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Handling of biological samples in the determination of the anti-neoplastic drug mitomycin C*

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Abstract: A study to ascertain suitable conditions for handling biological samples from patients, treated with the antibiotic mitomycin C (MMC), with the objective of improving the accuracy and reliability of the determination is described. Situations frequently occurring in medical practice are simulated to optimize procedures for reliable and reproducible sampling, sample treatment and determination of MMC.

Continuation of drug partitioning in whole blood after sampling can be prevented by immediate cooling in ice before the separation of plasma from cells. The adjustment of the pH of urine samples is shown to be particularly important since a low urinary pH causes decomposition of MMC; moreover, it may decrease extraction recovery. Furthermore, long-term exposure of samples to daylight induces drug decomposition.

Frozen storage of plasma and urine samples for periods greater than 3 weeks is to be avoided as this results in a considerable drop in MMC concentration. Repeated cycles of freezing and thawing are shown to have no effect upon the analytical results (6 cycles tested). The analysis of extracts of biological samples may take place up to at least 24 h after their preparation without measurable loss of analyte.

Keywords: *High-performance liquid chromatography; determination of anti-tumor agent mitomycin C; sample pre-treatment; uptake by red blood cells.*

Introduction

Chemotherapy is of increasing importance in the treatment of cancer. Many active drugs are being used nowadays, including the antibiotic mitomycin C (MMC), which has shown clinical activity in a number of neoplastic diseases [1]. MMC is considered to be the prototype of a class of antitumour drugs, the bioreductive alkylating agents, which require metabolic reduction to form a species capable of alkylating critical cellular

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components [2]. Knowledge of the actual mechanism of action is still limited, although considerable advances have been made in recent years [3–5]. Only a few clinical studies on the metabolism and pharmacokinetics of MMC have been performed, owing to the lack of a sensitive method of analysis. A number of high-performance liquid chromatographic (HPLC) assays for monitoring MMC concentrations in biological fluids have been published during the past years [6, 7]. Recently the authors have reported an extensive study of the pharmacokinetic behaviour of MMC in man [8], that employed a HPLC method reported previously [6].

Problems in the bioanalysis of MMC could be expected in view of the chemical instability of the drug [9]. This fact had to be taken into account not only during the analytical separation and detection but also during the sampling and sample treatment procedures, especially since analysis of the drug immediately after sampling was not common practice and furthermore was frequently performed at a different location. The aim of the present study was to define optimum conditions for the handling of blood and urine samples from patients treated with MMC in order to avoid artifacts in the bioanalysis and to increase the reliability of the pharmacokinetic data obtained. Because the distribution of MMC between plasma and red blood cells may have important implications for the separation of plasma and cells after withdrawal, drug partitioning was studied *in vitro* at different temperatures. The analytical recovery of MMC from plasma and urine samples was investigated during storage under different conditions, including the influence of repeated freezing and thawing of biological samples. Finally, MMC concentrations in extracts of biological samples, analysed promptly after preparation, were compared to the concentrations in the same extracts after short-term storage. These experiments, simulating circumstances common in clinical practice, permitted the determination of the optimum conditions for handling biological samples containing mitomycin C.

Experimental

Drugs and chemicals

MMC was a gift from the Bristol Myers Int. Co. (Syracuse, N.Y., USA). Porfiromycin (PM) was kindly supplied by Dr D.B. Borders (Lederle Laboratories, Pearl River, N.Y., USA). All other chemicals were obtained from standard sources and were of analytical reagent grade.

Procedures

Blood samples from healthy volunteers were collected in heparinized tubes. Plasma fractions were separated by centrifugation of whole blood samples for 15 min at 3000 rpm. Plasma was stored at −20°C prior to use. Red blood cells were always used immediately after isolation.

Uptake of MMC into red blood cells was studied by mixing an equal volume of Hank's Balanced Salt Solution (HBSS), containing 1 μg MMC ml^{-1}, with an equal volume of freshly isolated red blood cells, washed twice with HBSS after separation from plasma. The mixture was incubated under gentle agitation in a water-bath at either 20°C or 37°C, care being taken to exclude visible radiation. At $t = 1, 2, 3, 4, 5, 10, 20, 30$ and 60 min an aliquot of the incubation mixture was separated into supernatant and red blood cells by centrifugation (3000 rpm, 3 min) and the MMC concentration in the supernatant determined by HPLC.

Influence of storage conditions on the stability and analytical recovery of MMC from HBSS, plasma and urine was determined at a concentration of 1 μg ml^{-1} by adding known amounts of MMC to blank plasma, urine or buffer. The sample was stored in a refrigerator (4°C), freezer (−20°C) or water-bath (20°C, 37°C), protected from or exposed to visible radiation. The MMC concentration was determined by HPLC after predetermined periods.

HPLC determination

The determination of MMC was performed according to procedures described previously [6, 8]. Briefly, the drug was chromatographed on a μBondapak C_{18} column with a methanol–0.01 M phosphate buffer (pH = 6.0) (30:70 v/v %) and ultraviolet detection at 365 nm.

Results and Discussion

In vitro *uptake of MMC by red blood cells*

Incubation of MMC with red blood cells in HBSS at 37°C led to a decrease of the MMC concentration in the buffer as shown in Fig. 1. Initially, there was a rapid decay due to uptake of MMC by the cells and equilibrium was reached after about 5 min; however, the MMC concentration continued to decrease relatively slowly. This post-equilibrium slow decrease was also observed in an identical solution of MMC in buffer without red blood cells. Based on this observation the slow decay must be attributed to decomposition of the drug; a decomposition which is, however, only 1–2% per hour under the present experimental conditions. Uptake of MMC into red blood cells appeared to be slower at 20°C than at 37°C, but after equilibration the ratio of MMC concentration in the red blood cells to that in the buffer was the same at both temperatures. At 0°C the distribution rate appeared to be very slow; equilibrium was not reached within 1 h (data not shown in Fig. 1).

It may be concluded that within the temperature range studied (0°C–37°C) the rate of distribution but not the distribution ratio is temperature dependent. The latter

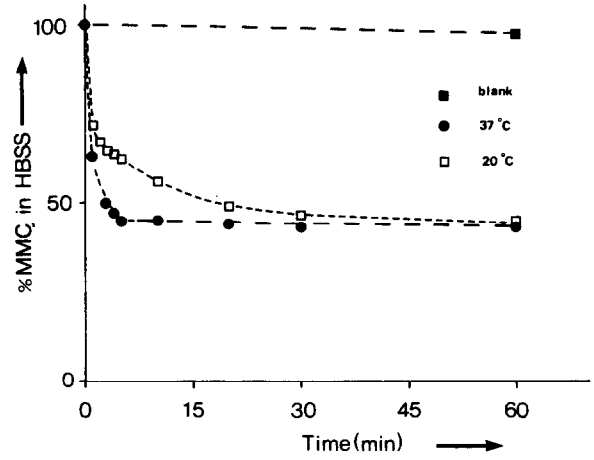

Figure 1
Incubation of MMC with red blood cells, suspended in HBSS. Blank = MMC in HBSS.

conclusion was confirmed by the following experiment in which MMC was incubated with red blood cells in HBSS at 37°C and the distribution ratio determined after 30 min. The incubate was then stored at 0°C for 3 h and again the distribution ratio was determined. No significant difference in distribution of MMC was found. Therefore, the separation of plasma and red blood cells after withdrawal of a whole blood sample from a patient need not necessarily be performed at a standardized temperature, in view of the observed effect of temperature on the distribution ratio of MMC. However, samples that are to be collected shortly after intravenous drug administration should be cooled to 0°C immediately, in order to slow down the distribution of MMC between blood cells and plasma, because for these samples partitioning is incomplete. The latter precaution is not important for samples collected ≥0.5 h after drug administration. In order to determine true *in vivo* concentrations of MMC it is recommended that blood samples after withdrawal are placed in ice and plasma and red blood cells separated at least within 30 min.

Influence of storage conditions on the stability and analytical recovery of MMC in plasma and urine

MMC is a drug which can easily undergo decomposition. Especially in acidic or alkaline solution it has a very short half-life. At neutral pH, MMC solutions can be stored for at least a week without serious degradation. However, care has to be taken to protect MMC solutions from long exposure to daylight as this will promote decomposition of the drug. This is illustrated by Fig. 2 which shows the decrease of the MMC concentration in a methanolic solution kept in daylight as compared to the decrease in a solution kept in the dark. It is obvious that it is advisable to protect MMC solutions, e.g. blood or urine samples, from daylight, at least during long-term storage. Plasma samples spiked with MMC showed no decrease in drug concentration during 2 h incubations at 4°, 20° and 37°C in the dark (Table 1).

The same result was found for urine samples containing MMC with a pH value of 6–8. For urine at lower pH values some decrease may occur due to decomposition of the drug. Therefore, control of pH of urine samples is advised, and if necessary, their neutralization. Another conclusion that may be drawn from this experiment is that

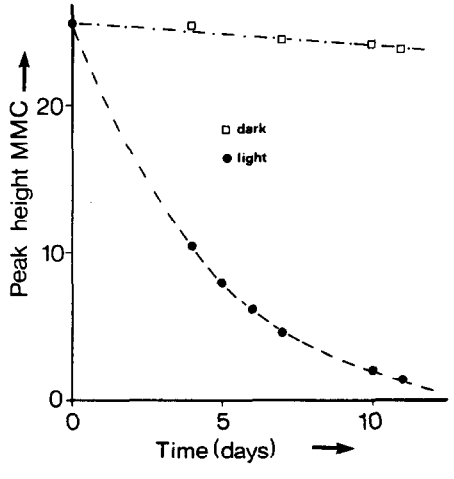

Figure 2
Influence of daylight on the stability of MMC in methanol.

Table 1
Incubation of MMC in plasma and urine*

Time (min)	Plasma 4°C	Plasma 20°C	Plasma 37°C	Urine 37°C pH 7.2
0	0.70 ± 0.01†	0.68 ± 0.01	0.71 ± 0.04	2.63 ± 0.04
15	0.65 ± 0.02	0.67 ± 0.02	0.67 ± 0.05	2.53 ± 0.10
30	0.70 ± 0.03	0.71 ± 0.01	0.67 ± 0.05	2.50 ± 0.06
45	0.68 ± 0.02	0.70 ± 0.03	0.67 ± 0.07	2.48 ± 0.18
60	0.67 ± 0.04	0.69 ± 0.03	0.67 ± 0.03	2.58 ± 0.04
120	0.68 ± 0.04	0.65 ± 0.02	0.67 ± 0.03	2.47 ± 0.09

* All samples were protected from light during incubation.
† Peak height ratio MMC/PM ± S.D. (n=4).

samples do not have to be placed in a refrigerator or freezer immediately after sampling and/or centrifugation.

Because samples could not always be analysed promptly after sampling, it was necessary to investigate under which conditions and for how long biological samples could be kept without affecting the MMC concentration. Physiological saline, plasma and urine samples spiked with MMC were stored at −20°C for four weeks. In each sample the MMC concentration was determined at regular intervals. The results are presented in Fig. 3. In physiological saline the MMC concentration was not influenced by storage under frozen conditions during at least four weeks. However, in plasma as well as in urine a decay of the MMC concentration was observed. This decrease appeared not to be due to decomposition of MMC as no decrease was observed during storage in frozen physiological saline. Most probably the decrease is the result of a lower extraction recovery, due to a change of the biological matrix. Frozen plasma samples as well as urine samples, stored for a longer period of time, frequently show an increasing amount of precipitate. It is probable that MMC is adsorbed on the precipitate, thereby resulting in a lower extraction recovery. In the case of urine, adjustment of the pH to 7–8 generally causes the precipitate to dissolve, resulting in normal analytical recoveries and reliable quantitative data. The adsorption on the plasma precipitate appeared to be irreversible. Based on these observations it is recommended that analysis of MMC in plasma and urine samples is performed within 3 weeks of collection of the samples and that the pH in urine samples is adjusted to about 7–8 to prevent precipitate formation.

Influence of freezing and thawing on the stability and analytical recovery of MMC in plasma and urine

When biological samples have to be transported from one department to another, the possibility that the samples may thaw out during transition cannot be excluded. This process was simulated by analyzing MMC in plasma samples after a number of freezing and thawing cycles. Repeated analysis of a batch of plasma spiked with MMC showed a peak height ratio of 0.74 ± 0.01 (n = 4) in fresh plasma and a ratio of 0.74 ± 0.02 (n = 4) after six freezing and thawing cycles. A similar result was found in the case of a urine sample (initial 1.30 ± 0.04 (n = 4), after six cycles 1.23 ± 0.04). It may be concluded that repeated freezing and thawing has no significant influence on the precision of the MMC determination.

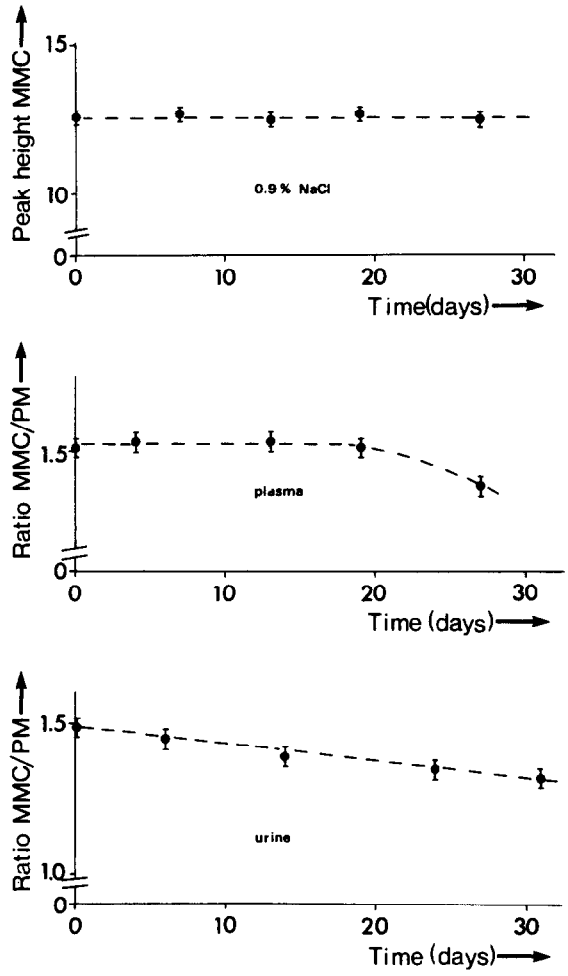

Figure 3
Recovery of MMC after storage in frozen saline, plasma and urine samples. Mean ± S.D. ($n = 3$).

Table 2
Stability of MMC in plasma extracts

Time	Storage conditions	Peak height ratio ± S.D. (n=4)
$t = 0$	4°C, dry, dark	0.99 ± 0.02
$t = 1$ day	20°C, dry	0.99 ± 0.01
$t = 1$ day	4°C, in methanol, dark	0.96 ± 0.02
$t = 1$ day	20°C, in methanol	0.98 ± 0.01

Stability of MMC in plasma extracts

In order to investigate whether plasma extracts have to be assayed immediately after preparation or can be stored for some time prior to analysis, we studied the stability of MMC in plasma extracts under different conditions. One plasma extract was divided into five portions, each of which was treated in a different way. The final results of the analysis are shown in Table 2. No significant differences were observed. A similar result was found if a particular extract was injected repeatedly over a period of 20 h. Accordingly it may be concluded that the final assay of an extract may be done up to at least 24 h after preparation of the extract.

Conclusions and Recommendations

Based on the experiments described, the following recommendations and guidelines should be taken into consideration when sampling and analyzing biological samples containing MMC:

(i) Whole blood samples should be placed in ice immediately after sampling; separation of plasma and red blood cells should take place within half an hour after collection of the samples;

(ii) MMC samples should be protected from long-term exposure to daylight in order to prevent analyte degradation;

(iii) The pH of urine samples should always be checked and, if necessary, adjusted to neutral values, as a low pH will induce MMC decomposition and the formation of precipitates upon which MMC may be adsorbed;

(iv) Plasma and urine samples need not necessarily be placed in a refrigerator or freezer immediately after preparation, provided analysis is carried out within a period of 2 h;

(v) Plasma and urine samples may be stored in a freezer at −20°C; however, analysis should take place within 3 weeks as longer storage at this temperature may induce considerable reduction of the MMC concentration;

(vi) Repeated freezing and thawing of plasma and urine samples does not influence the analytical results of the MMC assay;

(vii) Extracts of biological samples may be kept for at least 24 h, in the dark, either dry or dissolved in methanol at 4° or 20°C prior to analysis.

References

[1] D. Glaubiger and A. Ramu, in *Pharmacologic Principles of Cancer Treatment* (B. A. Chabner, Ed.), pp. 402–415. W. B. Saunders, Philadelphia (1982).
[2] H. W. Moore and R. Czerniak, *Med. Res. Rev.* **1**, 249–280 (1981).
[3] M. Tomasz and R. Lipman, *Biochemistry* **20**, 5056–5061 (1981).
[4] K. A. Kennedy, S. G. Sligar, L. Polomski and A. C. Sartorelli, *Biochem. Pharmacol.* **31**, 2011–2016 (1982).
[5] M. Tomasz, R. Lipman, J. K. Snyder and K. Nakanishi, *J. Am. Chem. Soc.* **105**, 2059–2063 (1983).
[6] J. den Hartigh, W. J. van Oort, M. C. Y. M. Bocken and H. M. Pinedo, *Anal. Chim. Acta* **127**, 47–53 (1981).
[7] U. R. Tjaden, J. P. Langenberg, K. Ensing, W. P. van Bennekom, E. A. de Bruyn and A. T. van Oosterom, *J. Chromatogr.* **232**, 355–367 (1982).
[8] J. den Hartigh, J. G. McVie, W. J. van Oort and H. M. Pinedo, *Cancer Res.* **43**, 5017–5021 (1983).
[9] W. J. M. Underberg and H. Lingeman, *J. Pharm. Sci.* **72**, 549–553 (1983).

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