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AUTOMATED ANALYSIS OF MITOMYCIN C IN BODY FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ON-LINE SAMPLE PRE-TREATMENT

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SUMMARY

A fully automated liquid chromatographic system for the bioanalysis of mitomycin C has been described. The isolation of the analyte from the biological matrix (plasma, ascites and urine) is performed using a continuous-flow system equipped with a dialysis membrane in order to remove proteins. The samples are concentrated on a reversed-phase pre-column and subsequently introduced on to a reversed-phase analytical column by applying column-switching techniques. The drug is detected by absorbance measurements at 360 nm. Using the described system up to 100 samples a day can be analysed with determination limits of the order of 1 ng/ml, with a linear dynamic range of at least three decades for plasma and urine samples. The procedure was applied to pharmacokinetic studies of ovarian cancer patients treated intraperitoneally with mitomycin C.

INTRODUCTION

Mitomycin C (MMC), still the only clinically applied representative of the mitomycin antibiotics, has been applied in clinical oncology since the late 1950s and has shown to be active against various tumours [1,2]. One of the clinical applications of MMC is ovarian malignancies [3] but, because of severe myelosuppression and gastrointestinal complications, the oral and intravenous administration of MMC leads to severe systemic toxicity. Regional administration by intraperitoneal infusion may result in a relatively high concentration of the drug at the tumour site and, therefore, a decreased systemic toxicity, depending on the ratio of total body clearance and peritoneal clearance, is expected. In order to evaluate the effectiveness, the tolerance and the pharmacokinetics of intraperitoneally applied MMC in resistant ovarian cancer, an appropriate bioanalytical assay that can be applied in routine analysis must be available.

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The determination and quantitation of MMC in biological fluids was first performed by means of microbiological assays [4,5]. The impossibility of these assays detecting metabolites and degradation products [6,7] and the cross-reactivity of the enzyme immunoassay [8,9] make these methods inferior to the more sensitive and selective liquid chromatographic (LC) [10–12] and high-performance differential pulse polarographic [13] methods that have been developed in recent years.

Proteins from the biological matrix may hamper the assay and can decrease the lifetime of the analytical column dramatically. Further, the plasma levels of MMC are normally very low because of rapid degradation and metabolization of the antitumour antibiotic [14,15]. A number of procedures have been described for the sample pre-treatment and for blocking the enzyme activity, but several of these methods, e.g., protein removal with acids, cannot be applied. The reason is the limited stability of MMC in acidic solutions [16,17]. Other investigated sample pre-treatment procedures for MMC, such as liquid–liquid extraction [18] or off-line liquid–solid extraction [10], are in general laborious or very time-consuming for large numbers of samples. For the analysis of MMC in rat serum a protein precipitation step with methanol was found to be sufficient as the only sample clean-up step [19] and Van Bennekom et al. [13] demonstrated that high-performance differential pulse polarography is capable of determining the solute in biological fluids without using a sample pre-treatment procedure. The conclusion is that most of the published bioanalytical LC procedures cannot be used as such [20,21].

Porfiromycin was introduced as an internal standard by Tjaden and co-workers [10,22] and today this MMC analogue is included as an internal standard in most of the described bioanalytical LC assays.

In this study a fully automated sample pre-treatment procedure was investigated by applying continuous-flow (CF) and valve-switching techniques. Valve switching is applied to allow the combination of relatively large volumes needed in CF techniques and reversed-phase (RP) LC.

The required sensitivity is achieved by using a modification of the assay as described by Tjaden et al. [10], which permits the determination of MMC in the urine of bladder cancer patients treated with intravesical MMC at levels down to 150 pg/ml, which is the lowest limit of determination reported sofar [23].

The combination of CF and LC in a fully automated assay improved both the reproducibility and the throughput of the samples, which is a necessity for large-scale pharmacological studies as carried out for the intraperitoneal administration of MMC. Owing to the improved reproducibility of the system, the addition of an internal standard is not necessary. The clinical pharmacokinetics of MMC following intravenous, oral, intrahepatic, intraperitoneal and intravesical administration and the pharmacokinetics of isolated liver perfusion and MMC behaviour during in vitro incubations with tumour cells are currently under investigation in our laboratories [24–26].

EXPERIMENTAL

Apparatus

The LC system consisted of a Model SP 8770 isocratic high-pressure pump (Spectra-Physics, Darmstadt, F.R.G.) combined with an LC–UV variable-wavelength detector (Pye Unicam, Cambridge, U.K.) operating at 360 nm. Computing integration of the chromatograms was performed with a Model C-R3A integrator (Shimadzu, Kyoto, Japan). The analytical column was a 100.0 m × 3.0 mm I.D. stainless-steel tube, slurry packed with an air amplifier booster pump (Model 122; Haskel, Burbank, CA, U.S.A.) with Nucleosil C_8 spherical particles of 5 μm (Macherey-Nagel, Düren, F.R.G.) dispersed in 1,1,1-trichloroethane using a pressure drop of 60 MPa. Stainless-steel pre-columns of 30.0 mm × 2.0 mm I.D. were manually packed with Polygosil C_8 material with 40–63 μm particles (Macherey-Nagel) and replaced daily.

In order to control the sample and reagent streams, the sampling was performed by using an automated sampler (Model 1000) equipped with a peristaltic pump (Model 2002), both from Skalar Analytics (Breda, the Netherlands), in order to control the sample and reagent streams. The autosampler tubes had a volume of 3.5 ml and were made of polystyrene (Model 1022; Skalar Analytics). A Model 75A laboratory matrix timer (Kipp Analytica, Delft, The Netherlands) was used to control the valve switching and to start the integration. Dialysis of the samples was executed by the application of a Perspex dialysis block (Model 5275; Skalar Analytics) equipped with a dialysis membrane (cellulose) with a pore diameter of about 1 nm and a molecular mass resistance (exclusion) of 10 000 (Skalar Analytics).

Chemicals

MMC was obtained from Kyowa Hakko Kogyo (Tokyo, Japan). The organic solvents were obtained from J.T. Baker (Deventer, The Netherlands) and were of analytical-reagent grade. 1,1,1-Trichloroethane came from Janssen Chimica (Beerse, Belgium) and all other reagents were of analytical reagent grade and were used as received. Throughout the study deionized water (Milli Q water purification system; Millipore, Bedford, MA, U.S.A.) was used.

Sample preparation and storage

Blood samples were taken during 24 h before and after the administration of MMC and collected in heparinized polyethylene tubes. After centrifugation (1000 *g*) of the samples for 10 min the supernatant was stored at 250 K until the analysis took place.

Urine samples from patients treated with MMC were collected in polyethylene vials during 18 h and stored at 250 K.

Ascites samples were gathered in polyethylene tubes during 6 h after the intraperitoneal administration of MMC through a catheter and stored at 250 K.

Assay procedures

Defrosted plasma samples of 2 ml were placed in the autosampler and aliquots of about 1.68 ml were transported by the peristaltic pump to the dialysis block

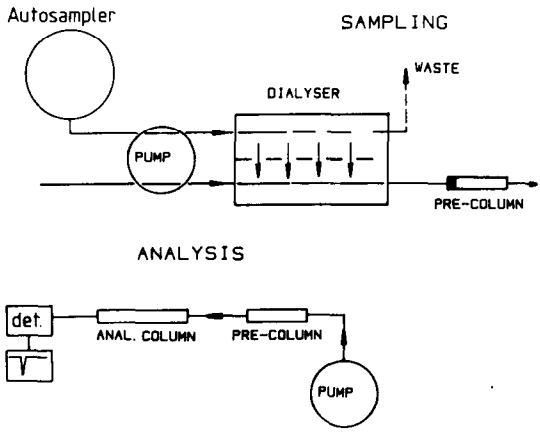

Fig. 1. Sampling and analysis of mitomycin C in plasma, urine and ascites samples.

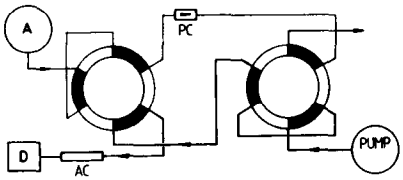

Fig. 2. Valve-switching unit for the analysis of mitomycin C samples. Loading of PC starts at A and follows the black blocks of the six-port valves. Elution of samples to the AC follows the white blocks of the valves. A, Autosampler and dialysis block; AC, analytical column; D, detector; PC, pre-column.

(Fig. 1). The sampling time was 4 min. Air segmentation (0.23 ml/min) was applied and improved by the addition of detergent (1% Teepol) to the donor stream (0.42 ml/min), which was pure water. Diffusion was subsequently performed with the described diffusion membrane and MMC was transferred to the acceptor stream (0.60 ml/min) of pure water. The pre-column was then loaded with the diffused MMC. The pre-column was kept under reduced pressure by a water-jet vacuum pump during the loading time. After 10 min, the six-port valve was switched and the pre-column was incorporated into the chromatographic system. MMC was eluted, in the back-flush mode, with acetonitrile–water (15:85) from the pre-column (Fig. 2) to the analytical column.

Stored ascites samples were diluted, just before the analysis, with 99 volumes of the donor stream solvent and aliquots of 2 ml of the resulting solutions were placed in the autosampler.

The stored urine samples were treated in the same way as described for the ascites samples. The only exception was that the defrosted samples were diluted with 9 volumes of the donor stream solvent instead of 99 volumes of this solvent.

For the plasma, urine and ascites samples, after every 25 samples a new calibration graph was established.

RESULTS AND DISCUSSION

Sample pre-treatment

Normally techniques based on dialysis (diffusion) of samples are relatively slow. It may take hours of dialysis before an equilibrium is reached between the

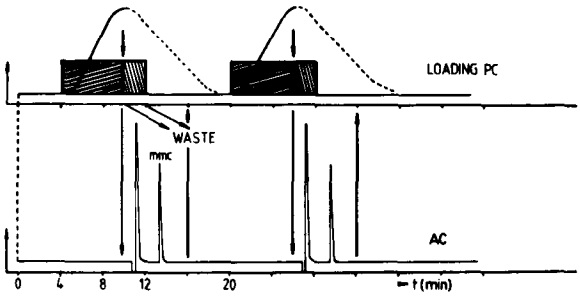

Fig. 3. Pre-concentration of mitomycin C samples. Top: $t=0$, start sample loading; $t=4$ min, start loading pre-column; $t=10$ min, switching valves; $t=10$–12 min, back-flushing of PC; $t=16$ min, start of second cycle. Bottom: what occurs on the analytical column at the same time.

two sample compartments of a dialysis system and the maximal achievable absolute recovery is 50%. However, using this system a relatively high diffusion gradient over the membrane is obtained. This increased diffusion gradient is caused by the increased contact time between the donor and acceptor stream in this system, realized by the application of flow for both solvents.

The amount of sample that is taken by the peristaltic pump is determined by the sampling time and the inner diameter of the applied tubing. Air segmentation in the donor stream prevents contamination of a sample by the next sample of MMC in the tubing and in addition improves the mixing in the sample compartment and consequently the diffusion.

Pre-concentration conditions

The pre-concentration of the MMC-containing acceptor stream on the pre-column was facilitated by holding the pre-column under reduced pressure during the total loading time of 10 min. After 10 min the optimal loading degree was achieved, because longer loading times resulted in a substantial increase in the total analysis time (Fig. 3) owing to the slow elution of the MMC samples from the pre-column.

The samples were eluted in the back-flush mode of the pre-column because elution of MMC in the forward-flush mode resulted in a rapid contamination of the analytical column (Fig. 2). Owing to the pressure shocks in the system, as a result of switching the valves, a gradual increase in the band widths of the peaks was observed.

The most important advantages of the described sample pre-treatment procedure for the determination of MMC in biological fluids were the very limited contamination of the LC system and the fact that the method could be used in routine analysis because up to 100 samples a day could be determined unattended, especially when the system is equipped with two alternating pre-columns in a box-car configuration.

The advantage of using two alternating pre-columns should be the possibility of injecting a new sample into the system every 10 min, because during equilibration of the first pre-column the second one could be loaded with the next MMC sample. By using one pre-column only four samples per hour can be injected (Fig.

3). However, as some memory effects occur when relatively high concentrations (over 75 ng/ml) were analysed, the CF-LC system was during this study equipped with only one pre-column.

Chromatographic conditions

For the analysis of MMC in biological fluids a number of chromatographic systems have been described. Most of these systems are based on reversed-phase separation [20,21] and only a few examples of the application of normal-phase systems have been described [6]. The limited stability of normal-phase systems and the incompatibility of these systems with the chosen sample pre-treatment procedure explains the choice of a reversed-phase system. Both octadecyl- and octyl-modified silica systems have been described [10,13,18,20,21]. However, the octyl-modified systems offer a better selectivity, which is essential if UV absorbance detection is applied.

Because MMC was the only analyte to be analysed, an isocratic eluent was used consisting of acetonitrile–water (15:85). For the same reason buffering of the mobile phase was not necessary.

The detection of MMC after an LC separation can be performed using UV or visible wavelength absorption detection, by electrochemical detection in the reductive mode [10] or by (off-line) mass spectrometric detection [27,28]. In spite of the higher selectivity of electrochemical and mass spectrometric detection, absorbance detection was applied because the necessary selectivity was achieved by the choice of the chromatographic system.

In general, absorbance detection is applied at 360–365 nm [17,29,30]. These wavelengths correspond to the absorption maximum of the mitosane structure. Sometimes the absorbance is also measured at 310 nm, the absorption maximum of the mitosene structure, but in our studies no mitosenes could be detected in the chromatograms. Therefore, detection was performed at 360 nm, which permits the detection of less than 150 pg/ml [10].

Chromatographic results

Representative chromatograms of the analysis of artificial solutions of MMC with the CF-LC systems are shown in Fig. 4. Fig. 4A is a blank chromatogram and Fig. 4B was obtained after the analysis of a 1.68-ml water sample containing 100 ng/ml MMC. The selectivity of the CF-LC procedure can be seen by comparison of Fig. 4A, B and 4C, D.

During the chromatographic analysis no metabolites and/or degradation products of MMC were found. The possibility of degradation of MMC during the analytical procedure was checked by repeated injections ($n=20$) of one standard artificial solution (100 ng/ml) of MMC and the injection of some isolated and purified mitosane degradation products [21], but no significant degradation products were observed.

Analytical variables

In order to investigate the linearity of the procedure, blank plasma, urine and ascites samples were spiked with different amounts of MMC to obtain concen-

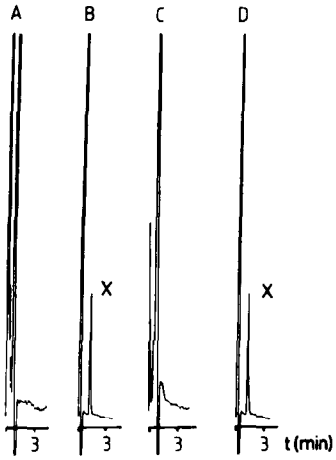

Fig. 4. Chromatograms obtained after the analysis of mitomycin C in water and plasma samples. (A) Blank water samples; (B) water sample spiked with 100 ng/ml MMC; (C) blank plasma sample; (D) plasma sample spiked with 100 ng/ml MMC. Detection is performed at 360 nm and samples of 1.68 ml are taken. The blank samples are recorded at 0.005 a.u.f.s. and the spiked samples are recorded at 0.02 a.u.f.s. ×, MMC.

trations of 5, 10, 50, 100 and 200 ng/ml. The samples were prepared immediately before the actual analysis. The calibration graphs showed good linearity, as can be seen in Table I for the analysis of 48 samples containing 5–200 ng/ml MMC. The linear regression calibration graphs for the plasma, urine and ascites samples were comparable to each other (all correlation coefficients were over 0.996), indicating that the linearity of the procedure was independent of the biological matrix.

The within-run precision was established for 40 samples at two different MMC concentrations (570 and 1140 ng/ml) in plasma. The samples were analysed within 24 h. The within-day coefficients of variation ranged from 4.4 to 2.9% for concentrations varying from 570 to 1140 ng/ml. The between-run precision was determined for three samples at six different concentrations (5, 10, 20, 50, 100 and 200 ng/ml) in plasma. The samples were again analysed immediately after

TABLE I

LINEAR REGRESSION (LEAST-SQUARES FIT) DATA FOR CALIBRATION GRAPHS OF MMC

For calculation of the calibration graphs peak-height ratios (x) are plotted against mitomycin C concentration in ng/ml (y); S_a is the standard deviation of the slope; S_b is the standard deviation of the intercept.

Matrix	Number of samples	Slope	S_a	y-intercept	S_b	Correlation coefficient (r)
Plasma	48	1.03	0.01	−0.31	0.94	0.9978
Plasma	48	1.04	0.01	−0.29	0.52	0.9998
Urine	48	0.98	0.02	0.20	0.57	0.9997
Ascites	48	0.94	0.01	0.21	0.32	0.9989

preparation. The between-day reproducibility ranged from 11.3% for MMC at the 5 ng/ml concentration level to 5.0% for MMC at 200 ng/ml.

The precision of the urine and ascites samples was checked by the analysis of a limited number of samples, but no significant deviations were found in comparison with the analysis of the plasma samples.

The recovery of the CF-LC method, inherent to the use of a dialysis membrane, is low. With the applied flow-rates of the donor and acceptor stream the recovery of the dialysis was about 25%. However, the recovery is dependent on these flow-rates, but it must be taken into account that a higher recovery implies an increase in the total analysis time.

Because the CF-LC apparatus is a fully automated system, this sample pre-treatment procedure is more precise and reproducible in comparison with the classical liquid–liquid and liquid–solid extractions.

A complication in the use of a dialysis membrane may be the degree of protein binding of the analyte, which may influence the recovery of the analyte. In principle, only analytes that are non- or only weakly bound (e.g., binding to human serum albumin) to serum proteins can be analysed without additional sample pre-treatment with this CF procedure.

In spite of the relatively low recovery of the method, the determination limit for the analysis of MMC in plasma samples, defined as peak-height response equal to three times the background noise (characterized by the top-to-top amplitude), was about 1 ng/ml. This determination limit could be lowered to less than 0.5 ng/ml by increasing the volume of the dialysed sample.

Interference studies

The possible interference of 5′-deoxyfluorouridine, 5-fluorouracil, doxorubicin, vincristine and bleomycin with the described procedure for the determination of MMC was studied. However, none of these cytostatic agents, which may be used in combination with MMC, interfered. The possible interferences with other endogenic or exogenic organic compounds was not studied.

Application of chromatographic procedure

The described procedure was applied to the determination of MMC in plasma, urine and ascites after the intraperitoneal administration of a single dose or repeated administration of 20 mg of the analyte.

In order to demonstrate the usefulness of the method, one example is shown. In Fig. 4C a chromatogram of a blank plasma sample is given and in Fig. 4D a similar chromatogram obtained after the analysis of a plasma sample spiked with 100 ng/ml MMC is shown.

To demonstrate the application of the method in pharmacokinetic studies, the concentration–time curve of MMC (Fig. 5) is given for a patient treated intraperitoneally four times (time interval of one month) with 20 mg of MMC. As can be seen, the sensitivity of the method is sufficient to allow pharmacokinetic studies, even 24 h after the administration of MMC.

For the analysis of MMC in ascites and urine samples of patients treated with MMC, a slightly modified procedure was applied. Because ascites contains 5–10

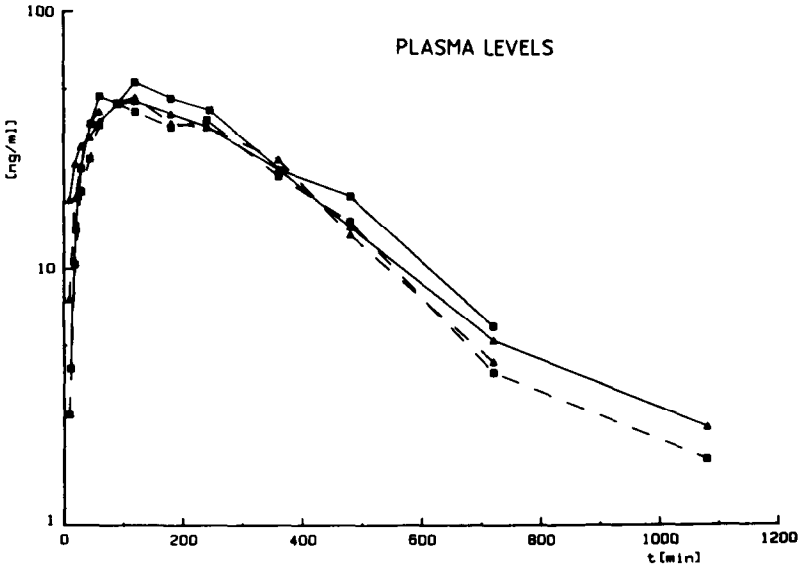

Fig. 5. Concentration versus time curve for mitomycin C. Plasma concentrations of MMC (ng/ml) of a patient treated intraperitoneally four times with 20 mg of MMC.

μg/ml MMC, the samples were diluted with 99 volumes of the donor stream solvent before they could be analysed with the described CF-LC system.

The urine samples were diluted with 9 volumes of the donor stream solvent in order to obtain MMC levels within the linear dynamic range of the developed procedure. An additional advantage of the dilution of the sample was that, although some interfering components were present in the matrix, their influence on the precision of the analysis was negligible.

Representative chromatograms of the analysis of MMC in urine and ascites samples are not shown here, because they were not significantly different from the given plasma chromatograms (Fig. 4C and D).

Owing to the low protein content of urine samples and the relatively high levels of MMC, it was not necessary to use the complete CF-LC apparatus for the quantitative analysis of these samples. No significant differences in the precision and accuracy were found whenever the analyte was measured with the complete CF-LC system or after injection directly on to the RP analytical column.

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