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Review

Mitomycin antitumour agents: a review of their physico-chemical and analytical properties and stability

J. H. BEIJNEN,[1]* H. LINGEMAN,[2] H. A. VAN MUNSTER[1] and W. J. M. UNDERBERG[1]

[1] *Department of Analytical Pharmacy, Subfaculty of Pharmacy, State University of Utrecht, Catharijnesingel 60, 3511 GH Utrecht, The Netherlands*
[2] *Division of Analytical Chemistry, Gorlaeus Laboratories, Center for Bio-Pharmaceutical Sciences, University of Leiden, P.O. Box 9502, 2300 RA Leiden, The Netherlands*

Abstract: The review enumerates the physico-chemical and analytical properties of mitomycin antitumour antibiotics, of which mitomycin C is the most important representative. After a short overview of the position of the compounds in oncology the following subjects will be discussed: structural features, prototropic properties, spectroscopy (UV-VIS, ORD, CD, IR, NMR, MS), chromatography and electrochemistry. The chemical stability and aspects of the mechanism of action of the compounds are also discussed. The last part of the review surveys the analysis of mitomycin C in biological fluids.

Keywords: *Mitomycins; physico-chemical and analytical properties; chemical stability; mechanism of action; analysis in biological materials.*

Introduction

The report of Hata and collaborators [1] in 1956 about the isolation of two pigmented compounds from *Streptomyces caespitosus*, which they named mitomycin A (MMA) and B (MMB), was the start of intensive research on this type of antibiotic. Wakaki *et al.* [2] isolated the related mitomycin C (MMC) from the same bacterial strain. Work by Urakawa *et al.* [3, 4] showed another pigment, 10-decarbamoyloxy-9-dehydromitomycin B, produced by the same *Streptomyces* species. 1a-*N*-Methylmitomycin C, porfiromycin (PM), was first obtained from cultures of *Streptomyces ardus* [5, 6]. Apart from MMA, MMB, MMC and PM, a related compound, mitiromycin (MT), could be detected in the ethyl acetate extract of the aqueous filtrate of the fermentation broth of *Streptomyces verticillatus* [7].

MMA and MMB are highly active antibacterial and antitumour agents but, unfortunately, they are also very toxic. PM and MT are much less potent. MMC has been

*To whom correspondence should be addressed.

proven to be superior in antitumour potency to other mitomycins and this has prompted the selection of MMC for further application. Much research has been devoted to the development of MMC analogues [8–15] possessing more favourable effectiveness and/or less toxicity, but none of the derivatives shows a better therapeutic potential. Therefore, MMC is still the only representative from the family of mitomycin antibiotics which is currently clinically used. Possibly, 7-*N*-(*p*-hydroxyphenyl)mitomycin C (M-83) [16–19] turns out to be a useful antineoplastic drug.

During the early sixties initial enthusiasm for MMC [20] quickly waned when it became apparent that the drug induces severe delayed and cumulative myelosuppression and gastrointestinal complications, which further impeded its utility. However, with the advent of high dose intermittent schedules and innovative regimens, by which the bone marrow and gastrointestinal toxicity have become manageable and acceptable without attenuation of therapeutic efficacy, the usefulness of MMC has been clearly demonstrated. At present, clinical indications of MMC include adenocarcinomas of the stomach, pancreas and colon [21]. For the treatment of these neoplasms the drug constitutes an essential basis of important combination regimens such as MA (mitomycin C, adriamycin) and the triple drug combinations FAM (5-fluorouracil, adriamycin, mitomycin C) and MOB (mitomycin C, vincristine, bleomycin) [21]. Furthermore, MMC has been successfully applied as a single agent to patients suffering from superficial carcinoma of the urinary bladder [22, 23]. Clinical aspects of MMC chemotherapy have been amply reviewed and have been the subject of books and conferences [21, 24–27] and will not be elaborated further in this paper. The revived interest in MMC has resulted in a large number of investigations in the field of drug level monitoring [28–33], dosage scheduling [21, 34, 35], targetting to tumour cells [36–38], syntheses and development of drug analogues [39–41] and prodrugs [42–50], drug stability [51–53] and mode of action [21, 54–56]. The last-mentioned is an especially fascinating item, as MMC is a very unique cytostatic in forming covalent bonds and cross-linkings with DNA after it has been activated by reduction or acid.

Thorough knowledge about the physico-chemical and analytical properties of MMC is of paramount importance for successful research and optimal use of this antibiotic. In this report an attempt is made to overview the most important properties of MMC and its congeners. Prototropic, spectroscopic, chromatographic and electrochemical properties as well as drug stability will be discussed. The last part of the review compiles the analysis of MMC in biological materials.

Structures of mitomycins

Mitomycins are generally subdivided into two groups: mitosanes and mitosenes. These trivial definitions have been proposed by Webb *et al.* [57]. The basic structures and numbering system are given in Fig. 1.

Mitosanes

Spectroscopic and chromatographic techniques and extensive degradation studies have been served as the basis for the assignment of the complete structures of the naturally occurring mitomycins and their conversion products [57–63]. X-ray crystallographic analysis of suitable derivatives of MMA [64, 65], MMB [66] and MMC [67, 68] confirmed the structural determinations. Direct interconversion of MMA, MMC and PM with one another [7, 57, 60, 69–72] shows their stereochemical relationships which led to the

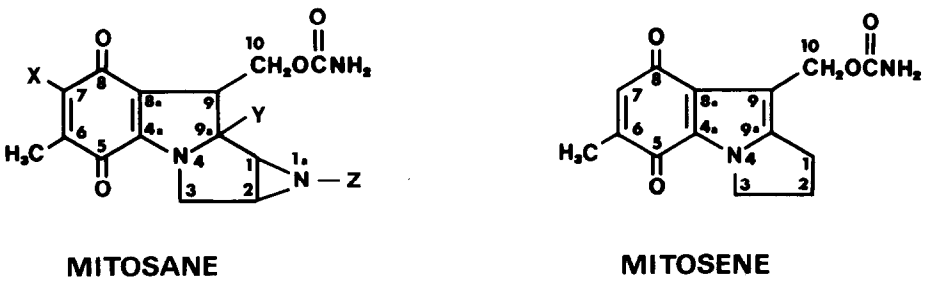


Figure 1
Structures of mitosane and mitosene as defined by Webb *et al.* [57].

assumption that the absolute configurations of MMA, MMC and PM are identical. However, the first supposed configurations at C1 (R), C2 (R), C9a (S) and C9 (R) [65] were hard to bring into agreement with the biosynthesis of mitomycins [73, 74]. This was the motive for Shirahata and Hirayama [75] to reinvestigate the absolute configuration of MMC by X-ray crystallographic analysis using the heavy atom method. They provided conclusive evidence that the absolute configuration of MMC should be reversed into C1 (S), C2 (S), C9a (R) and C9 (S). Recently Hornemann and Heins [76], using circular dichroism spectroscopy, demonstrated that both MMA and MMB have the same chirality at C1 and C2. The final correct structures are depicted in Fig. 2, showing that MMB differs stereochemically from the other mitomycins at C9.

From the naturally occurring mitomycins several semisynthetic mitosanes have been derived, such as 10-decarbamoyl derivatives [4, 13], C9 epimerized derivatives [15], C9a substituted- [12, 77, 78], C7 substituted- [8, 10, 79–81] and C7, N1a disubstituted mitosanes [9, 12, 71, 81, 82].

Mitosenes

The mitosenes, as defined by Webb *et al.* [57], differ structurally from mitosanes in lacking C7, C9a substituents and the 1,2 fused aziridine ring system and in the presence of the unsaturated 9,9a bond (Fig. 1). These indoloquinones are obtained when mitomycins undergo an acid-catalysed or reductive degradation [57–59, 62, 83–88]. Treatment of mitomycins with dilute acid generates two diastereomeric mitosenes, as illustrated in Fig. 3 for MMC, with a hydroxyl function at the 1-position and an amino function at the 2-position as conclusively evidenced by spin decoupling experiments on their diacetate derivatives [84]. Congeners with the hydroxyl and amino group reversed

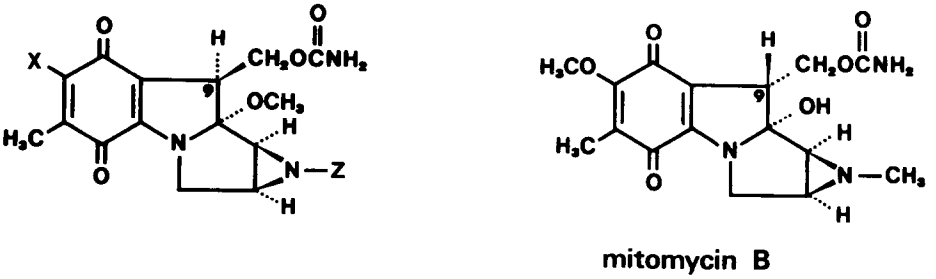


Figure 2
Structures of mitomycin A (X = H_3CO; Z = H), mitomycin C (X = H_2N; Z = H); porfiromycin (X = H_2N; Z = CH_3) and mitomycin B [75, 76].

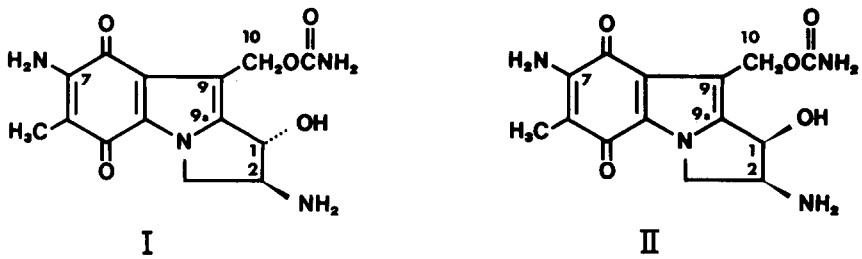


Figure 3
Structures of 1,2 *trans*-2,7-diamino-1-hydroxymitosene (**I**) and 1,2 *cis*-2,7-diamino-1-hydroxymitosene (**II**).

in position have never been found. Based on the observation that apomitomycin A (1,2 *cis*-1-hydroxy-2-amino-7-methoxymitosene) was able to form a stable cyclic carbamate when treated with phosgene and that it is converted to a 1-oxo-7-methoxymitosene upon semipinacolic deamination with nitrous acid [58, 89], it was established to have the 1,2 *cis*-configuration [84]. The 1,2 *trans*-aminohydrin (iso-apomitomycin A) yielded upon deamination a 1,2 diol [84, 89]. Configurations of other mitosenes were derived from interconversions with apomitomycin A [85]. Aziridine ring opening is supposed to occur without epimerization at the C2 atom and thus the 2-amino function should have the same absolute configuration as its mitosane precursor [84]. Apart from the 1-hydroxy-2-aminomitosenes and synthesized mitosenes [90] a great number of other mitosenes have been described in studies where mitomycins were subjected to acid or reductive conditions, among which 10-decarbamoylmitosenes [12, 13, 57, 59–62, 71, 78, 91, 92], mitosenes substituted with nucleophiles at position 1 or at positions 1 and 10 [87, 93–99], a 2,7-diaminomitosene unsubstituted at C1 [86, 100] and aziridinomitosenes [76, 101–103] bearing an intact 1,2 fused aziridine function.

Prototropic Properties

Mitomycins contain a number of prototropic functions. In the mitosanes the aziridine function and the 4-nitrogen are always present, whereas in MMC and PM the 7-amino group is also potentially basic [104]. Moreover, due to keto-enol tautomerism MMC and PM also exhibit acidic properties (pKa 12.44) [104]. In MMA and MMB the latter two prototropic groups are absent, whereas in PM and MMB the pKa of the aziridine group deviates somewhat from those of the other mitosanes, due to methylation [52]. In the mitosenes the aziridine ring is cleaved, resulting in the occurrence of a much more basic primary (MMA, MMC) or secondary (MMB, PM) aliphatic amino group (pKa 7) [59, 61, 62]. In the 7-hydroxymitosanes and -mitosenes, an enolic hydroxy function with acidic properties exists (pKa 4) [61, 62, 81]. Determinations of the pKa values of the various functions have been performed spectrophotometrically [52, 62, 81], titrimetrically [59, 61, 62, 105] and kinetically, from the pH–rate constant relationships in degradation studies [51, 104, 106].

The most important prototropic function in the mitosanes is the aziridine group, the properties of which not only govern to a substantial extent the degradation patterns and rates in various media [51, 52, 59, 62, 81, 106], but also seems to be involved in the initial alkylation of DNA, as suggested by the pH dependence of this reaction [105]. Different ways of determination yield different values of the pKa of the aziridine group [51, 59, 105, 106]. The correct way for the pKa determination is the mathematical treatment of

the pH–rate constant relationships, obtained in degradation studies in the appropriate pH ranges [51, 106]. The pKa of the protonated aziridine moiety in MMC has been determined to be 2.8 [51]. Interaction of the lone electron pair of the aziridine ring with the electron-poor quinone system may account for the relative low pKa value [51].

Spectroscopy

Ultraviolet-visible (UV-VIS) spectrophotometry

The absorption spectrum of MMC in methanol, identical to that of PM, is characterized by maxima at 216 nm (ϵ = 21 880), 360 nm (ϵ = 21 380) and 555 nm (ϵ = 234) which is in agreement with the chromophores of aminobenzoquinones [57, 59, 60]. The mitosane absorption spectrum displays important changes when the 7-amino substituent is replaced by a methoxy function as in MMA (λ_{max}: 218 nm (ϵ = 18 500), 320 nm (ϵ = 13 900), 520 nm (ϵ = 3900)) [57]. For MMC Beer-Lambert's law is obeyed to a concentration up to 1.5×10^{-5} M in an aqueous buffer pH 10 at 290 nm. At higher concentrations the standard graph deviates from linearity. This phenomenon has been attributed to intermolecular interactions [104]. The planar quinone moiety may be responsible for the tendency to self-aggregation, resembling the "stacking" behaviour of anthracyclines in solution [107, 108]. At pH values over 10 the absorption maximum of MMC at 360 nm rapidly shifts to 295 nm, due to the conversion of MMC into its deprotonated enolic mesomer [104].

In acidic solution the MMC spectrum undergoes extended hypsochromic shifts due to the conversion into mitosenes, allowing UV spectrophotometry to be used for the quantitation of the degradation rate [51, 52, 62, 83, 106, 109]. The hypsochromic shift is unexpected as it is generally adopted that an increase in conjugation (mitosane → mitosene) should result in a bathochromic shift. The UV-VIS absorption spectra of 2,7-diamino-1-hydroxymitosenes show maxima at 248 nm (ϵ = 8913), 309 nm (ϵ = 6026), 343 nm (shoulder; ϵ = 2089) and 562 nm (ϵ = 537) [103]. The spectral changes due to MMC degradation in alkaline solution are normally small, except when the degradation takes place at very high alkalinity [81].

By recording the differential UV spectrum of the second derivative spectra of a guanine–mitosene adduct and a mitosene reference and comparison of this difference spectrum with second derivative spectra of substituted guanines, Verdine and Nakanashi [110] demonstrated that MMC alkylates guanine at the 7-position.

Other UV-VIS spectrophotometrical data of mitosane [7, 39, 49, 57, 59–62, 80, 81, 95] and mitosene derivatives [57, 59–62, 78, 86, 87, 93, 95, 96, 101, 103, 106, 111–113] are available.

Infra-red (IR) spectroscopy

The IR spectra of mitomycins and their derivatives were generally determined by the KBr pellet technique and used for identification purposes. IR data of MMA [7, 60], MMB [7], MMC [7, 59, 60], PM [80], MT [7, 63], related mitosanes [3, 4, 7, 12, 13, 15, 77, 80, 81, 83, 101] and mitosenes [12, 13, 59–61, 83, 87, 94, 96] have been reported.

The rather complex structure of the compounds is the reason that only a limited number of assignments of IR data have been published so far. In Table 1 some characteristic vibrations of the mitomycin molecule (and derivatives) are summarized. The characteristic carbonyl vibration of the carbamate structure is often used as evidence for the presence or non-presence of this side chain. Just as differential second-derivative

Table 1
Infrared data of mitomycin derivatives

Wave number (cm^{-1})	Vibration	Ref.
1770–1775	C7-enolic acetate of acetylated derivatives	[61, 81]
1750	Hydroxylic acetate vibration of acetylated derivatives	[61]
1700–1750	Carbonyl vibration of carbamate function	[3, 4, 12, 13, 60, 86, 103, 124]
1595/1640–1660	Quinone carbonyl vibration of mitosenes	[61, 83, 86, 94, 103, 124]
1628/1649	Quinone carbonyl vibration of MMB	[117]
1250–1330	Ester vibration of carbamate function	[63, 124]
1200	Aziridine ring structure vibration	[101]

UV spectroscopy [110], differential second-derivative Fourier Transform IR spectroscopy can be applied for the determination of the linkage site of guanine–mitosene adducts [110, 114].

Optical rotation

Due to the presence of four asymmetric centres (C1, C2, C9, C9a) MMC is optically active. Hornemann *et al.* [77] have published the optical rotatory dispersion spectrum of MMC and demonstrated the usefulness of this spectropolarimetric technique in achieving stereochemical information. In other studies the specific rotation of mitomycins or its derivatives is mostly measured at one fixed wavelength and serves for structure characterization [7, 57, 59, 69, 94, 95, 101].

Circular dichroism (CD) spectroscopy

Since mitosanes and mitosenes simultaneously absorb electromagnetic radiation and are optically active, the compounds fulfil the two prerequisites for CD activity. CD spectropolarimetry has been demonstrated to be a nonambiguously analytical technique for the assignment of the C1, 2 stereochemistry of mitosenes for which at first ^{1}H NMR was a risky alternative [85, 99]. Using the 1,2 *cis*- and 1,2 *trans*-2,7-diamino-1-hydroxymitosenes (Fig. 3) as reference compounds [59, 84, 85], Tomasz *et al.* [96, 99] noticed that the sign of the 520 nm CD Cotton effect correlates with the C1 configuration. A positive sign of the CD Cotton effect is associated with a C1 (S) configuration and a negative sign with a C1 (R) configuration. The general applicability of the use of the sign of the CD signal in the longer wavelength region was demonstrated for several *cis/trans* mitosene pairs, among which are covalent nucleoside–mitosene adducts [99]. It appeared that the sign of the CD signal in the longer wavelength region is only dependent on the C1 configuration and is unaffected by C1, C2, C7 and C10 substituents [99].

Nuclear magnetic resonance (NMR) spectrometry

^{1}H and ^{13}C NMR are well documented methods in the structure elucidation and in configuration determination of mitomycins and derivatives.

Lown and Begleiter [115] presented ^{13}C as well as ^{1}H NMR spectra of MMC and confirmed the presence of 18 MMC protons, including the three exchangable nitrogen protons. The most important features of these spectra for structure elucidation are:

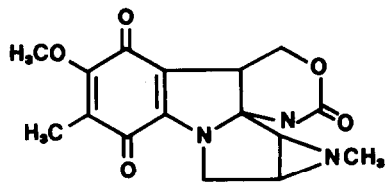
(i) the difference in chemical shift of the C3 and C_3' proton due to the electron-withdrawing effect of the C5 carbonyl function;
(ii) the small J value of the C1 and the C2 proton, due to the influence of the aziridine ring:
(iii) the different J values of the two C10 protons with respect to the C9 proton:
(iv) the 90° dihedral angle of the C3 and the C2 proton ($J = 0$).
However, some of the ^{13}C NMR interpretation results of Lown and Begleiter [115] have been corrected in a later study by Keller and Hornemann [116]. According to the results obtained with the interpretation of the MMC spectra, the interpretation of comparable mitosane spectra is not very complicated [3, 4, 8–10, 15, 39, 77, 94, 117].

Structure elucidation of mitosenes with the use of NMR is also described by several authors [53, 57, 59, 61, 78, 85–87, 90, 94, 100, 101]. Not only the structures of hydrolysis products but also the rather complicated structures of nucleotide–mitosene adducts could be elucidated with the use of NMR spectroscopy [96–98, 113]. The most notable difference in the ^1H NMR spectra between mitosanes and mitosenes is the disappearance of the C9 proton signal and the appearance of the C10 protons as one singlet in the mitosenes [85]. According to Taylor and Remers [85] the differences in the vicinal coupling constants ($J_{1/2}$) of 1,2 *cis*- and *trans*-mitosene pairs are not sufficiently characteristic to be singularly applicable for the assignment of the *cis* or *trans* configuration. However, with the use of high-field ^1H NMR, Hornemann *et al.* [78] were able to assign *trans* stereochemistry of acetylated mitosenes in certain cases. An interesting aspect in the NMR spectra of acetylated mitosenes is the appearance of a second set of signals for the C1 and C2 protons due to two conformations which cannot convert into each other as a result of steric crowding of the acetate groups [53, 78].

Mass spectrometry (MS)

A systematic examination of the electron impact (EI) MS behaviour of mitomycins was published by Van Lear [102], showing that characteristic, interpretable, fragmentation patterns provide definite structure information of substituents at the positions N1a, C7 and C9a. Molecular ions, though weak, were always present. An interesting peak at *m/e* 70 was observed in the spectra of the 1a-*N*-methylmitomycins, MMB and PM, which was absent in the spectra of MMC and MMA. The 1a-*N*-methylaziridinyl origin of this fragment could be demonstrated by recording the spectrum of 1a-*N*-trideuteromethylMMA which yielded a fragment shifted by three mass units to *m/e* 73. The observations of Van Lear were confirmed later [79, 81, 112]. In general MS analysis (EI, chemical ionization (CI), field desorption (FD)) of mitosanes [3, 4, 15, 77, 79, 81, 118, 119] is not surrounded with difficulties. Interestingly, Gaulden *et al.* [120] recently reported about the presence of impurities in a lot of the MMC used, tracked down by direct insertion probe MS. For the structure elucidation of MT (Fig. 4) a high resolution mass spectrum provided essential structural information [63].

EI-MS of the mitosene degradation products differs substantially from their mitosane precursors and is hampered by low volatility. This problem could be overcome by acetylation of the substances and inserting them as methanolic solutions on a Vespel rod probe tip. It is striking that most of the studied 1,2 *cis* mitosenes yielded molecular ions, but not the *trans* isomers, which also showed diminished peak intensities [112]. EI-MS analysis of more lipophilic synthetic mitosenes did not necessitate a preceding derivatization step [90, 121]. For the MS analysis of the polar, low volatile mitosenes the soft ionization techniques such as FD [78, 86–88, 94, 106, 109, 122] thermospray-



Mitiromycin

Figure 4
Structure of mitiromycin [63].

ionization MS [87, 88] and californium-252 plasma desorption MS [77] are obvious choices. To our knowledge, fast atom bombardment (FAB) MS and MS in the negative ion mode have not yet been used in mitosane and mitosene analysis.

Electrochemistry

The current interest in the electrochemical behaviour of mitomycins arises from several causes:

(i) Polarographic and chemical reduction resembles the activation reduction step by cellular reductases and therefore may extend the insight into the *in vivo* activation mechanism of mitomycins [12–14, 103, 123–126];

(ii) A correlation between the polarographic half wave potential of mitomycins and their biological activities has been demonstrated which may serve as a starting point for the synthesis of new derivatives [8, 9, 12–14, 71, 127];

(iii) Optimization of polarographic detection in HPLC benefits from knowledge of the electrochemistry of mitomycins [128, 129].

Rao *et al.* [103, 124] investigated thoroughly the electrochemical behaviour of MMB, MMC, their mitosene degradation products and an aziridinomitosene in aqueous solution by polarographic and cyclic voltammetry. The authors proposed that at pH 7 the initial reduction of MMC at −368 mV ($E_{1/2}$) is a reversible two-electron two-proton reduction to its hydroquinone (compound B, Fig. 5). This unstable species rapidly ($t_{1/2}$~0.07 s) loses the C9a methoxy function, generating a double bond between C9a and C9. The resulting indolohydroquinone (compound C, Fig. 5) undergoes fast (<1 s) aziridine ring opening and is then immediately oxidized to its indoloquinone (mitosene) form at −368 mV. This mitosene is reversibly reduced to its hydroquinone by a two-electron two-proton reduction at a more negative potential ($E_{1/2}$ = −468 mV). Cyclic voltammetry provided the data that irreversible chemical processes followed reduction [103, 124]. Electrochemical studies of Andrews *et al.* [125] have demonstrated that, following a two-electron reduction of MMC in dimethylformamide, 10-decarbamoyl-mitosenes occur. Structure–activity studies of mitomycin analogs have revealed that, for optimal antitumour activity, a balance of functional groups capable of alkylating, a favourable quinone reduction potential and the right hydrophilic/lipophilic properties of the compounds are required [8–10, 12–14, 71, 82, 127, 130]. Thorough knowledge of the contributions of each of these parameters, among which is the electrochemical behaviour, will support a more rational design of mitomycin analogs.

Chemical and enzyme-activated reduction of MMC in the presence of various nucleophiles (e.g. water, inorganic phosphate, nucleotides, DNA) have been shown to produce, after reexposure to air, nucleophile–mitosene adducts where the nucleophile is

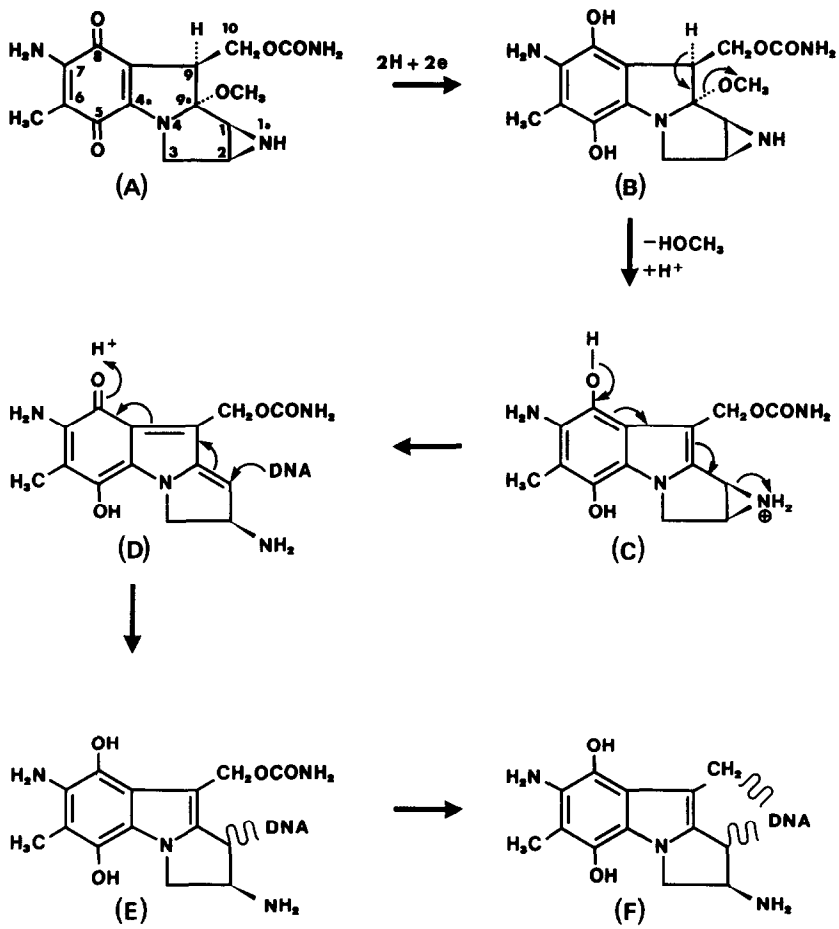


Figure 5
Reductive activation and DNA alkylation of mitomycin C [132].

covalently attached at C1 [86–88, 93–98, 113]. Hornemann *et al.* [95] were able to reveal the second electrophilic reactive site at C10. Reduction of MMC with sodium dithionite in the presence of the nucleophile potassium ethylxanthate afforded after reoxidation, apart from the monoadduct 2,7-diamino-1-ethylxanthylmitosene, also the bisadduct 2,7-diamino-1,10-diethylxanthyl-decarbamoylmitosene [95]. These findings lend great credibility to the action of MMC as a bioreductive mono- and bifunctional alkylation agent, as proposed by Iyer and Szybalski [131] and Moore [56, 132]. The mechanism is depicted in Fig. 5. Evidence in favour of this scenario was also provided by the identification of 2,7 diaminomitosene, obtained after reduction–reoxidation of MMC, which is presumed to have arisen from tautomerization of the quinone methide intermediate D (Fig. 5) [86, 100]. The first step of the metabolic activation of MMC involves reduction and the question arises whether this process occurs by a two-electron reduction, as shown in Fig. 5, or by a one-electron reduction, yielding the MMC semiquinone free radical anion. Evidence in favour of the one-electron pathway has been reported [122, 126, 131, 133, 134] and the existence of the semiquinone, i.e. in

microsomal incubations [135], could be demonstrated with electron paramagnetic resonance spectroscopy [101, 122, 125, 135] and by cyclovoltammetric analysis of MMC in dimethylformamide [125]. It is proposed that the semiquinone provides for the initial, noncovalent contact, presumably of an intercalative type, between MMC and DNA [133, 136]. Further reduction in the close vicinity of the nucleic acid may result in alkylating, as outlined in Fig. 5. However, from a chemical point of view, further reduction to the hydroquinone is not a requisite, as the one-electron reduction can also generate an activated MMC form capable of alkylation [122].

Successive oxidation–reduction cycling of the (DNA-bound) semiquinone (and hydroquinone) species under oxygenated conditions can produce reactive oxygen species which may cause DNA strand scission and membrane lipid peroxidation [137–142]. The importance of this effect at pharmacological drug levels is not yet clear [143].

Chromatography

For the separation and isolation of the mitomycins and their derivatives, chromatography is an indispensable analytical tool [144, 145].

Thin-layer chromatography (TLC)

Before the introduction of high-performance liquid chromatography (HPLC), thin-layer chromatography was the method of first choice. Nowadays TLC has lost importance, although it still provides simple and rapid solutions for many separation problems. TLC analysis of mitomycins is generally performed with silica gel as the stationary phase. However, a great diversity of mobile phases are employed [59, 85, 93]. The existence of the different systems cannot be always rationalized, just as it is hard to recommend a certain system, which, of course, is strongly dependent on the desired performance. Paper chromatography [7, 59, 69, 70, 77, 93, 133] and chromatography on aluminium oxide [10, 39], cellulose [86, 93] and C_{18} phase-bonded silica [95] coated plates have also been applied. The objectives for TLC analysis are rather diverse, including structure characterization of mitosanes [69, 72, 79, 118] and mitosenes [78, 86, 93, 109, 112, 122] and the control of reaction patterns [3, 8–10, 15, 79]. With the use of TLC 1,2 *cis*- and 1,2 *trans*-2,7-diamino-1-hydroxymitosene, as well as other diastereomeric mitosene pairs, can be separated effectively [84, 85, 106]. The quantitation of the individual isomers was achieved by removal of the zones containing the analyte and subsequent spectrophotometrical determination of the eluates of the layer material [85, 111]. It is noteworthy that the elution order of the *cis* and *trans* mitosenes is strongly depending on the composition of the mobile phase [85, 122] while, using the same mobile phase, the order of elution may reverse by acetylation of the isomers [78]. A similar reversal effect has been observed with HPLC analysis of *cis* and *trans* mitosenes and their acetylated derivatives [112].

Gel filtration chromatography (GFC)

For the characterization and isolation of hydrolytic and reductive degradation products of MMC, among which are nucleotide–mitosene adducts, GFC gave satisfactory separation. Even *cis* and *trans* mitosene isomers have different elution volumes. Columns loaded with Sephadex G-25 material and 0.02 M NH_4HCO_3 as eluent have often been preferred [86, 93, 96, 99]. An advantage of this eluent is that after evaporation in vacuum no salts remain and the isolated products can be subjected

directly to spectroscopic techniques. Within the scope of MMC prodrug development [146–148] Sephadex columns have also proved to be valuable for molecular size determinations of high molecular weight MMC conjugates and to confirm the existence of covalent linkages between MMC and the macromolecules. GFC is also applied for the separation of MMC bound to immunoglobulins and free MMC [36–38].

High-performance liquid chromatography (HPLC)

With respect to the reported HPLC methodologies for the analysis of mitomycins and their derivatives, a subdivision can be made into reversed-phase (RP), normal-phase (NP) and ion-pair (or ion-exchange) (IP) RP chromatography.

Liquid chromatography (LC) in the isocratic RP-mode is most frequently applied and is suitable for drug level monitoring [28, 29, 33, 129, 145, 149–157], drug degradation studies [81, 106, 109, 158–162] and metabolic profiling studies [96, 122]. All RP systems are largely similar, using C_{18} [28, 29, 33, 81, 96, 128, 129, 144, 149–154, 156–163] or C_8 [32, 155] alkyl-bonded silica gel stationary phases and mobile phases composed of an aqueous buffer and methanol [28, 29, 33, 81, 128, 149–156, 159–163] or acetonitrile [32, 96, 129, 144, 155, 158] as organic modifier. As MMC is susceptible to degradation in acidic as well as in alkaline solution the pH of the eluent should be within the range 4–8 in order to prevent on-column degradation [81]. RP-LC systems for the analysis of MMA, MMB, MMC, PM and their corresponding mitosene degradation products are described as well [106, 109, 160]. Interestingly, the peak shapes of the *trans* isomers are much better than those of the *cis* diastereomers. Acetylation of the hydroxyl function at C1 and the basic amino group at C2 greatly improves the peak symmetry [112]. For the analysis of MMC and potential metabolites, which possess a wide range of polarities, Andrews *et al.* [112] developed a linear gradient system and utilized a radially compressed C_{18} column. They demonstrated later the applicability of the assay in analysing enzymatically activated degrading MMC samples [122]. Gradient elution in therapeutic drug level monitoring [32] offers no advantages over the available isocratic systems [145]. NP-LC systems have also been reported for the analysis of mitomycins [30, 129, 164], but in comparison with RP chromatography the latter evidently prevails [129]. Polar conversion products of MMC showed very broad tailing peaks using the NP chromatographic procedure [164].

In order to retain acidic and basic degradation products of MMC the technique of IP chromatography has proved to fulfil [81, 109, 158].

Detection in mitomycin chromatography

In general, irradiation with UV light of 254 nm is suitable for the detection of the spots of mitomycins and their mitosene derivatives on thin-layer plates coated with a fluorescence indicator containing sorbent.

Most column LC assays utilize UV detection at 360/365 nm for MMC. The high molar absorptivity (ϵ = 21 380 [60]) and the relative long UV absorption wavelength guarantee high sensitivity and selectivity. According to Tjaden *et al.* [129], the detection limit for MMC at 360 nm is about 150 pg (S/N = 3). As MMC degradation products as well as potential metabolites possess the 7-aminomitosene chromophore, dual wavelength scanning at 365 and 313 nm is imperative.

The favourable reduction potential of the 7-aminoquinoid chromophore of MMC ($E_{1/2}$ = −368 mV [103]) also allows polarographic detection [128, 129]. Using a hanging mercury drop electrode 250 pg of MMC could be detected [129]. The advantage of the

electrochemical detection mode is that a totally different type of selectivity is obtained from the UV detection, which may be favourable in the search for MMC metabolites. On the other hand, polarographic detection is hampered by the presence of oxygen in the mobile phase and sample solution and is rather sensitive to the noise level of the solvent delivery system, demanding that extensive experimental precautions be taken [128]. Surveying the available detection modes, for MMC, shows that HPLC analysis with UV detection at 365 nm is by far the method of first choice, especially for routine analysis [129]. For the structure elucidation of ethyl monothiocarbonate–mitosene adducts, Bean and Kohn [87] utilized the sophisticated thermospray LC/MS combination developed by Blakley and Vestal [165].

Stability

Much research has been devoted to the chemical stability of mitomycins. The reasons for this interest are:

(i) Degradation studies have contributed to an important extent to the structure elucidation of the complex mitomycin structure [57–62];

(ii) Mitomycins are unstable compounds and this demands knowledge about the chemical stability for proper handling of the compounds;

(iii) Mitomycins can be activated to alkylate DNA at low pH without the presence of reducing agents [105, 114, 166].

As drug stability depends strongly upon storage conditions the following subjects come up for discussion: stability in aqueous solution, stability in pharmaceutical preparations and stability in biological materials.

Stability in aqueous solution

MMC and related mitomycins are not stable to storage in solution. Under mild acidic conditions the C9a function is cleaved, forming a 9–9a double bond, and the aziridine ring is opened, yielding the diastereomeric aminohydrin mitosene pair (Fig. 3) [59, 84, 85, 89, 93, 109, 111]. In the presence of phosphate buffer ions 1,2 *cis*- and 1,2 *trans*-2,7-diamino-1-phosphate mitosene are also found [93, 122]. Similarly, acetylated mitosenes have been observed when MMC is degraded in an acetic acid–acetate buffer or glacial acetic acid [59, 85, 109]. Under prolonged acid treatment the C7 amino function is hydrolysed to give the 1,7-dihydroxy-2-aminomitosene [57, 59]. More drastic acidic conditions leads to hydrolysis of the C10 carbamate, furnishing the C10 decarbamoyl derivative, designated as mitomycinone [58, 59, 61]. In alkaline solution the C7 amino group is hydrolysed to give an acidic enolic hydroxyl group, while the rest of the mitosane skeleton remains intact [81]. According to Garrett [62], consecutive alkaline degradation reactions lead to cleavage of the C10 carbamate. Strong alkaline conditions destroy the quinone chromophore [81]. The above-described degradation pattern also applies to MMA, MMB and PM [60–62, 80, 81, 83–85, 89, 105, 106].

The kinetics of the degradation of MMA [51, 106], MMB [106], MMC [51, 52, 106, 109, 148, 155, 158, 159, 163] and PM [51, 52, 62, 83, 106], as well as the influences of buffers, ionic strength and temperature, are well documented in the literature. Comparison of these data of different research groups shows good agreement. In buffers the degradation obeys pseudo first-order kinetics and general acid–base catalysis has been demonstrated. Maximum stability of MMC is observed in neutral solutions. At lower and higher pH values the observed rate constants increase proportionally to $[H^+]$

and [OH^-], respectively. The linear relationship levels at pH <3 due to aziridine protonation.

Elucidation of the acid-catalysed degradation mechanism has been one of the greatest challenges in mitomycin chemistry. The principal reason for this interest is that MMC is capable of alkylating DNA at low pH without the presence of any reducing agents or enzymes [105, 114, 166].

With the use of deuterium incorporation methods [53] and based on studies on the acid hydrolysis of aziridine-containing structural analogs of MMC [167], it is recently demonstrated that the mechanism of acid degradation, as illustrated in Fig. 6, is most acceptable. It is assumed that protonation of the C9a methoxy group, by which it becomes an excellent leaving group, initiates the degradation in acid. Loss of the C9 hydrogen yields an aziridinomitosene from which the aziridine ring opens after protonation to give intermediate Im (Fig. 6). The protonation degree of the C2 amino

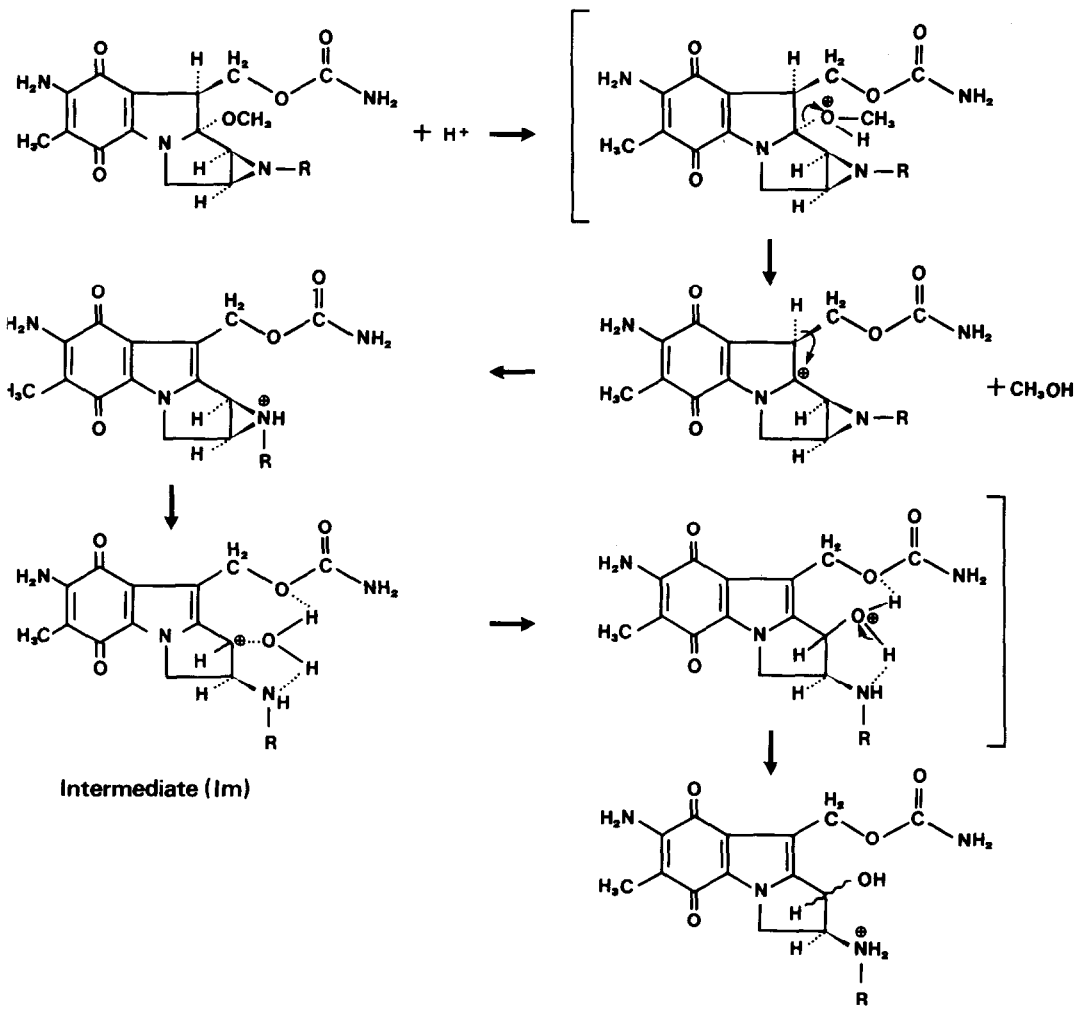


Figure 6
Acid hydrolysis mechanism of mitomycin C (R = H) and porfiromycin (R = CH_3) [52].

group of this species (Im) controls the stereochemistry of the resulting mitosenes which arise after nucleophilic water attack at the C1 carbonium ion [109].

The reductive activation (Fig. 5) and the acid activation (Fig. 6) of MMC show a strong resemblance. The parallel strongly appears from the observations that both activation pathways can lead to the same mitosene degradation products [86, 93, 96, 114, 122, 125]. In both pathways aziridine ring opening is preceded and elicited by cleavage of the C9a methoxy group and in both cases an electrophilic center at C1 is proposed. An important difference between the two mechanisms is that in the acid activation of MMC no second alkylating center at C10 is generated. However, it is observed that the propensity of interstrand cross-linking of DNA was enhanced as the intracellular pH in aerobic EMT6 tumour cells was lowered [168]. Further research is needed to explain this paradox. Interesting in the scope of acid activation are the observations that the pH of interstitial fluid of tumour tissue is somewhat lower than in normal tissues [169, 170] which may promote selective toxicity of MMC for tumours just as hypoxia in tumour cells (connected with the reductive activation) [171, 172]. Furthermore, the effectiveness of MMC in the treatment of gastric cancer may be related to the acid activation.

Stability in pharmaceutical preparations

MMC is commercially available in a lyophilized form containing mannitol (Mutamycin®) or sodium chloride (Mitomycin-C Kyowa®) as excipients. According to the manufacturer, the drug is stable in this solid state for at least two years at room temperature [173]. After dissolution MMC is administered intravenously or intravesically. The stability of MMC in most commonly used infusion fluids is limited [160, 162, 174, 175]. This must be taken into account when the drug solutions are stored or are to be infused over an extended period of time [34, 35]. Studies dealing with the stability of MMC in infusion fluids are unanimous in that the pH of the solution is the crucial parameter and should not be lower than 7 [160, 162]. From the point of chemical stability storage of MMC at room temperature in unbuffered samples (pH 7) [160] and buffered samples (pH 7.75) [162] for 5 and 15 days, respectively, is justified.

The results from studies where the stability of MMC is measured as cytotoxic activity [176, 177] should be handled with caution when no information is given about the activity of degradation products.

Stability in biological materials

Metabolic and chemical changes of MMC can occur when the drug is exposed to all kind of biological tissues and fluids. Stability investigations in this field have been initiated with the objective:

(i) To obtain insight into the bioreductive activation mechanism [96, 131, 178, 179];

(ii) To compare the stability of MMC with that of MMC prodrugs under physiological conditions [49, 148, 163];

(iii) To find appropriate conditions for storage of biological samples [32, 180];

(iv) To follow MMC stability under experimental *in vitro* assay conditions [161, 181].

In order to avoid misinterpretations in the bioanalysis, MMC plasma samples must be handled properly prior to analysis. According to van Bennekom *et al.* [180] and van Hazel and Kovach [32], plasma samples can be stored at −20° and −70°C, respectively, for at least one month without any loss of MMC.

MMC is thought to be one of the most suitable anticancer drugs for instillation therapy of bladder tumours [21, 26]. However, an important drawback of the therapy is its high cost. After the retention period of the drug in the bladder (approx. 2 h) a major part of the MMC is voided. MMC appears to be relatively stable in urine at body temperature and retains antineoplastic activity when stored in urine [151, 182]. On account of these observations authors [151, 182] have put forward the possibility of recycling and reusing of MMC if, of course, sterility and an appropriate drug concentration are guaranteed. Many *in vitro* test systems (incubations with tumour cells) are used for evaluating the antitumour potency of cytostatics. In order to interpret the results reliably it is essential to be well posted in the subject of chemical and biological drug stability [183]. It appears that MMC is fairly stable under the assay conditions [161, 181]. After a seven days' incubation period at 37°C in cell culture media supplemented with serum, 70–80% of the original drug concentration was recovered. On the other hand, the 7-methoxymitomycins MMA and MMB are far less stable and degraded rapidly ($t_{1/2}$ 1–2 h) into their 7-hydroxy analogs [161].

Analysis of MMC in biological materials

The therapeutic effectiveness and toxicity of MMC are strongly dependent upon dosage and dosage schedule. In order to obtain insight into the pharmacokinetics, indispensible for optimizing chemotherapy, selective and sensitive determination methods for MMC in biological samples (plasma, urine, tumour tissues, etc.) are needed. The bioanalysis is hindered by low blood/plasma levels, MMC instability and frequent application of combination therapies. Microbiological [30, 33, 46, 48, 148, 178, 179, 184–187], immunological [188, 189], polarographic [180] and HPLC assays [28–33, 129, 149, 150–157] are used.

Microbiological assays

For a long time microbiological assays have been the only available determination technique for MMC in biological materials. Using the cup-plate method and *Escherichia coli B* as the test organism, it is possible to detect MMC plasma concentrations down to 2–10 ng ml^{-1} [30, 46, 148, 178]. Furthermore, a good relation is observed between the bioassay and specific HPLC analysis methods [30, 33]. The sensitivity and the fact that no sample pre-treatment is required is to the credit of the microbiological method. However, the impossibility of detecting metabolites and degradation products, the long analysis time and the restricted precision make the microbiological assays inferior to modern, more sensitive, LC methods.

Immunological assays

An enzyme immunoassay for MMC is described by Fujiwara and co-workers [188] using an enzymatic labelling of MMC with β-galactosidase. The limit of detection is 4 ng ml^{-1} in a 50 μl serum sample. No interference of commonly used antineoplastic drugs with the immunoassay was observed. Cross-reactivity with other mitomycins (MMA, MMB, PM and 1a-*N*-acetylMMC) has been demonstrated [189] but is not important from a therapeutical point of view, as these compounds are never used at the same time.

The enzyme immunoassay has also been applied successfully to the bioanalysis of 7-*N*-(*p*-hydroxyphenyl)mitomycin C (M-83) [190].

Polarographic assays

High-performance differential pulse polarography appears the most favourable polarographic technique [180]. After sample pre-treatment (liquid–solid extraction) the lowest detectable amount was 25 ng ml^{-1} using 2 ml samples.

High-performance liquid chromatographic assays

The most suitable quantitation methods for MMC in biological fluids are based on HPLC separation systems with spectrophotometric detection. An overview of the available assays is presented in Table 2. Some form of sample clean-up is generally necessary to achieve a determination method with sufficient selectivity, sensitivity, accuracy and reproducibility [145]. For the analysis of MMC in rat serum a protein precipitation step with methanol was found sufficient as clean-up [152]. Other methods utilize liquid–liquid extraction or liquid–solid extraction as the purification step. The partition coefficient [49, 153] and solubility [49] of MMC in organic solvents are important parameters if liquid–liquid extraction is used as the clean-up procedure, in

Table 2
Survey of published methods for the analysis of MMC in biological fluids

Matrix	Sample pre-treatment*	Internal standard†	Determination limit‡ (ng ml^{-1})	Chromatography	Ref.
Serum, urine, ascites	1.1.	no	40 (serum)	RP	[149]
Plasma	1.1.	no	1	RP	[153]
Plasma	1.1.	no	1	RP	[29]
Plasma, urine, bile, ascites	1.1.	yes	1	RP	[28]
Serum, plasma urine	1.s.	yes	5, 50 (urine)	RP	[129]
Plasma, urine	1.s.	yes	4 (plasma)	RP (gradient)	[32]
Plasma, urine	1.s.	no	2 (plasma) 500 (urine)	RP	[155]
Plasma, urine	1.1.	yes	1 (plasma) 200 (urine)	NP	[30]
Plasma, urine	1.1. (plasma)	no	1 (plasma)	RP	[33]
Serum	1.s.	no	10	RP	[150]
Serum	deproteination with methanol	no	10	RP	[152]

*1.1., Sample pre-treatment by liquid–liquid extraction; 1.s., sample pre-treatment by liquid–solid extraction.

†The internal standard is porfiromycin.

‡Determination limits for 25 μl [152], 1.0 ml [28, 29, 33, 149, 150, 153, 155], 2.0 ml [30, 129] and 2.5 ml [32] samples.

order to obtain high recoveries. Chloroform/2-propanol [28, 29, 33, 153] and chloroform/2-propanol/ethyl acetate [30] mixtures gave the best results. Recoveries for plasma and serum samples higher than 90%, in the concentration range 1–2000 ng ml^{-1}, can be achieved [28, 30, 153]. In order to prevent MMC degradation the evaporation temperature of the extraction solvent should not exceed 40°C [153]. Furthermore, it is found that the dissolution of MMC from evaporated samples in the eluent, containing 30% methanol, was incomplete and irreproducible. This problem could be overcome by using pure methanol, but this confined the maximal injectable amount to 10 μl [153]. Addition of desipramine improved the dissolution step [155].

As the evaporation of the organic extract is rather time-consuming, liquid–solid extraction procedures have been developed employing Sep Pak C_{18} [150, 151, 155], Porapak Q [32] and Amberlite XAD-2 [129] cartridges. The recoveries are in general somewhat lower than with the classic liquid–liquid extractions. Concentration-dependent recoveries [32, 155] may warrant the use of an internal standard for which PM is very convenient. Investigators differ in opinion about giving a preference to liquid–liquid or liquid–solid extraction sample clean-up [191, 192].

Until now MMC metabolites in human plasma have not been detected except for two unidentified peaks in HPLC chromatograms [153, 193]. Metabolites are probably of the mitosene type and the more pronounced polar character of these compounds may prevent effective extraction with the usual solvents.

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