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1 **A Validated High Performance Liquid Chromatography – Diode Array Detector**

2 **Method for the Quantification of Mitomycin C in Plasma, Peritoneal Fluid and Urine**

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26 **Abstract**

27 Mitomycin C (MMC) is a quinone-containing alkylating agent, that has been extensively
28 studied in preclinical and clinical work due to its antitumor activity. A thoroughly validated
29 high performance liquid chromatography-diode array detector method is provided to quantify
30 MMC in plasma, peritoneal fluid and urine. Porfiriomycin served as internal standard. The
31 mobile phase for the plasma, peritoneal fluid and urine analysis consisted of 27% MeOH and
32 73% 20mM ammoniumacetate buffer (pH 6.5) and 9% ACN and 91% 20mM phosphate buffer
33 (pH 6.5) respectively. The residue from 100 μ L ACN deproteinated plasma was dissolved in
34 250 μ L mobile phase. Peritoneal fluid and urine were diluted 10-fold in their respective mobile
35 phases. UV detection was performed at 365nm. Quantification of MMC was achieved over a
36 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
37 peritoneal fluid; 0.25-5 μ g/mL and 5-100 μ g/mL in urine. The limit of quantification was
38 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
39 further validated for selectivity, specificity, inter- and intraday precision and accuracy (\leq 15%),
40 extraction recovery and stability. MMC remains stable in the different biofluids for 20 days
41 (short-term stability) at 4 $^{\circ}$ C, -27 $^{\circ}$ C and -80 $^{\circ}$ C and for 80 days (long-term stability) at -80 $^{\circ}$ C.

42

43 **Keywords**

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

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49 **Introduction**

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

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

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

82 **Materials and methods**

83 **Safety considerations**

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

90 **Chemicals and reagents**

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

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

103 **Calibration standards and validation standards**

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

110 *Plasma*

111 MMC calibration standards in human plasma were prepared by serially diluting the MMC
112 standard stock solution in pooled drug-free plasma to reach final concentrations of 0.05, 0.1,
113 0.5, 1, 5, 10, 20 and 50 $\mu\text{g/mL}$. Quality control samples were prepared by diluting the MMC
114 standard stock solution with pooled drug-free plasma to reach final concentrations of 0.15, 2,
115 5, 16 and 25 $\mu\text{g/mL}$. Calibration standards and quality control samples were freshly prepared
116 each day.

117 *Peritoneal fluid and Urine*

118 Calibration standards in peritoneal fluid and urine were prepared by serially diluting the MMC
119 standard stock solution in drug-free peritoneal fluid and urine to reach final concentrations of
120 0.1, 0.25, 0.5, 1, 5, 10, 50 and 100 $\mu\text{g/mL}$. Quality control samples were prepared by diluting
121 the MMC standard stock solution with drug-free peritoneal fluid and urine to reach final

122 concentrations of 2, 5, 20 and 80 $\mu\text{g}/\text{mL}$. Calibration standards and quality control samples
123 were freshly prepared each day.

124 **Sample preparation**

125 *Plasma*

126 Human heparin plasma was obtained by centrifuging blood at 3000 x g for 15 min at 21°C. To
127 95 μL plasma sample volume, 5 μL of the internal standard working solution containing 10
128 $\mu\text{g}/\text{mL}$ porfiromycin was added. Protein precipitation was performed with 1 mL ACN. After
129 thoroughly mixing on a vortex-mixer for 10 seconds, the mixture was centrifuged at 3000 x g
130 for 10 minutes at 21°C. Subsequently, the supernatant was transferred to a clean glass tube and
131 evaporated to dryness under a steady stream of N_2 at 39°C. The residue was dissolved in 250
132 μL mobile phase and transferred to dark HPLC vials for injection.

133 *Peritoneal fluid and Urine*

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

139 **Instrumentation and chromatographic conditions**

140 The HPLC system consisted of a Hitachi LaChrom Elite (VWR) system equipped with a
141 Hitachi L-2200 autosampler, a Hitachi L-2100/2130 pump, a Hitachi L-2300 column oven and
142 a Hitachi L-2455 DAD (VWR, Leuven, Belgium). The analytical column was a reversed phase
143 Polaris 3 C18-A, 150 x 3.0 mm, 3 μm particle size (Agilent Technologies, Brussels, Belgium).
144 The mobile phase for the plasma and peritoneal fluid analysis consisted of 27% MeOH in 73%
145 20 mM ammoniumacetate buffer (pH 6.5). The mobile phase for the urine analysis consisted

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

153 **Method validation**

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

156 *Linearity*

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

160 *Limit of Detection*

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

163 *Limit of Quantification*

164 Limit of quantification (LOQ) was defined as the concentration of MMC at which the signal-
165 to-noise ratio corresponded to at least 10, that could be measured with an acceptable precision
166 and accuracy. Precision was expressed as the relative standard deviation (RSD%); calculated
167 as $((SD/mean) \times 100)$. The accuracy was expressed as the relative error (RE%); calculated as
168 $((\text{found concentration} - \text{theoretical concentration})/\text{theoretical concentration}) \times 100$.

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

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

197 **Case study**

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

204 **Results**

205 **Chromatography**

206 The total runtime for the plasma (Figure 2a) and peritoneal fluid (Figure 2b) analysis was 12
207 min. MMC eluted with a retention time of 6.9 min and the internal standard eluted with a
208 retention time of 9.4 min. The total runtime for the urine (Figure 2c) analysis was 30 min, with
209 a retention time of 14.9 min and 23.0 min for MMC and porfiromycin respectively. All peaks
210 exhibit a symmetric shape with only limited tailing. MMC displays two signals at 3.97 min and
211 6.83 min when measured in the plasma and peritoneal fluid mobile phase (Figure 2d) and at
212 6.21 min and 15.34 min when measured in the mobile phase for the urine analysis (Figure 2e).

213 **Validation**

214 *Linearity*

215 Calibration curves of MMC in plasma, peritoneal fluid and urine were divided into two sections
216 to cover a large linear range. The HPLC-DAD method covered a linearity range of 0.05 – 5
217 $\mu\text{g/mL}$ and 5 – 50 $\mu\text{g/mL}$ in plasma with typical standard curves of $y = 2.66x - 0.041$ with a
218 coefficient of determination (r^2) of 0.9998 and $y = 2.76x - 0.818$, $r^2 = 0.9997$ respectively.
219 Linearity in peritoneal fluid ranged from 0.1 – 5 $\mu\text{g/mL}$ and 5 – 100 $\mu\text{g/mL}$ with typical standard
220 curves of $y = 0.11x - 0.0017$, $r^2 = 0.9999$ and $y = 0.11x - 0.0021$, $r^2 = 0.9997$ respectively.
221 Linearity in urine ranged from 0.25 – 5 $\mu\text{g/mL}$ and 5 – 100 $\mu\text{g/mL}$ with typical standard curves
222 of $y = 0.31x - 0.0331$, $r^2 = 0.9995$ and $y = 0.34x - 0.2699$, $r^2 = 0.996$ respectively.

223 *Limit of detection*

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

225 *Limit of Quantification*

226 The LOQ was 0.05 $\mu\text{g/mL}$ MMC in plasma (n=10; RSD: 5.53%, RE: -15.07%), 0.1 $\mu\text{g/mL}$
227 MMC in peritoneal fluid (n=10; RSD: 2.09%, RE: 8.30%) and 0.25 $\mu\text{g/mL}$ MMC in urine (n =
228 10; RSD: 6.45%, RE: 14.97%).

229 *Specificity and Selectivity*

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

235 *Precision and Accuracy*

236 The inter- and intraday precision and accuracy were investigated at different MMC levels; 2,
237 16 and 25 $\mu\text{g/mL}$ MMC in plasma; 2, 20 and 80 $\mu\text{g/mL}$ MMC in peritoneal fluid and urine
238 (Table I). Both the RSD and RE are less than 8.94% for the HPLC-DAD analysis of all
239 biofluids. As MMC concentrations in plasma are very low in a cancer patient treated with
240 MMC-based HIPEC (see section case study), an additional level of 0.15 $\mu\text{g/mL}$ MMC in plasma
241 was evaluated for accuracy and precision. RSD and RE were less than 9.72%. Inter- and
242 intraday precision and accuracy were also validated at a fourth level, 5 $\mu\text{g/mL}$ MMC, i.e. at the
243 intersection of the calibration curves in the respective biofluids, using both calibration curves
244 (Table II). Both the RSD and RE are less than 9.58% for all biofluids.

245 *Extraction recovery*

246 The extraction recovery from plasma, peritoneal fluid and urine using the sample preparation
247 methods described in the materials and methods section is presented in Table III. In summary,
248 the recovery was assessed at three different MMC levels; 1, 10 and 50 $\mu\text{g/mL}$ in plasma; 1, 50
249 and 100 $\mu\text{g/mL}$ in peritoneal fluid and urine. The reported recoveries range from 82.13 –
250 99.44%.

251 *Stability*

252 The short-term and long-term stability at 4°C, -27°C and -80°C of MMC in plasma, peritoneal
253 fluid and urine is summarized in Table IV. Stability was assessed at three different MMC levels;
254 2, 16 and 25 $\mu\text{g/mL}$ MMC in plasma; 2, 20 and 80 $\mu\text{g/mL}$ MMC in peritoneal fluid and urine,
255 and expressed in terms of recovery. For all biofluids, recovery ranged from 76.61 – 112% after
256 20 days of storage at all defined storage conditions. As to long-term stability; when stored at
257 4°C and -27°C, recoveries ranged from 26.52 – 93.25% with high SDs. When stored at -80°C,
258 recoveries of all biofluids ranged from 76.74 – 103%. Freeze-thaw stability was not investigated
259 as part of this validation study. For the application of conducting pharmacologic research

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

263 **Case study**

264 The concentration versus time curve of MMC in plasma, peritoneal fluid and urine is depicted
265 in Figure 3. MMC was quantified in the respective biofluids sampled at each 15-min time
266 interval during the HIPEC procedure. The peritoneal fluid concentration curve demonstrates
267 the administration of MMC in three phases, ‘Triple Dosing Regimen’, and the subsequent
268 decrease in MMC concentration. The concentration in peritoneal fluid ranged from 6.02 – 9.90
269 $\mu\text{g/mL}$. Plasma concentration, 15 min after initial administration of MMC, was 0.08 $\mu\text{g/mL}$ and
270 increased to 0.31 $\mu\text{g/mL}$ after the 90-minute HIPEC procedure. MMC concentration in urine
271 ranged from 0.25 – 9.95 $\mu\text{g/mL}$. Representative chromatograms of the MMC and internal
272 standard signals, 15 minutes after intraperitoneal chemotherapy administration in the different
273 biofluids of the case study’s patient, are presented in Figure 4.

274 **Discussion**

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

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

284 temperatures higher than 40°C will result in the decomposition of MMC and can therefore not
285 be utilized [21]. In the optimization part of this assay, we explored protein precipitation as
286 plasma sample preparation, followed by evaporation at 39°C under a steady stream of N₂. The
287 improvement we report relative to the protein precipitation method described by Joseph *et al.*
288 [14] is the time needed for evaporation after precipitation with ACN, i.e. 20 minutes for 36
289 samples as compared to 4-5 hours for 20 samples. For the urine analysis, we explored SPE
290 using different stationary phases; Bond Elut Plexa (30 mg, 3 mL, Agilent Technologies, USA)
291 and ProElut NH₂ (500 mg, 3mL, Dikma Technologies, USA) as sample preparation method.
292 However, SPE sample preparation did not result in an acceptable LOQ and extraction recovery.
293 The above-mentioned sample preparation methods were all optimized aiming at the use of a
294 uniform mobile phase and chromatographic conditions for all biofluids. However, for the urine
295 analysis, the use of 27% MeOH and 73% 20 mM ammoniumacetate buffer (pH 6.5) did not
296 result in acceptable selectivity, specificity and sensitivity. A mobile phase consisting of 9%
297 ACN and 91% 20mM phosphate buffer (pH 6.5) with a runtime of 30 minutes was necessary
298 to achieve good baseline separation for the MMC and internal standard signals in urine and to
299 eliminate interference from contaminants. We can conclude that precipitation with ACN as
300 sample preparation for plasma and a 10-fold dilution in mobile phase followed by filtration
301 through a 0.45 µm syringe filter as sample preparation for peritoneal fluid and urine resulted in
302 limited to no loss of sample and that good baseline separation was achieved used the above-
303 mentioned mobile phases.

304 MMC displays two signals when measured in the mobile phases of the biofluids (Figure 2d and
305 2e). From liquid chromatography – mass spectrometry (LC-MS) analysis, we know that both
306 signals have the same parent ion (m/z 335) and generate the same fragment ion (m/z 242).
307 Therefore, it can be postulated that two stereoisomers of MMC are present in the reference
308 standard and are retained differently on the chromatographic column. Throughout the validation

309 of the HPLC-DAD method, we performed measurements on the signal with a retention time of
310 6.83 min (plasma and peritoneal fluid analysis) and 15.34 min (urine analysis); i.e. the
311 stereoisomeric form of MMC most present in the reference standard.

312 The above described method was validated and proven to be robust in terms of linearity,
313 selectivity, specificity, accuracy, precision and extraction recovery. Regarding stability,
314 recovery of the peritoneal fluid analysis after long-term storage at -80°C was lower than
315 reported for the other biofluids, i.e. 76.74 - 79.35% versus 94.10 - 103%. However, recovery
316 is still in the acceptable range and therefore we confirm that long-term storage of all biofluids,
317 up to 80 days, should be conducted at -80°C. The LOQ we report in plasma is higher than the
318 LOQ reported for the HPLC-UV method by Joseph *et al.* [14]. However, the presented LOQ's
319 in the different biofluids allow to perform pharmacokinetic studies in which patients receive
320 the 'Dutch High Dose Mitomycin C Regimen, Triple Dosing Regimen', the most widely used
321 HIPEC MMC dosing regimen. The latter is again confirmed by previous pharmacokinetic
322 research reporting on the concentration of MMC administered during HIPEC. Sugarbaker *et al.*
323 treated patients with low dose MMC, 12.5 mg/m² in males and 10 mg/m² in females using a
324 single administration and diluted this dose in varying volumes of carrier solution. The
325 concentration reported in plasma ranged from 0.01 µg/mL at time point zero to approximately
326 0.5 µg/mL and in peritoneal fluid from 5 µg/mL to approximately 10 µg/mL [22]. Van der
327 Speeten *et al.* treated patients with a combination therapy of 15 mg/m² doxorubicin and 15
328 mg/m² MMC. MMC plasma concentration ranged from 0.1 µg/mL at time point zero to 0.25
329 µg/mL, MMC urine concentration ranged from 0.1 µg/mL to approximately 5 µg/mL and from
330 5 µg/mL to approximately 10 µg/mL in peritoneal fluid [4].

331 **Conclusion**

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

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

338

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345

346 **Conflict of interest statement**

347 The authors declare that they have no conflict of interest.

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444 **Tables**445 **Table I. Interday and intraday precision (RSD%) and accuracy (RE%) in plasma,**
446 **peritoneal fluid and urine.**

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

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456 **Table II. Interday and intraday precision (RSD%) and accuracy (RE%) of 5 µg/mL MMC**
 457 **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

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467 **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

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

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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

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

501 **Figure legends**

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503 **Figure 1. Chemical structure of mitomycin C and porfiromycin.**

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505 **Figure 2. Selectivity and specificity of the HPLC-DAD mitomycin C analysis in plasma,**

506 **peritoneal fluid and urine** (a) HPLC-DAD analysis of MMC (6.84 min) and the internal

507 standard, porfiromycin (9.28 min), in plasma. Plasma spiked with the LOQ, 0.05 µg/mL MMC

508 and 0.5 µg/mL porfiromycin (blue). Drug-free plasma (green). (b) HPLC-DAD analysis of

509 MMC (7.01) and the internal standard, porfiromycin (9.50 min), in peritoneal fluid. Peritoneal

510 fluid spiked with the LOQ, 0.1 µg/mL MMC and 10 µg/mL porfiromycin (blue). Drug-free

511 peritoneal fluid (green). (c) HPLC-DAD analysis of MMC (14.85 min) and the internal

512 standard, porfiromycin (22.98 min), in urine. Urine spiked with the LOQ, 0.25 µg/mL MMC

513 and 5 µg/mL porfiromycin (blue). Drug-free urine (green). (d) HPLC-DAD analysis of MMC

514 and the internal standard, porfiromycin, in 27% MeOH / 73% 20 mM ammoniumacetate buffer

515 (pH 6.5). The mobile phase of the plasma and peritoneal fluid analysis was spiked with 5 µg/mL

516 MMC and 1 µg/mL porfiromycin (9.28 min). MMC displays two signals at 3.97 min and 6.83

517 min. (e) HPLC-DAD analysis of MMC and the internal standard, porfiromycin, in 9% ACN /

518 91% 20 mM phosphate buffer (pH 6.5). The mobile phase of the urine analysis was spiked with

519 5 µg/mL MMC and 1 µg/mL porfiromycin (23.75 min). MMC displays two signals at 6.21 min

520 and 15.34 min.

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522 **Figure 3. Concentration versus time graph of intraperitoneal mitomycin C, after**

523 **administering ‘Triple Dosing Regimen’ during a 90-minute hyperthermic intraperitoneal**

524 **perioperative chemotherapy procedure** At each 15-minute time interval, plasma (red),

525 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.

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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.**