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DETERMINATION OF MITOMYCIN C IN PLASMA, SERUM AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ULTRAVIOLET AND ELECTROCHEMICAL DETECTION

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SUMMARY

The performance of a number of normal phase and reversed-phase systems, with ultraviolet detection at 360 nm, has been investigated with respect to their applicability to pharmacokinetic studies of mitomycin C (MMC). The reversed-phase system developed was also combined with a polarographic detector in order to compare the sensitivity and selectivity of ultraviolet and electrochemical detection.

A simple isolation procedure, based on the adsorption of MMC on a non-ionogenic resin, has been developed. The developed assay is applied to a pharmacokinetic study from which some examples are given.

INTRODUCTION

The mitomycin antibiotics, which are derived from *Streptomyces caespitosus*, were discovered in 1956 [1]. Mitomycin C (MMC), having anti-tumor properties, was isolated in 1958 [2] and has the structure shown below. MMC is currently used in the treatment of various tumours. In order to obtain an insight into its pharmacokinetics and to relate its concentration in blood with therapeutic effectiviness, in order to optimize the schedule of administration,

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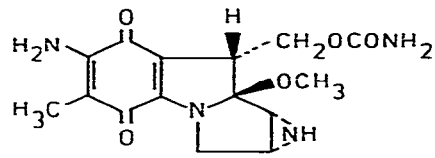

mitomycin C (MMC)

the determination of MMC and its possible metabolite(s) is of great importance.

For the determination of MMC in body fluids there are several analytical techniques, such as ultraviolet (UV) spectrophotometry [3], fluorometry, polarography [4—6], liquid chromatography [7—9] and bacterial growth inhibition [3]. UV spectrophotometry and bacterial growth inhibition were applied to the analysis of samples from dogs and rats in a pharmacokinetic study [3]. However, a detailed description of the pharmacokinetic parameters was not possible, due to the lack of sensitivity and selectivity of the method. Polarography, which is a fairly sensitive method, can only be applied if there are no interfering compounds, i.e. compounds with the same half-wave potential(s). Although to our knowledge no active metabolites have been reported so far, it should be noted that MMC decomposes rapidly under acidic and alkaline conditions. This implies that, prior to a polarographic technique, a separation of MMC from its (electrochemically active) degradation products might be necessary.

A few papers dealing with the high-performance liquid chromatographic determination of MMC have recently been published [7—10]. MMC can be chromatographed in the normal phase as well as in the reversed-phase mode. In order to be able to observe any degradation products that could be metabolites, we developed a normal phase system. The main degradation product of MMC is formed by cleaving off the carbamate moiety of the molecule. The resulting molecule is much less polar than MMC itself. Expecting low concentrations of this compound, one should apply normal phase chromatography. In such a phase system the degradation product will be eluted before MMC, which means that the detectability is better than in a reversed-phase system, in which MMC is eluted first.

For the quantitative determination of MMC in plasma, serum and urine, we developed a reversed-phase system, that is readily applicable to routine analysis. Moreover, reversed-phase systems are more suitable for electrochemical detection than normal phase systems. After slow infusion of MMC one can expect very low concentrations. Electrochemical detection might fulfil the demands for low limits of detection. In general, liquid adsorption systems do not allow direct injection of biological samples. We therefore developed a relatively simple isolation procedure, based on adsorption on Amberlite XAD-2 resin.

EXPERIMENTAL

Apparatus

Two liquid chromatographs were used, which were constructed from commercially available and custom-made parts and consisted of a thermostatted

glass eluent reservoir, a high-pressure pump (solvent delivery system, Model 6000A, Waters Assoc., Milford, MA, U.S.A., and Model 740 B, Spectra Physics, Santa Clara, CA, U.S.A.), a thermostatted column (stainless steel precision-bore tubing, 3.0 mm I.D., 6.35 mm O.D., length 100 mm), an injection device (Model U6K, Waters Assoc., and Model 7125, Rheodyne, Riviera Beach, FL, U.S.A.), a variable wavelength detector (LC-UV 3, Pye-Unicam, Cambridge, Great Britain) operating at 360 nm, a flat-bed recorder (BD 8, Kipp & Zoon, Delft, The Netherlands) and a computer integrator (Autolab, System I, Spectra Physics). Columns were packed by means of an air amplifier booster pump (DSTV-122, Haskel Inc., Burbank, CA, U.S.A.) as described elsewhere [11]. For the electrochemical experiments a polarographic detection system consisting of a polarographic cell (PAR 310, E.G. & G. Instruments, Princeton, NJ, U.S.A.), an interface (Bruker SMDE-Interface, Bruker, Brussels, Belgium) and a polarograph (Model E 100, Bruker) was used.

Chemicals and materials

MMC was obtained either as Mutamycin® from Bristol-Myers B.V. (Weesp, The Netherlands) or as Mitomycine from Kyowa (Tokyo, Japan). Porfiromycin was kindly donated by Upjohn (Ede, The Netherlands).

All organic solvents were of analytical grade (Merck, Darmstadt, G.F.R.) and water was obtained from a Milli-Q Water Purification System (Millipore, Bedford, MA, U.S.A.). Hypersil-MOS (5 μm) was purchased from Ahrin (The Hague, The Netherlands), Servachrom Amberlite XAD-2 (100—200 μm) from Brunschwig Chemie, B.V. (Amsterdam, The Netherlands). The normal-phase support material (silica SI-60) was prepared by grinding silica SI-60 (Merck) with a particle size range of 63—200 μm in a rotating mortar and classifying the ground material by means of an air classifier (Alpine MZR, Augsburg, G.F.R.).

Chromatography

The capacity ratios were calculated from the retention times of MMC and of an unretained compound, for which toluene was used in the normal-phase systems and potassium periodate in reversed-phase systems. The theoretical plate height was determined from its retention time and half the peak width at 0.6 of the peak height.

Polarographic detection

Oxygen was removed from the mobile phase and the samples by bubbling through oxygen-free nitrogen, which was presaturated with the mobile phase.

Preparation of the biological samples

Plasma samples. During 24 h blood samples were taken before and after administration (intravenous and intra-arterial) of MMC. They were collected in heparinized tubes and centrifuged at 1000 *g* for 5 min. The plasma samples were stored at 243°K until analysis.

Serum samples. After clotting of the blood samples, the serum was decanted, centrifuged at 1000 *g* for 5 min and stored at 243°K until analysis.

Urine samples. The urine was collected during 24 h after administration and stored at 243°K until analysis.

Isolation

MMC was isolated from plasma, serum and urine by means of an Amberlite XAD-2 resin, of which 100 mg (100—200 μm) were transferred to a Pasteur pipette in which a plug of cotton wool had been inserted. The resin was pretreated with methanol (three aliquots of 3 ml) and water (10 ml). Then 2 ml of the sample were brought onto the resin. The XAD-2 was washed with water (three aliquots of 2 ml), after which MMC was eluted with methanol (three aliquots of 2 ml). The methanol fractions were collected in a conical flask and the methanol was evaporated at reduced pressure at a temperature of about 330°K. The residue was dissolved in 200 μl of mobile phase by thoroughly mixing it on a whirlmixer for 2 min. Aliquots of 10—100 μl were injected onto the column.

If an internal standard was used, porfiromycin was added to the sample as an aqueous solution (100 μl of a solution of about 1 μg/ml).

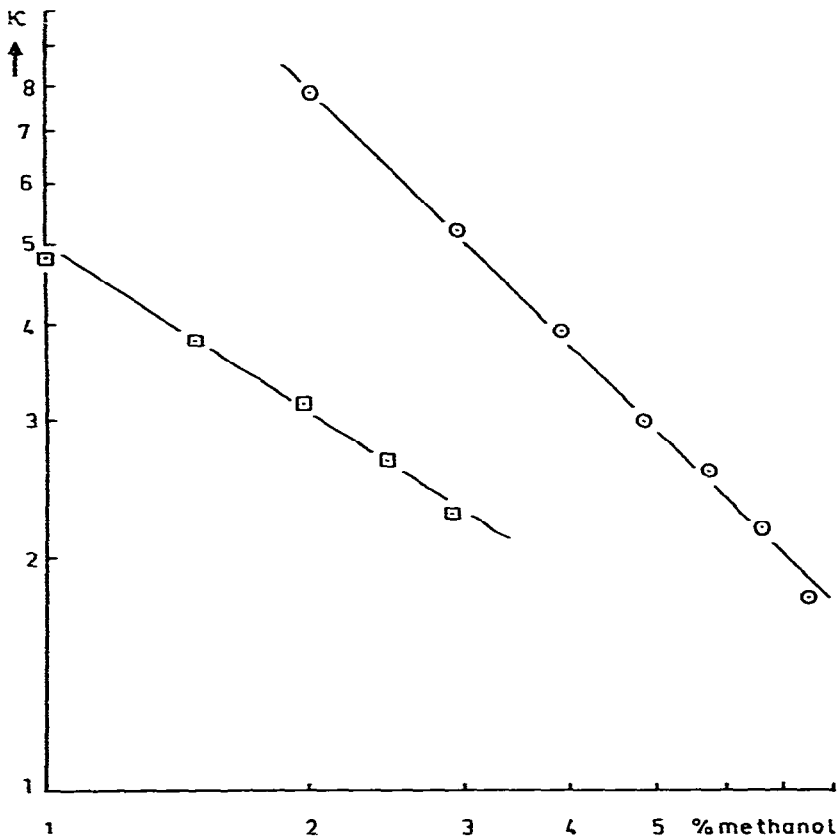

Fig. 1. Log—log plot of the relationship between the capacity ratio of MMC and the methanol content of the mobile phase in normal phase chromatography. (○), Ethyl acetate; (□), acetonitrile.

RESULTS AND DISCUSSION

Normal-phase system

The influence of the methanol (MeOH) content of the mobile phase (ethyl acetate and acetonitrile, respectively) on the capacity ratio of MMC was investigated first. In Fig. 1 the logarithm of the capacity ratio is plotted against the logarithm of the methanol content of the mobile phase. There is a remarkable linear relationship between the logarithms of these quantities, both with ethyl acetate (EtAc) and acetonitrile (ACN). The dependence of the capacity ratio, κ_{MMC}, on the methanol content, C_{MeOH}, as a percentage, is given by the following equations:

$$\log \kappa_{MMC}\,(\text{EtAc}) = -1.098 \log C_{MeOH} + 1.234 \quad (1)$$

$$\log \kappa_{MMC}\,(\text{ACN}) = -0.682 \log C_{MeOH} + 0.689 \quad (2)$$

The percentage goodness of fit is 99.8 and 99.6, respectively. It should be noted that eqns. 1 and 2 are only valid for a limited range of methanol content of the mobile phase.

The phase system was also optimized with respect to column efficiency. Although phase systems based on ethyl acetate and methanol showed better efficiencies than with acetonitrile and methanol, the MMC peak shape was very asymmetric. By adding water to the mobile phase to deactivate the silica and to obtain a more stable phase system, the peak symmetry improved. By adding dichloromethane next to water we obtained the best efficiencies. The methanol content was diminished to keep sufficient retention. In Table I some theoretical plate numbers are given, showing the effect of adding water and dichloromethane on column efficiency.

TABLE I

COLUMN EFFICIENCIES

$\langle v \rangle$ = 1.5 mm/sec, length 10 cm.

Mobile phase*		κ_{MMC}	N_{MMC}
EtAc—MeOH	(97 : 3)	5.2	520
EtAc—MeOH—H_2O	(97 : 3 : 1)	4.1	1640
EtAc—MeOH—H_2O	(97 : 3 : 2)	2.8	1830
EtAc—MeOH—H_2O—DCM	(97 : 2 : 1 : 1)	5.3	3280

*EtAc = ethyl acetate, MeOH = methanol, DCM = dichloromethane.

Reversed-phase system

The capacity ratio of MMC was measured as a function of the pH and the acetonitrile content of the mobile phase. In the pH range 5—8 the capacity ratio was constant, while at lower pH values it increased. However, at these pH values MMC decomposes rapidly, which means that in practice the capacity ratio cannot be affected by the pH of the mobile phase.

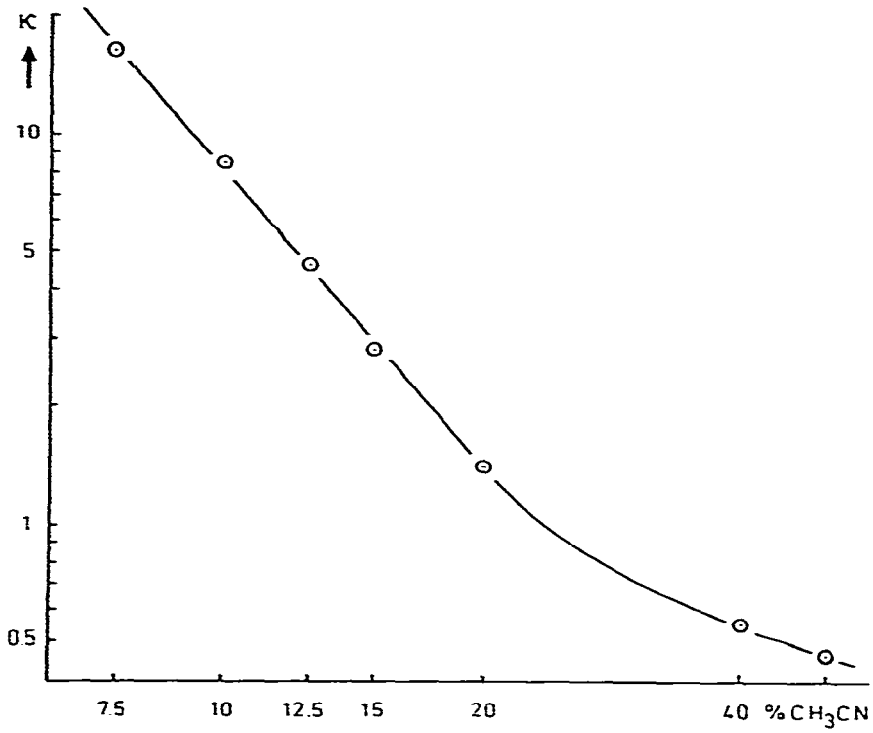

Fig. 2. Log—log plot of the relationship between the capacity ratio of MMC and the acetonitrile content of the mobile phase in reversed-phase chromatography.

In Fig. 2 the influence of the acetonitrile content of the mobile phase on the capacity ratio of MMC is shown by plotting $\log \kappa_{MMC}$ against the logarithm of the acetonitrile content. Between 7.5 and 20% acetonitrile $\log \kappa_{MMC}$ is, just as in the normal-phase systems, proportional to the logarithm of the acetonitrile content, C_{ACN}. In this range eqn. 3 is valid:

$$\log \kappa_{MMC} = -2.533 \log C_{ACN} + 3.445 \quad (3)$$

The percentage goodness of fit is 99.95%.

Although the efficiency of the reversed-phase system is slightly lower than the normal-phase system, the former allows electrochemical detection, is more stable and is less expensive than the latter. For these reasons we applied the normal phase system only to qualitative investigations in view of the possible occurrence of metabolites or degradation products. The reversed-phase system was used for the routine analysis of MMC.

Polarographic detection

It is known from the literature [4, 5] that MMC is polarographically active. This can also be concluded from its molecular structure: the quinone group can be reduced electrochemically to the corresponding hydroquinone.

The polarographic detection system used can be applied in the hanging mercury drop electrode (HMDE) mode as well as in the static mercury drop electrode (SMDE) mode. In the HMDE mode only one mercury drop is used as electrode during one chromatographic run, while in the SMDE mode the electrode is refreshed frequently, whereby the drop is forced to grow rapidly and reaches its maximum size long before the drop is knocked off and the current is sampled. So, at the moment of sampling, the drop size is constant and is said to be static. This results in a negligible charging current, normally caused by the increase of the drop size area.

For the detection of MMC, SMDE is expected to be more favourable than HMDE, because of the adsorption of MMC to the mercury surface. This adsorption might cause a decrease in the effective surface area of the mercury drop, resulting in a lowered sensitivity for the HMDE mode, while in the SMDE mode this effect can be neglected by the frequently refreshed electrode. Nevertheless, HMDE is observed to offer much better signal-to-noise ratios than SMDE, resulting in detection limits of about 250 pg and 15 ng, respectively. This unexpected result is probably caused by the increased noise level in the SMDE mode, due to fluctuations in the drop size areas as obtained with the applied detection systems.

With the detection system three different drop sizes have been used — small, medium and large, corresponding to about 50, 100 and 200 mg of mercury per drop. Due to the fact that the noise level is more or less proportional to the

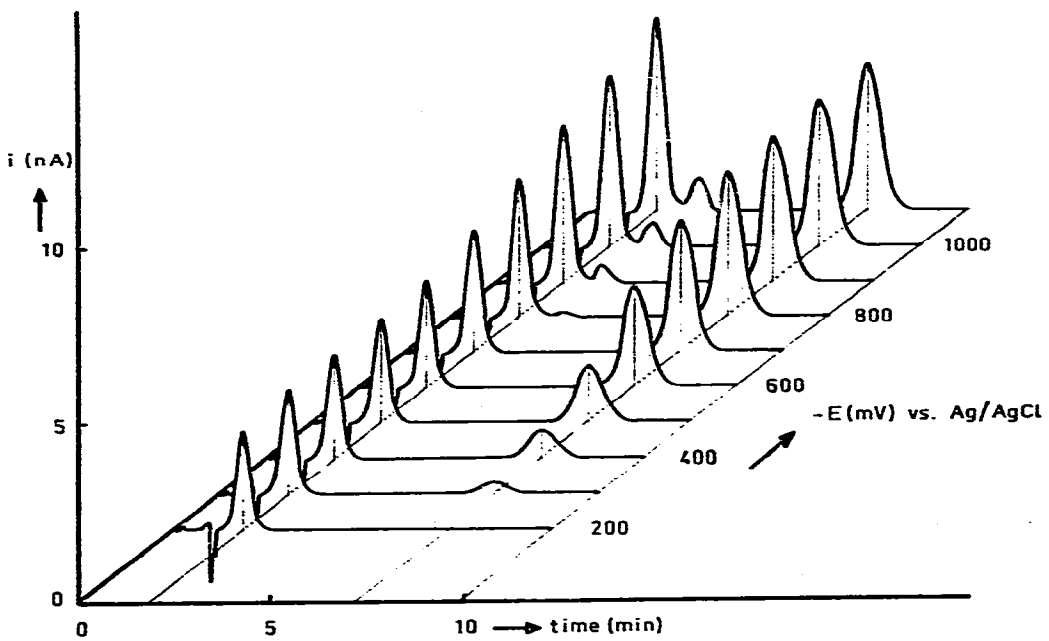

Fig. 3. Chromatoamperogram of MMC. Oxygen, about 2 min; MMC, about 7 min. Conditions: stationary phase, Hypersil-MOS (5 μm); mobile phase, 12% acetonitrile in phosphate buffer (0.05 *M*, pH = 7); flow-rate, 0.5 ml/min.

mercury surface area [12], while only a small part of the drop is used as reduction electrode, the signal-to-noise ratio of the smallest drop size is most favourable.

In Fig. 3 the relation is given between the reduction current, the time of analysis and the potential applied to the mercury electrode. It can be seen that at a potential of about —600 mV versus the silver/silver chloride reference electrode an adequate signal for MMC is obtained. Note the oxygen peak in the chromatograms at about 2 min, which is caused by the presence of remaining oxygen in the sample. Fig. 4 shows a chromatogram of an extract from plasma spiked with MMC. Porfiromycin was used as internal standard.

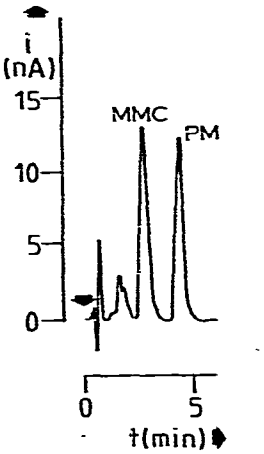

Fig. 4. Chromatogram of an extract of plasma, spiked with mitomycin C (MMC) and porfiromycin (PM) (concentration about 100 ng/ml). Detection: hanging mercury drop electrode (small size) (see text). Conditions: stationary phase, Hypersil-MOS (5 μm); mobile phase, 10% acetonitrile in phosphate buffer (0.05 *M*, pH = 7); flow-rate, 2.0 ml/min.

Isolation

The isolation of MMC from a biological matrix by means of the procedure described above enables one to concentrate the compound tenfold. By dissolving the residue in less than 200 μl of the mobile phase this concentrating can be enhanced, although there is loss of reproducibility. The recovery of MMC isolated from plasma, serum and urine was determined by treating samples spiked with amounts varying from 5 to 500 ng/ml. The peak areas were compared with those obtained by direct injection of the same amounts dis-

TABLE II

RECOVERY OF MMC (5—1000 ng/ml)

Matrix	Recovery (%)	C.V. (%, *n*=5) (5—1000 ng/ml)
Water	97.4	2.0—0.4
Plasma	84.8	2.1—0.6
Serum	84.3	2.5—0.6
Urine	86.4	4.3—1.1

solved in the mobile phase. In Table II the recoveries are given for MMC isolated from different biological samples. From the table it can be seen that even without an internal standard highly reproducible results can be obtained. In practice, porfiromycin appeared to be a suitable internal standard for UV detection as well as for polarographic detection.

Precision and linearity

The precision and linearity of the determination of MMC by HPLC, preceded by adsorption on XAD-2, were investigated by determining biological samples spiked with amounts of MMC varying from 5 to 1000 ng/ml for plasma and serum and from 50 to 1000 ng/ml for urine with UV detection as well as with electrochemical detection (ElCD). In Table III the equations for the calibration curves and their corresponding correlation coefficients are summarized for UV detection as well as for ElCD. The limits of detection, based on a signal-to-noise ratio of 3, are about 150 pg and 250 pg for HPLC—UV and HPLC—ElCD, respectively.

TABLE III

PRECISION AND LINEARITY

Matrix	Concentration (ng/ml)	Detection	Slope	Intercept	Correlation coefficient
Water	5—1000	UV*	18.7 mV·sec/ng	2.3 mV·sec	0.9999
Plasma	5—1000	UV	15.9	5.1	0.9981
Serum	5—1000	UV	16.0	3.0	0.9962
Urine	50—1000	UV	16.2	1.8	0.9934
Water	5—1000	ElCD**	1.15 nC/ng	0.26 nC	0.9987
Plasma	5—1000	ElCD	0.98	5.2	0.9931
Serum	5—1000	ElCD	0.99	3.7	0.9942
Urine	50—1000	ElCD	1.00	8.7	0.9914

*UV detection at 360 nm.

**Electrochemical detection at —700 mV vs. Ag/AgCl.

Application to biological specimens

In order to demonstrate the usefulness of the method two elimination curves and a cumulative excretion curve for MMC after a single dose are given. For the pharmacokinetic studies UV detection is applied because this detection is more suited for routine analysis.

Fig. 5 shows the chromatogram of an extract of plasma from a patient who received 20 mg of MMC intravenously. The blood sample was taken 2 h after administration and treated as described above. Fig. 6 gives the plasma concentration—time course for this patient. Fig. 7 shows the chromatogram of an extract of urine from this patient, collected during 24 h. Although there are a number of urine peaks in the chromatogram, it can be seen that only small interfering compounds are present in the chromatogram and that, due to the relatively high MMC concentrations occurring in urine it can be determined by applying the same isolation procedure. Moreover, it appears that porfiromycin

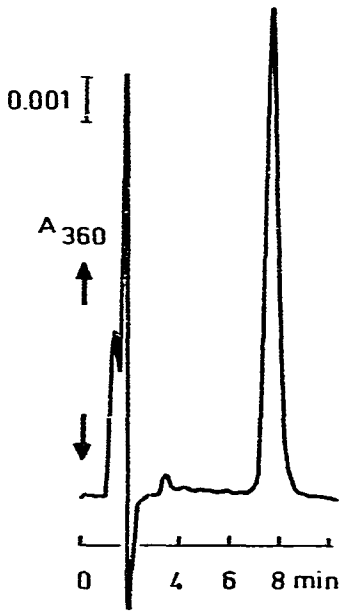

Fig. 5. Chromatogram of an extract of plasma from a patient who received 20 mg of MMC intravenously. MMC peak corresponds to 25 ng. Conditions: stationary phase, Hypersil—MOS (5 μm); mobile phase, 10% acetonitrile in water; flow-rate, 0.5 ml/min.

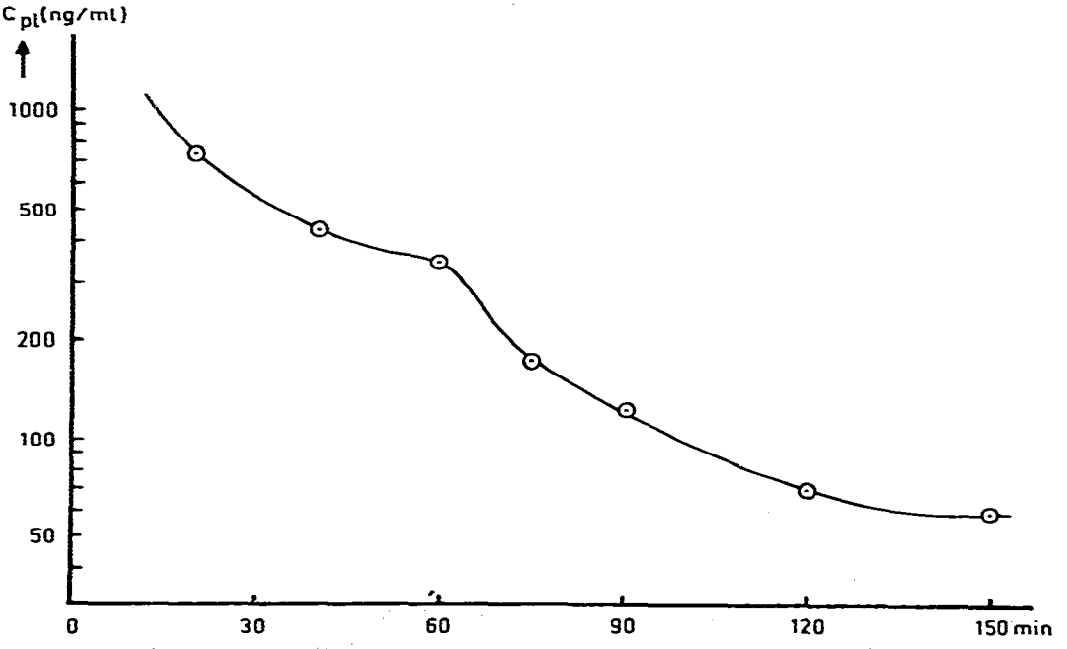

Fig. 6. Plasma concentration—time course for a patient receiving 20 mg of MMC intravenously.

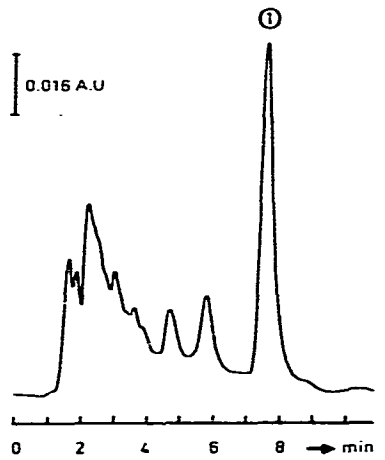

Fig. 7. Chromatogram of an extract of urine from a patient receiving 20 mg of MMC intravenously. Urine was collected during 24 h. MMC peak corresponds to 400 ng. Conditions: see Fig. 5.

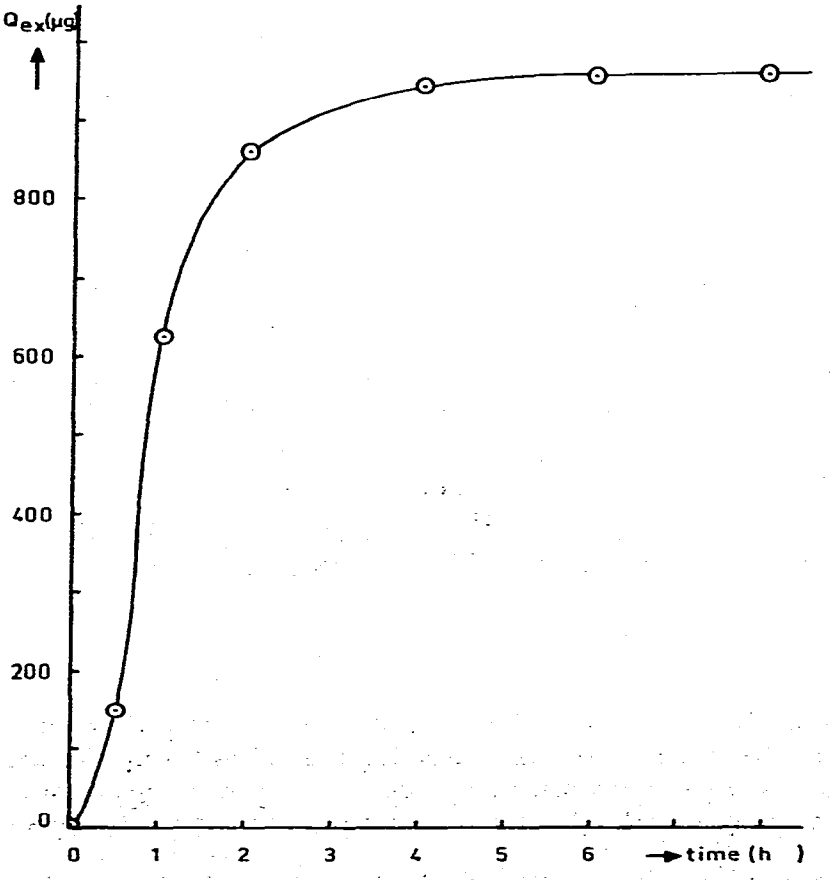

Fig. 8. Cumulative excretion curve for a patient receiving 20 mg of MMC intravenously.

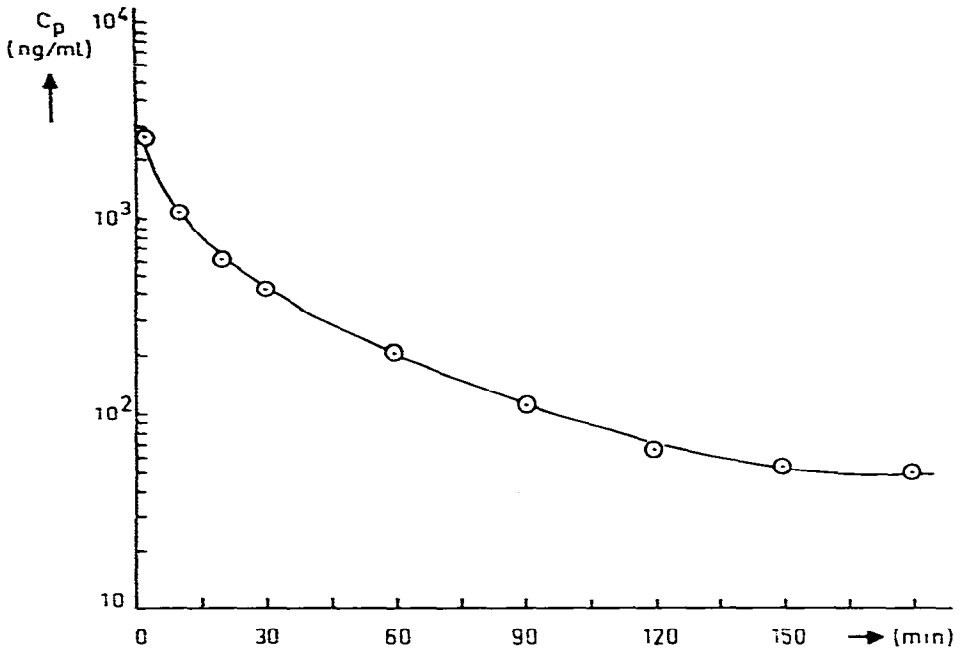

Fig. 9. Plasma concentration—time course for a rat receiving 100 μg of MMC intravenously.

is a good internal standard, because it is eluted after MMC and thus after the interfering urine compounds.

Fig. 8 gives the cumulative excretion curve for a patient who received 20 mg of MMC intravenously. It appears that only a small amount of the administered drug is recovered in the urine collected during the first 8 h after administration.

In Fig. 9 the plasma concentration—time course for a rat receiving 100 μg of MMC intravenously (jugularis externa) is shown. Blood samples were taken by means of a cannula that was inserted into the arteria carotis. In this experiment the maximum sample volume was only 300 μl, demonstrating the possibility of handling small sample volumes with the described method.

From both Fig. 6 and Fig. 9 it can be seen that the pharmacokinetics of MMC are rather complicated and cannot be described by a simple one- or two-compartment model.

CONCLUSIONS

It can be concluded that HPLC with UV detection as well as with polarographic detection can be applied to the determination of MMC in plasma, serum and urine. Because of the extensive provisions needed for polarographic detection (removal of oxygen), UV detection is more suited for routine analysis.

Until now no metabolites or degradation products have been determined in biological specimens.

The pharmacokinetic study of MMC is being continued and will be reported in due course.

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