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Sensitive and convenient high-performance liquid chromatographic method for the determination of mitomycin C in human plasma

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Abstract

An improved high-performance liquid chromatographic assay for the cytostatic drug mitomycin C in plasma is presented. The principal steps are precipitation of plasma proteins with acetonitrile, lyophilization of the supernatant and reversed-phase chromatography on a Hypersil ODS 5 μm column with 0.01 *M* NaH_2PO_4 buffer (pH 6.5)–methanol (70:30, v/v) in isocratic mode. At a flow-rate of 1.3 ml/min a column pressure of 180–220 bar resulted. Porfiromycin served as internal standard. UV detection was performed at 365 nm. Quantitation limit based on a coefficient of variation <10% in intra- and inter-day assay was 5 μg/l mitomycin C, detection limit based on a signal-to-noise ratio of 3 was 1 μg/l. Recovery was 100% and linearity was shown for the whole range of concentration (1–500 μg/l). None of the five drugs used during chemoembolisation interfered with the assay in vitro. The assay meets the requirements for pharmacokinetic studies of mitomycin C in patients as regards sensitivity and ease of use. © 1997 Elsevier Science B.V.

Keywords: Mitomycin C

1. Introduction

Chemoembolisation is a well established method in the treatment of hepatic metastasis. The most frequently used chemotherapeutic drug for this application is mitomycin C (MMC) (Fig. 1A), a bioreductive alkylating agent first isolated from *Streptomyces caespitosus* by Wakiki et al. in 1958 [1].

Intraarterial chemotherapy can produce much higher tumor drug concentrations and lower systemic availability than intravenous administration. Additional embolisation of the tumor supporting vessels can reduce blood flow, so drug exposure may be prolonged, tumor drug uptake increased and systemic availability of the cytostatic drug further reduced.

Various clinical studies are currently investigating the pharmacokinetic properties and local availability of MMC after different procedures of intraarterial chemoembolisation or proximal arterial occlusion by a balloon catheter. These studies require an analytical method for MMC in biological samples which should be highly sensitive but also as quick and simple as possible.

The present study describes an improved high-

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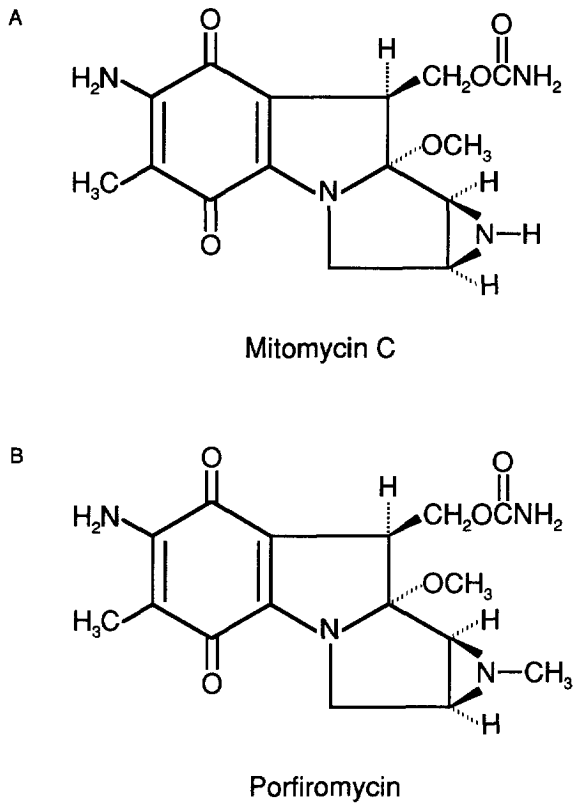

Fig. 1. Structures of mitomycin C [A] and porfiromycin [B].

performance liquid chromatographic (HPLC) method that matches these requirements closely.

2. Experimental

2.1. Instrumentation

The method was developed using a Merck–Hitachi HPLC system (Merck, Darmstadt, Germany) consisting of an AS-4000 autosampler, a D-6000 interface, a L-6200 intelligent pump and a T-6300 column-thermostat. The storage rack for the vials in the autosampler was cooled to 5°C by a WK-5 cryo-thermostat (Colora Meβtechnik, Lorch, Germany). UV detection was performed at 365 nm with a L-4250 UV–Vis detector (Merck). Separations were done on a 250×4 mm Hypersil ODS 5 μm column (Chromatographie Service, Langerwehe, Germany). The mobile phase was made up of an aqueous solution of 0.01 *M* NaH_2PO_4 buffer (pH 6.5)–methanol (70:30, v/v). The HPLC system was operated at 30°C in an isocratic mode. The flow-rate was set to 1.3 ml/min resulting in a column pressure of 180–220 bar.

2.2. Chemicals

The following chemicals were used for the analytical procedure: HPLC grade acetonitrile (LiChrosolv, acetonitrile gradient grade, Merck), HPLC grade methanol (Rotisolv HPLC, methanol gradient grade, Carl Roth, Karlsruhe, Germany), purified water (Milli-Q Plus, Millipore, Molsheim, France), NaH_2PO_4 (NaH_2PO_4 for molecular biology) and 1 *M* NaOH (Merck).

Mitomycin C (mitomycin 2 Medac for intravenous and intraarterial injection, Ch. B.: 949 ADK) was from Medac (Hamburg, Germany) and porfiromycin (porfiromycin for investigational and laboratory purposes, Lot. No. 8458-THP-49-10) was from Upjohn Laboratories (Kalamazoo, MI, USA) (Fig. 1B).

Drugs normally administered to patients during intraarterial chemoembolisation and proximal arterial occlusion in the treatment of hepatic tumors and metastasis were tested for interference with the analytical procedure in vitro:

Lorazepam (Tavor, Wyeth-Pharma, Münster, Germany), dexamethasone (Fortecortin, Merck), mezlocillin (Baypen, Bayer, Leverkusen, Germany), pethidine (Dolantin, Hoechst, Frankfurt, Germany), ondansetrone (Zofran, Glaxo Wellcome, Hamburg, Germany), were all used as their injectable pharmaceutical preparations.

2.3. Sample preparation

Previously spiked and frozen (−20°C) plasma samples were thawed, stirred (vortex, Bender and Hobein, Zurich, Switzerland) for 10 s and centrifuged (Eppendorf 5402 centrifuge, Hamburg, Germany) for 5 min at 15 800 *g* and 4°C. Then 500 μl of plasma was pipetted (Eppendorf micropipette) into plastic vials, 50 μl of a solution containing porfiromycin (PM) (4000 μg/l) in methanol–water (50:50, v/v) was added as internal standard and 1 ml acetonitrile was added for protein precipitation. The vials were then stirred for 1 min and centrifuged for

10 min at 15 800 *g* and 4°C. The supernatant was transferred into plastic vials and lyophilized in a vacuum centrifuge (Hetovac VR-1, Heto Lab Equipment, Allerod, Denmark). Lyophilization lasted about 4–5 h for 20 samples. After lyophilization the residue was reconstituted in 150 μl elution medium [0.01 *M* NaH_2PO_4 (pH 6.5)–methanol, 70:30, v/v] and transferred into HPLC autosampling vials. Fifty microliters were used for chromatography.

2.4. Assay validation

Drug free plasma (0.5 ml) was spiked with aliquots from the stock solution of MMC (0.21 g/l) or its aqueous dilutions (5000 μg/l, 500 μg/l, 50 μg/l) to obtain plasma concentrations from 1–500 μg/l. The samples were treated as described above. Linearity and variability were determined for the MMC/PM ratio. The lowest concentration with a coefficient of variation (C.V.) <10% in intra- and inter-day assay was regarded as the lower limit of quantitation. The detection limit was defined as a signal-to-noise ratio of 3. Correlation between the spiked and the measured concentrations was tested by linear least-squares regression analysis. Variability was estimated as the standard deviation in percent of the mean value of the respective concentration. The intra-day (intra-assay) variability was determined by replicate measurements ($n=7$ on the same day) of six different concentrations (concentrations shown in Table 1) processed in replicates on five days. The inter-day (inter-assay) variability was determined by assaying a specimen containing 2, 5, 10 and 500 μg/l MMC on 10 different days during a period of one month. Recovery rates were determined by comparing the areas for MMC (500 μg/l, 100 μg/l) and PM (400 μg/l) obtained from plasma standards with those obtained by direct injection of the aqueous solutions.

Table 1
Intra-assay variability of the mitomycin C/porfiromycin ratio on 5 different days

Conc. (μg/l)	C.V. (%)				
	Day 1	Day 2	Day 3	Day 4	Day 5
2	11.27	16.41	25.24	19.89	24.79
5	8.23	7.95	8.34	7.41	9.72
10	3.9	4.85	5.32	5.82	5.72
20	4.36	3.91	4.49	4.23	4.98
100	3.55	1.22	2.14	3.27	3.64
500	3.27	1.92	3.73	3.62	2.43

C.V.=coefficient of variation ($n=7$ for each concentration on each day).

2.5. Interference

Drugs which may be used during chemoembolisation or proximal arterial occlusion (lorazepam, dexamethasone, mezlocillin, pethidine, ondansetrone) were tested for interference with the MMC assay. The drugs were added to samples of human plasma to obtain concentrations in excess of concentrations occurring under clinical conditions. The visual appearance of chromatography was assessed and the areas for MMC and PM obtained from plasma samples with and without the respective drug were compared.

3. Results

Fig. 2A shows a chromatogram of a plasma sample containing 500 μg/l MMC after preparation as described above. It demonstrates the peaks of MMC and PM at retention times of 4.92 min and 6.64 min, respectively. The peaks are clearly separated and well shaped. Fig. 2B shows a chromatogram of a drug free plasma extract.

Intra-assay variability at 5 different days (Table 1) ranges from 1.9–3.7% at the maximum concentration (500 μg/l) investigated. At 5 μg/l it ranges from 7.4–9.7% and at a concentration of 2 μg/l the intra-assays varied between 11.3% and 25.2%. The inter-assay variability (Table 2) was 3.0%, 8.7% and 19.9% for 500 μg/l, 5 μg/l and 2 μg/l on 10 different days. According to the above definition a concentration of 5 μg/l MMC turned out to be the lower limit of quantitation. The detection limit was 1 μg/l. The correlation between the MMC/PM area ratio and the spiked MMC concentration is shown in Fig. 3. The correlation coefficient (r) and the coefficient of determination (r^2) are both higher than 0.999. Recovery rates for MMC (500 μg/l, 100 μg/l) and PM (400 μg/l) were 100% each.

None of the five drugs tested (lorazepam, dexamethasone, mezlocillin, pethidine, ondansetrone) in-

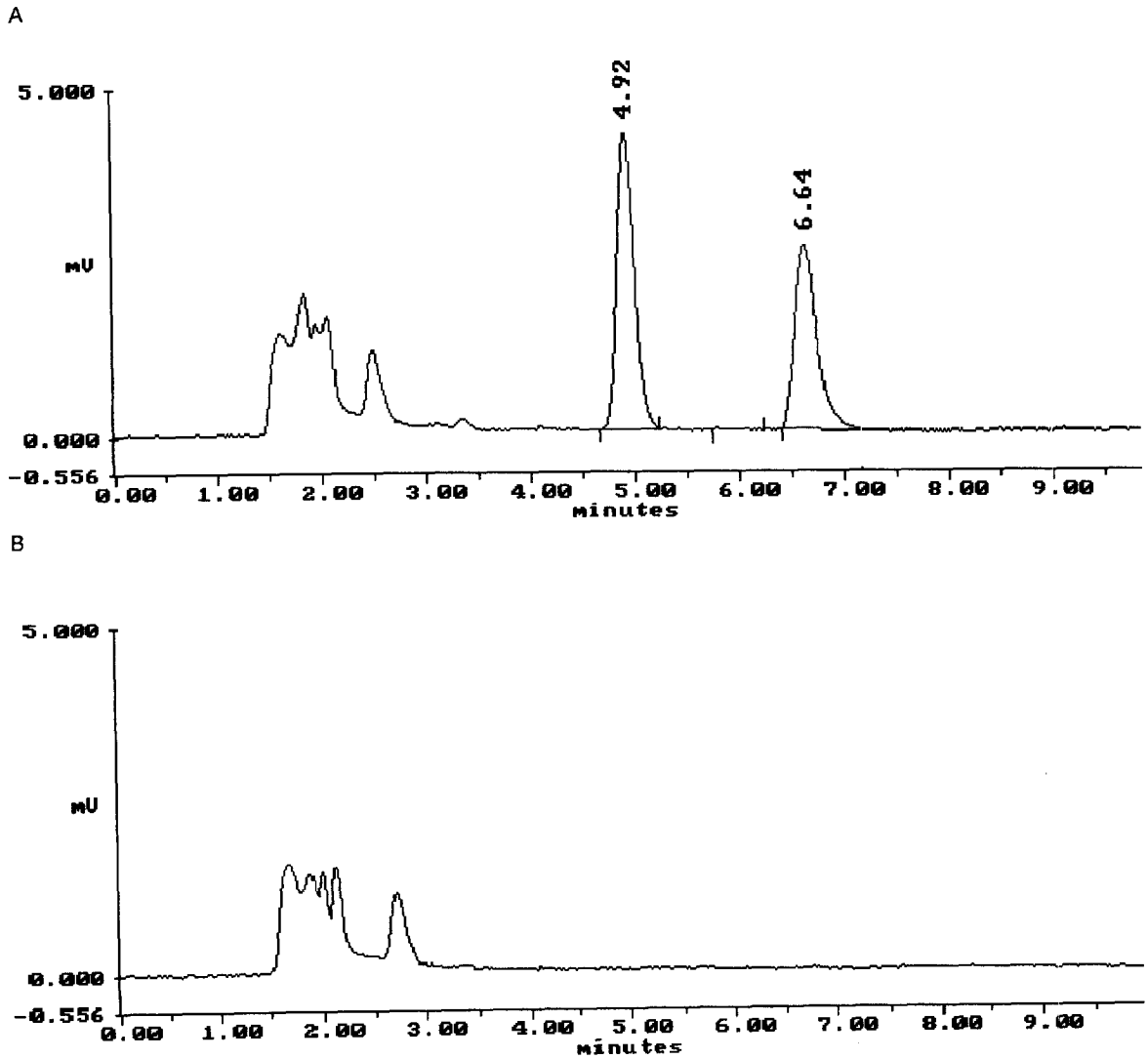

Fig. 2. HPLC chromatogram of a spiked plasma sample containing mitomycin C (500 μg/l, t_R=4.92 min) and porfiromycin (400 μg/l, t_R =6.64 min) (A) and a drug free plasma extract (B).

terfered with the MMC assay. Fig. 4 shows a concentration–time curve of MMC in human plasma after chemoembolisation. Ethical approval of the IRB and written informed consent of the patients were obtained. Pharmacokinetic evaluation of our data revealed a terminal elimination half-life of 59.0±23.7 min for MMC after chemoembolisation with spherex [2] which corresponds to the figures reported in the literature [3] quite well.

4. Discussion

HPLC methods for the analysis of mitomycins and their derivatives can be divided into reversed-phase (RP), normal phase and ion-exchange chromatography.

Liquid chromatography in the isocratic RP-mode is most frequently applied and is suitable for drug monitoring [4–17]. All RP systems are largely

Table 2
Inter-assay variability of the mitomycin C/porfiromycin ratio on 10 different days

Conc. (μg/l)	C.V. (%)
2	19.87
5	8.74
10	6.08
500	2.97

C.V.=coefficient of variation (n=1 for each concentration on each day).

similar, using either C_{18} [4–7,9–14,16–27] or C_8 [15,28] alkyl-bonded silica gel stationary phases and mobile phases consisting of an aqueous buffer and methanol [4–6,9–16,19–24,26] or acetonitrile [7,15,18,25,27,28]. Most column LC assays use UV detection at 360/365 nm for MMC because the high molar absorptivity (ϵ=21 380) [29] and the relatively long UV absorption wavelength guarantee high sensitivity and selectivity.

So far our method corresponds to the methods normally used in literature. The improvements we are proposing refer to the sample preparation. Sample purification is generally necessary to achieve a determination method with sufficient selectivity, sensitivity, accuracy and reproducibility [8]. For the analysis of MMC in rat serum a protein precipitation step with methanol was found sufficient as clean-up [12]. However, a determination limit of 10 μg/l restricted the use of this method. Other methods utilize liquid–liquid extraction for purification. Chloroform–2-propanol [4–6,13] and chloroform–2-propanol–ethyl acetate [30] mixtures gave the best results. Recoveries higher than 90% in the concentration range 1–2000 μg/l and a detection limit of 1 μg/l could be achieved but evaporation of the extraction medium required several hours resulting in a long turnaround time for the assays [10]. In order to eliminate this disadvantage, liquid–solid extraction procedures have been developed. Eksborg et al. [15], Tjaden et al. [7] and Buice et al. [10] used Amberlite XAD-2 resin or C_{18} Sep-Pak cartridges in order to extract the drug from the biological matrix. The recovery and sensitivity are in general somewhat lower than in liquid–liquid extractions. Buice et al. [10] reported a determination limit of 10 μg/l which may be not sufficient for pharmacokinetic studies of MMC in patients. The isolation procedure of Tjaden et al. [7], which included the preparation of Pasteur

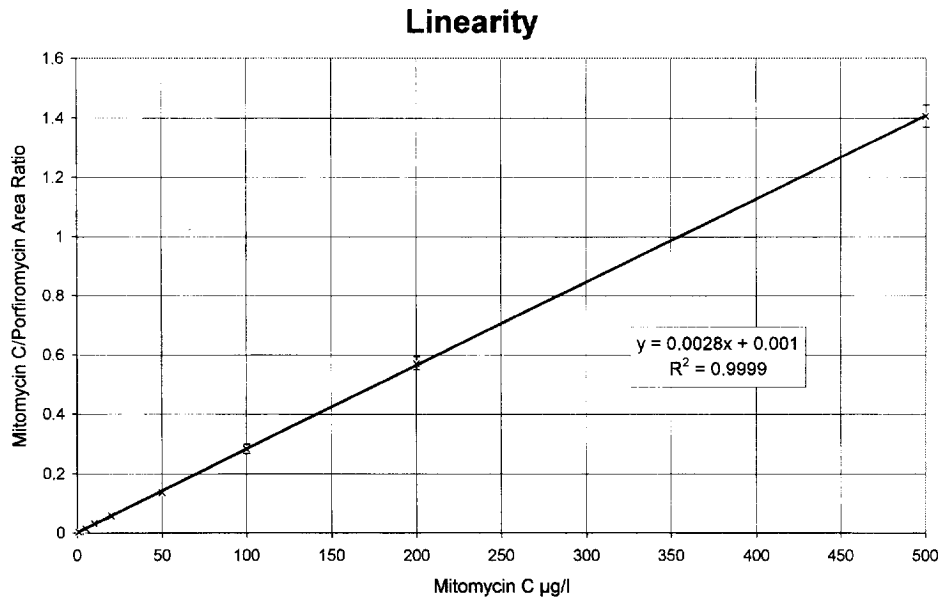

Fig. 3. Correlation of the mitomycin C/porfiromycin area ratio versus spiked mitomycin C concentration. Each point represents mean±S.D. (n=5).

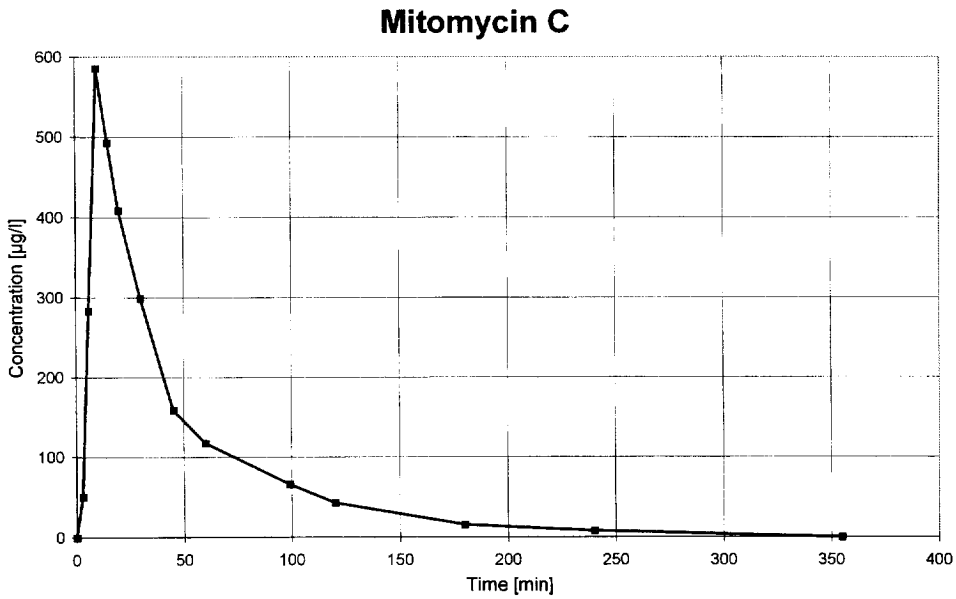

Fig. 4. Plasma concentration–time curve of mitomycin C after chemoembolisation; 20 mg mitomycin C combined with 400 mg spherex starch particles in 20 ml saline were applied in a short infusion over 4 min.

pipettes containing an ion-exchange resin, was found to be time consuming [10] and whether it was an improvement compared to liquid–liquid extraction is uncertain. Eksborg et al. [15] showed considerable variations in recovery (71.5–102.9%). The minimum recovery was found at the minimum concentration and the recovery seemed to increase with the MMC concentration tested. The most rapid and most sophisticated sample preparation was described by Choi et al. [31]. They reported an on-line extraction of MMC and PM from blood and urine using an injector-loop column packed with Porapak Q. But with a detection limit of 25 μg/l this method may be not sufficient for pharmacokinetic studies of MMC in patients.

The present work proposes protein precipitation with acetonitrile, which is easy to do and yields a sample quality suitable for HPLC analysis. The lower limit of quantitation – 5 μg/l with a coefficient of variation <10% in intra- and inter-day assay – is sufficient for pharmacokinetic studies of MMC in patients. The new method makes it possible to prepare up to 40 samples for HPLC in an 8-h shift. The subsequent HPLC analysis is performed automatically overnight with a processing time of 30 min per sample.

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