitoneal administration of mitomycin C to cancer patients, the biological samples are only thawed once before analysis and discarded afterwards. Therefore, freeze-thaw stability was not relevant for the particular application.

Case study

The concentration versus time curve of MMC in plasma, peritoneal fluid and urine is depicted in Figure 3. MMC was quantified in the respective biofluids sampled at each 15-min time interval during the HIPEC procedure. The peritoneal fluid concentration curve demonstrates the administration of MMC in three phases, ‘Triple Dosing Regimen’, and the subsequent decrease in MMC concentration. The concentration in peritoneal fluid ranged from 6.02 – 9.90 µg/mL. Plasma concentration, 15 min after initial administration of MMC, was 0.08 µg/mL and increased to 0.31 µg/mL after the 90-minute HIPEC procedure. MMC concentration in urine ranged from 0.25 – 9.95 µg/mL. Representative chromatograms of the MMC and internal standard signals, 15 minutes after intraperitoneal chemotherapy administration in the different biofluids of the case study’s patient, are presented in Figure 4.

Discussion

The majority of pharmacokinetic studies reported today draw conclusions from analytical analysis that were developed without a supporting validation. To address this issue, we have demonstrated the use of a thoroughly validated, rapid and sensitive HPLC-DAD method to quantify MMC in plasma, peritoneal fluid and urine.

In literature, several sample preparation methods have been described for MMC quantification in plasma and urine, including liquid-liquid extraction [21], protein precipitation [14] and SPE methods [17]. The evaporation of organic solvents during liquid-liquid extraction methods requires high temperatures and when performed at ambient temperature are often time-consuming (presumably in the hours range). Moreover, sample preparation methods involving

temperatures higher than 40°C will result in the decomposition of MMC and can therefore not be utilized [21]. In the optimization part of this assay, we explored protein precipitation as plasma sample preparation, followed by evaporation at 39°C under a steady stream of N₂. The improvement we report relative to the protein precipitation method described by Joseph *et al.* [14] is the time needed for evaporation after precipitation with ACN, i.e. 20 minutes for 36 samples as compared to 4-5 hours for 20 samples. For the urine analysis, we explored SPE using different stationary phases; Bond Elut Plexa (30 mg, 3 mL, Agilent Technologies, USA) and ProElut NH₂ (500 mg, 3mL, Dikma Technologies, USA) as sample preparation method. However, SPE sample preparation did not result in an acceptable LOQ and extraction recovery. The above-mentioned sample preparation methods were all optimized aiming at the use of a uniform mobile phase and chromatographic conditions for all biofluids. However, for the urine analysis, the use of 27% MeOH and 73% 20 mM ammoniumacetate buffer (pH 6.5) did not result in acceptable selectivity, specificity and sensitivity. A mobile phase consisting of 9% ACN and 91% 20mM phosphate buffer (pH 6.5) with a runtime of 30 minutes was necessary to achieve good baseline separation for the MMC and internal standard signals in urine and to eliminate interference from contaminants. We can conclude that precipitation with ACN as sample preparation for plasma and a 10-fold dilution in mobile phase followed by filtration through a 0.45 µm syringe filter as sample preparation for peritoneal fluid and urine resulted in limited to no loss of sample and that good baseline separation was achieved used the above-mentioned mobile phases.

MMC displays two signals when measured in the mobile phases of the biofluids (Figure 2d and 2e). From liquid chromatography – mass spectrometry (LC-MS) analysis, we know that both signals have the same parent ion (m/z 335) and generate the same fragment ion (m/z 242). Therefore, it can be postulated that two stereoisomers of MMC are present in the reference standard and are retained differently on the chromatographic column. Throughout the validation

of the HPLC-DAD method, we performed measurements on the signal with a retention time of 6.83 min (plasma and peritoneal fluid analysis) and 15.34 min (urine analysis); i.e. the stereoisomeric form of MMC most present in the reference standard.

The above described method was validated and proven to be robust in terms of linearity, selectivity, specificity, accuracy, precision and extraction recovery. Regarding stability, recovery of the peritoneal fluid analysis after long-term storage at -80°C was lower than reported for the other biofluids, i.e. 76.74 - 79.35% versus 94.10 - 103%. However, recovery is still in the acceptable range and therefore we confirm that long-term storage of all biofluids, up to 80 days, should be conducted at -80°C. The LOQ we report in plasma is higher than the LOQ reported for the HPLC-UV method by Joseph *et al.* [14]. However, the presented LOQ's in the different biofluids allow to perform pharmacokinetic studies in which patients receive the 'Dutch High Dose Mitomycin C Regimen, Triple Dosing Regimen', the most widely used HIPEC MMC dosing regimen. The latter is again confirmed by previous pharmacokinetic research reporting on the concentration of MMC administered during HIPEC. Sugarbaker *et al.* treated patients with low dose MMC, 12.5 mg/m² in males and 10 mg/m² in females using a single administration and diluted this dose in varying volumes of carrier solution. The concentration reported in plasma ranged from 0.01 µg/mL at time point zero to approximately 0.5 µg/mL and in peritoneal fluid from 5 µg/mL to approximately 10 µg/mL [22]. Van der Speeten *et al.* treated patients with a combination therapy of 15 mg/m² doxorubicin and 15 mg/m² MMC. MMC plasma concentration ranged from 0.1 µg/mL at time point zero to 0.25 µg/mL, MMC urine concentration ranged from 0.1 µg/mL to approximately 5 µg/mL and from 5 µg/mL to approximately 10 µg/mL in peritoneal fluid [4].

Conclusion

We have developed a thoroughly validated HPLC-DAD method to quantify MMC in plasma, peritoneal fluid and urine. As compared to what previously has been reported, our HPLC-DAD

method does not require the use of complex chromatographic conditions, expensive and time-consuming sample preparation methods. Moreover, good specificity and sensitivity could be achieved resulting in the successful application of our analytical method in a pharmacokinetic case study of a cancer patient, receiving MMC-based HIPEC.

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Conflict of interest statement

The authors declare that they have no conflict of interest.

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Tables

Table I. Interday and intraday precision (RSD%) and accuracy (RE%) in plasma, peritoneal fluid and urine.

<i>n</i> =10		Validation parameter	Theoretical concentration MMC (µg/mL)		
			2	16	25
Plasma	Interday	Mean observed concentration (µg/mL) ± SD	1.94 ± 0.15	16.15 ± 1.22	24.42 ± 1.72
		RSD (%)	7.71	7.56	7.05
		RE (%)	-2.86	0.92	-2.31
	Intraday	Mean observed concentration (µg/mL) ± SD	1.86 ± 0.13	16.02 ± 0.58	26.36 ± 1.16
		RSD (%)	6.92	3.65	4.38
		RE (%)	-7.20	0.10	5.43
			2	20	80
Peritoneal fluid	Interday	Mean observed concentration (µg/mL) ± SD	1.98 ± 0.13	19.53 ± 1.32	78.70 ± 6.18
		RSD (%)	6.40	6.75	7.86
		RE (%)	-0.93	-2.35	-1.62
	Intraday	Mean observed concentration (µg/mL) ± SD	2.00 ± 0.13	17.70 ± 0.55	86.20 ± 7.70
		RSD (%)	6.70	3.13	8.94
		RE (%)	0.24	-11.48	6.36
Urine	Interday	Mean observed concentration (µg/mL) ± SD	1.95 ± 0.14	20.56 ± 1.65	82.75 ± 6.95
		RSD (%)	7.03	8.02	8.40
		RE (%)	-2.47	2.80	3.43
	Intraday	Mean observed concentration (µg/mL) ± SD	1.94 ± 0.10	20.41 ± 0.84	86.25 ± 1.41
		RSD (%)	5.01	4.10	1.64
		RE (%)	-2.97	2.04	7.82

Table II. Interday and intraday precision (RSD%) and accuracy (RE%) of 5 µg/mL MMC assessed in the different calibration curves in plasma, peritoneal fluid and urine.

<i>n=10</i> Validation parameter			Theoretical concentration MMC (µg/mL)	
			5	
			Calibration curves	
			0.05 - 5	5 - 50
Plasma	Interday	Mean observed concentration (µg/mL) ± SD	5.05 ± 0.10	5.04 ± 0.48
		RSD (%)	2.06	9.58
		RE (%)	0.92	0.84
	Intraday	Mean observed concentration (µg/mL) ± SD	5.18 ± 0.18	5.11 ± 0.22
		RSD (%)	3.44	4.26
		RE (%)	3.69	2.16
			0.1 - 5	5 - 100
Peritoneal fluid	Interday	Mean observed concentration (µg/mL) ± SD	5.00 ± 0.02	4.95 ± 0.26
		RSD (%)	0.45	5.17
		RE (%)	0.01	-1.04
	Intraday	Mean observed concentration (µg/mL) ± SD	5.09 ± 0.05	5.05 ± 0.06
		RSD (%)	0.96	1.22
		RE (%)	1.71	0.94
			0.25 - 5	5 - 100
Urine	Interday	Mean observed concentration (µg/mL) ± SD	5.01 ± 0.01	5.13 ± 0.34
		RSD (%)	0.12	6.67
		RE (%)	0.13	2.65
	Intraday	Mean observed concentration (µg/mL) ± SD	4.85 ± 0.07	5.14 ± 0.07
		RSD (%)	1.42	1.27
		RE (%)	-2.98	2.82

Table III. Extraction recovery from plasma, peritoneal fluid and urine.^a

	Theoretical concentration MMC (µg/mL)	Extraction recovery (%) ± SD
Plasma	1	82.32 ± 6.16
	10	87.57 ± 2.50
	50	88.42 ± 6.64
Peritoneal fluid	1	99.44 ± 3.13
	50	96.55 ± 5.06
	100	96.90 ± 3.57
Urine	1	86.16 ± 0.78
	50	93.20 ± 0.77
	100	86.24 ± 0.83

^a For each concentration, three independent samples were analyzed (n=3).

Table IV. Short-term and long-term stability of MMC analysis in plasma, peritoneal fluid and urine at 4°C, -27°C and -80°C.^a

		Temperature (°C)	Recovery (%) ± SD (<i>n</i> = 3)		
			Theoretical concentration MMC (µg/mL)		
			2	16	25
Plasma	Short-term (20 days)	4	96.96 ± 2.85	93.72 ± 0.14	94.47 ± 2.83
		-27	103 ± 6	101 ± 2	92.91 ± 1.34
		-80	112 ± 6	103 ± 0.19	91.30 ± 2.77
	Long-term (80 days)	4	69.46 ± 6.40	47.37 ± 8.96	89.18 ± 1.28
		-27	93.25 ± 4.54	77.02 ± 28.28	91.64 ± 5.34
		-80	96.50 ± 3.55	103 ± 10	98.48 ± 1.11
		2	20	80	
Peritoneal fluid	Short-term (20 days)	4	96.96 ± 2.85	93.72 ± 0.14	81.69 ± 0.75
		-27	103 ± 6	101 ± 2	95.54 ± 0.28
		-80	112 ± 6	103 ± 0.2	99.41 ± 1.07
	Long-term (80 days)	4	64.83 ± 9.25	87.01 ± 19.23	78.11 ± 9.34
		-27	65.37 ± 3.15	73.14 ± 0.88	77.09 ± 1.04
		-80	78.35 ± 0.87	76.74 ± 1.60	79.35 ± 0.57
Urine	Short-term (20 days)	4	76.61 ± 1.15	89.83 ± 1.06	91.17 ± 0.81
		-27	95.67 ± 5.29	100 ± 1	96.68 ± 0.63
		-80	110 ± 1	109 ± 1	99.32 ± 0.42
	Long-term (80 days)	4	27.96 ± 0.49	38.57 ± 1.39	53.93 ± 3.15
		-27	26.52 ± 9.21	60.96 ± 2.84	40.97 ± 32.82
		-80	94.10 ± 1.27	96.90 ± 0.82	99.35 ± 0.86

^a For each concentration, three independent samples were analyzed (n=3).

Figure legends

Figure 1. Chemical structure of mitomycin C and porfiromycin.

Figure 2. Selectivity and specificity of the HPLC-DAD mitomycin C analysis in plasma, peritoneal fluid and urine (a) HPLC-DAD analysis of MMC (6.84 min) and the internal standard, porfiromycin (9.28 min), in plasma. Plasma spiked with the LOQ, 0.05 µg/mL MMC and 0.5 µg/mL porfiromycin (blue). Drug-free plasma (green). (b) HPLC-DAD analysis of MMC (7.01) and the internal standard, porfiromycin (9.50 min), in peritoneal fluid. Peritoneal fluid spiked with the LOQ, 0.1 µg/mL MMC and 10 µg/mL porfiromycin (blue). Drug-free peritoneal fluid (green). (c) HPLC-DAD analysis of MMC (14.85 min) and the internal standard, porfiromycin (22.98 min), in urine. Urine spiked with the LOQ, 0.25 µg/mL MMC and 5 µg/mL porfiromycin (blue). Drug-free urine (green). (d) HPLC-DAD analysis of MMC and the internal standard, porfiromycin, in 27% MeOH / 73% 20 mM ammoniumacetate buffer (pH 6.5). The mobile phase of the plasma and peritoneal fluid analysis was spiked with 5 µg/mL MMC and 1 µg/mL porfiromycin (9.28 min). MMC displays two signals at 3.97 min and 6.83 min. (e) HPLC-DAD analysis of MMC and the internal standard, porfiromycin, in 9% ACN / 91% 20 mM phosphate buffer (pH 6.5). The mobile phase of the urine analysis was spiked with 5 µg/mL MMC and 1 µg/mL porfiromycin (23.75 min). MMC displays two signals at 6.21 min and 15.34 min.

Figure 3. Concentration versus time graph of intraperitoneal mitomycin C, after administering ‘Triple Dosing Regimen’ during a 90-minute hyperthermic intraperitoneal perioperative chemotherapy procedure At each 15-minute time interval, plasma (red), peritoneal fluid (blue) and urine (green) were sampled. Concentration of MMC in peritoneal

526 fluid ranged from 6.02 – 9.90 µg/mL. Concentration of MMC in plasma ranged from 0.08 -
527 0.31 µg/mL. Concentration of MMC urine ranged from 0.25 – 9.95 µg/mL.

528

529 **Figure 4. Representative chromatograms of the mitomycin C and internal standard**
530 **signals, 15 minutes after administering ‘Triple Dosing Regimen’ hyperthermic**
531 **intraperitoneal perioperative chemotherapy** (a) HPLC-DAD analysis of MMC (6.73 min)
532 and the internal standard, porfiromycin (9.11 min), in plasma. (b) HPLC-DAD analysis of
533 MMC (6.77) and the internal standard, porfiromycin (9.15 min), in peritoneal fluid. (c) HPLC-
534 DAD analysis of MMC (12.39 min) and the internal standard, porfiromycin (19.50 min), in
535 urine.