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Determination of mitomycin C, 2,7-diaminomitosene, 1,2-*cis*- and 1,2-*trans*-1-hydroxy-2,7-diaminomitosene in tumour tissue by high-performance liquid chromatography

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ABSTRACT

A high-performance liquid chromatographic method is described for the determination of mitomycin C (MMC) and its metabolites 2,7-diaminomitosene (2,7-DM), 1,2-*cis*-1-hydroxy-2,7-diaminomitosene (cis-hydro) and 1,2-*trans*-1-hydroxy-2,7-diaminomitosene (trans-hydro) in tumour tissue. N-1a-Methylmitomycin C (porfiromycin, PM) was used as an internal standard. Two factors were critical in resolving the metabolites: pH and buffer ionic strength, where the retention times of the four components were affected in the order 2,7-DM ≫ cis-hydro ≫ trans-hydro ≫ MMC. The optimal isocratic conditions (flow-rate 1 ml/min) were 18 m*M* sodium phosphate pH 5.8–methanol (74:26) and a column temperature of 40°C on a Spherisorb ODS-2 column (25 cm × 4.6 mm I.D.). Liquid–liquid extraction [twice with chloroform–propan-2-ol–ethyl acetate (2:2:1)] is described for tumour tissue. Recoveries varied depending on the component: MMC, 71.9 ± 12.4%; PM, 85.5 ± 27%; 2,7-DM, 51.7 ± 5.4%; cis-hydro, 52.0 ± 16.8%; trans-hydro, 62 ± 8%. When applied to the analysis of a rat mammary carcinoma treated intra-tumourally with 450 μg of MMC five drug-related "metabolite" peaks were detected. Three of these co-chromatographed with standards of 2,7-DM, cis- and trans-hydro, and had identical absorption maxima to their respective standards, with the possible exception of trans-hydro.

INTRODUCTION

Mitomycin C (MMC, Fig. 1) remains an important component in combination chemotherapy regimens for the treatment of breast and prostate cancer and is probably the drug of first choice for local intravesical administration in superficial bladder cancer [1]. MMC requires quinone reduction, preferentially under anaerobic conditions, before drug activation occurs resulting in cross-linking of DNA and other covalent adducts [2,3]. This mechanism is referred to as bioreductive alkylation and it opened up the pos-

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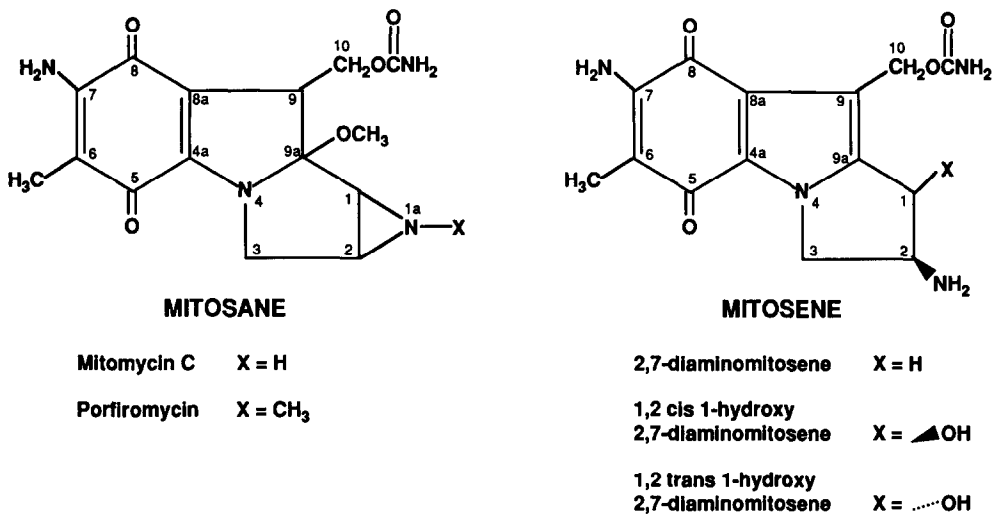

Fig. 1. Structures of mitomycin C, its three primary metabolites and porfiromycin, the internal standard.

sibility that MMC might exhibit selectivity to hypoxic cells present in solid tumours which are difficult to treat with either radiation or convential cytotoxic drugs (for review see ref. 4).

Three primary metabolites of MMC can be produced as a consequence of quinone reduction: 2,7-diaminomitosene (2,7-DM, Fig. 1) and the stereoisomers 1,2-*cis*- and 1,2-*trans*-1-hydroxy-2,7-diaminomitosene (cis-hydro and trans-hydro, Fig. 1) [5]. Formation of three products is due to the fact that an intermediate in drug activation (the quinone methide) can exhibit both electrophilic characteristics to give rise to cis- and trans-hydro and nucleophilic characteristics to yield 2,7-DM [6]. The eventual proportions of primary metabolites produced in an *in vitro* incubation is highly pH-dependent [5] and is likely to be influenced by many other factors *in vivo* such as oxygen tension, enzymology and level of free radical detoxification enzymes and scavangers. High-performance liquid chromatographic (HPLC) methods have been previously reported for the detection of MMC and its primary metabolites *in vitro* in relatively simple incubations with purified enzymes or microsomes where no or limited sample preparation was required [5,7–9]. When more complex techniques have been applied to HPLC analysis of biological fluids and tissues from whole animal studies and clinical pharmacokinetic studies either no metabolites were detected or only hints of metabolites were suggested [10–16].

In this paper a new, rapid and efficient HPLC separation is described together with a tissue liquid–liquid extraction method for the *in vivo* determination of MMC and its three primary metabolites in tumour tissue. Application of this methodology will hopefully provide insights into the role of metabolic activation in the *in vivo* antitumour mechanism of action of MMC.

EXPERIMENTAL

Chemicals and drug standards

All methanol and propan-2-ol were HPLC reagent grade and were from Rathburn (Walkerburn, UK). Sodium dihydrogenphosphate, disodium hydrogenphosphate (both Aristar grade), orthophosphoric acid, potassium chloride, chloroform and ethyl acetate (all Analar grade) were from BDH (Poole, UK). Water was deionised and double-distilled in a quartz glass still. All other chemicals were of the highest grade commercially available and were used as received.

MMC was from Kyowa Hakko Kogyo (Tokyo, Japan) and was received through the auspic-

es of John Kelly, Martindale Pharmaceuticals (Farillon House, Romford, UK). Porfiromycin (PM), used as an internal standard, was a kind gift from Professor Alan Sartorelli, Department of Pharmacology, Yale University (New Haven, CT, USA). 2,7-DM was a kind gift from Professor Maria Tomasz, Department of Chemistry, Hunter College (New York, NY, USA). Cis- and trans-hydro were synthesised in house by treatment of MMC with 0.1 *M* hydrochloric acid for 25 min at room temperature. Under these conditions the *cis* and *trans* isomers are produced at a molar ratio of 3:1 [17], which was confirmed by HPLC in this present study. The isomers were purified by column chromatography as a mixture and the 3:1 molar ratio was then used to calculate the concentration of each species. Drug and metabolite standards were reconstituted in a small volume of methanol and were diluted in HPLC mobile phase (see below). Methanolic standards were normally stored at 4°C and all samples were never subjected to repeated cycles of freezing and thawing. Calibration curves were constructed by injecting 100 μl of an individual standard solution over a concentration range of 0.1–100 μg/ml to allow separate curves to be generated for each of the four main components under investigation. The limit of detection was set at a peak height-to-baseline noise ratio of 3:1.

Apparatus

HPLC was performed with two different sets of apparatus. One system consisted of a Hewlett Packard Model 1090 liquid chromatograph equipped with a diode-array detector as described previously [18]. This was used principally for the collection of spectral data on the HPLC peaks generated from both solutions of standards and extracts of tumour specimens either spiked with standards or treated *in vivo* with MMC. The other system was comprised of an L-6000 solvent delivery system, an AS-2000 autosampler, an L-4200 UV–VIS detector (all from Merck-Hitachi, Poole, UK) set at 310 nm and, connected in series, a Model 440 absorbance detector (Waters Assoc., Northwich, UK) set at 360 nm for dual-wavelength detection. Both detectors were linked to computing integrators. This system was used in routine validation experiments.

Chromatographic conditions

The stationary phase was Spherisorb ODS-2 (5 μm particle size) obtained prepacked in 25 cm × 4.6 mm I.D. stainless-steel columns from Phase Separations (Deeside, UK). Other stationary phases investigated were: LiChrosorb RP-18 (7 μm); μBondapak C_{18} (10 μm); Apex-1 octadecyl (5 μm); Nova-pak C_{18} (5 μm); YMC Basic (C_8, 5 μm); and Dynamax, 300 Å pore size C_{18} (5 μm). All were packed in 25 cm × 4.6 mm I.D. columns with the exception of μBondapak and Nova-pak which were packed in 30 cm × 3.8 mm I.D. columns. The mobile phase consisted of 18 m*M* sodium phosphate buffer pH 5.8–methanol (74:26). Elution was isocratic at a flow-rate of 1 ml/min and the column was maintained at a constant temperature of 40°C. Mobile phase components were filtered before used (0.2-μm filter, Waters-Millipore, Northwich, UK) and were continuously sparged with helium during chromatography.

Extraction of MMC and its metabolites from tumour tissue

The animal/tumour model used throughout was the WAB/NOT strain of rat and the syngeneic, undifferentiated mammary carcinoma Sp 107, a tumour that originally arose spontaneously in a female rat [19]. For *in vivo* studies, MMC was administered by direct intra-tumoural (i.t.) injection at a dose of 450 μg. In control extractions, tumours were collected from non-drug-treated animals, and 100 μl of a methanolic solution of MMC or one of its three mitosene metabolites were added to 1 ml of tumour homogenate. PM, at either 5 or 50 ng in 10 μl of methanol, was added per ml of homogenate as an internal standard to all samples. After tumours were collected, they were immediately frozen to −60°C by placing them in solid CO_2. Tumours were stored at −20°C for never more than two months prior to analysis and were only partially thawed in ice prior to homogenisation. To a known weight of tumour tissue was added 154 m*M* KCl in propor-

tions 1:2 (w/v). A homogenate was then produced using a high-speed Silverson laboratory mixer with a cutting blade [obtained from Fisons Scientific Equipment (FSE), Loughborough, UK]. A portion of this homogenate (1 ml, plus 10 μl of PM and in control extractions 100 μl of MMC or mitosenes) was extracted with 5 ml of chloroform–propan-2-ol–ethyl acetate (2:2:1) for 15 min with vigorous vortex-mixing using a Buchler vortex evaporator and 50-ml tapered glass centrifuge tubes (FSE). After extraction, samples were centrifuged at 1000 *g* for 15 min at 4°C. The solvent layer was retained for evaporation, the aqueous phase was then re-extracted with a further 5 ml of solvent mixture as above. After re-extraction, samples were again centrifuged and the solvent layer from this second extraction was combined with the solvent layer from the first extraction for evaporation to dryness under a stream of nitrogen. Residues were reconstituted in 300 μl of mobile phase by vortex-mixing for at least 2 min after which they were centrifuged at 15 000 *g* in a microfuge (Model 5414, Eppendorf, Germany) and finally filtered before 100 μl were subjected to HPLC analysis as above.

RESULTS AND DISCUSSION

High-performance liquid chromatography

Fig. 2 shows an example of the separation with standards, and Table I summarises its chromatographic characteristics. Due to slight peak tailing

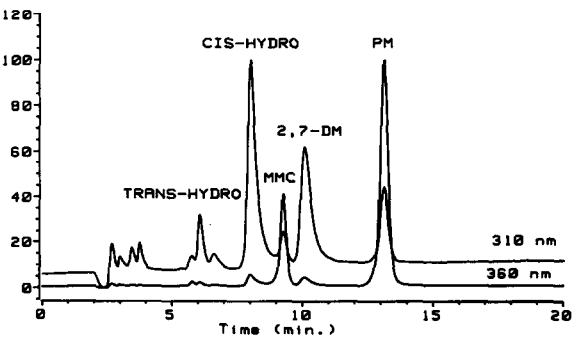

Fig. 2. Chromatograms of a mixture of standards of mitomycin C, its metabolites and porfiromycin monitored at two different wavelengths: 310 and 360 nm. Chromatographic conditions as in Experimental. Peaks: trans-hydro = 1,2-*trans*-1-hydroxy-2,7-diaminomitosene (1.25 μg/ml, t_R = 6.08 min); cis-hydro = 1,2-*cis*-1-hydroxy-2,7-diaminomitosene (3.75 μg/ml, t_R = 8.14 min); MMC = mitomycin C (5 μg/ml, t_R 9.27 min); 2,7-DM = 2,7-diaminomitosene (5 μg/ml, t_R = 10.09 min); PM = porfiromycin (10 μg/ml, t_R = 13.12 min). Peaks have been normalised against the largest peak, PM.

the efficiency of 2,7-DM and cis-hydro were reduced compared to the other components. This phenomenon has been noted previously where *trans*-mitosene products were shown to yield better peak symmetry than their *cis* isomer counterparts [20]. The limit of detection was reduced for the mitosenes because of their lower molar extinction coefficent (ε = 6026 at 309 nm *versus* ε = 21380 at 360 nm for MMC) [21].

The factors that influence the separation of MMC and its mitosene metabolites were studied. Two main determinants were identified: mobile phase pH (see Fig. 3) and buffer ionic strength

TABLE I

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF MITOMYCIN C AND ITS METABOLITES

Component	Retention time (n=20) (min ± % C.V.)	k'	Efficiency (plates/m)	Calibration curve linearity over range 0.1–100 μg/ml (r^2)	Detection limit on column (ng)	Detection limit after extraction (ng/g tumour)
MMC	9.27 ± 1.45	4.2	23 580	0.999	2	20
2,7-DM	10.10 ± 0.78	4.6	11 440	1.000	10	100
Trans-hydro	6.07 ± 1.48	2.4	16 000	0.998	10	100
Cis-hydro	8.01 ± 2.6	3.5	11 480	0.999	10	100
PM	13.11 ± 1.13	6.3	33 760	–	–	–

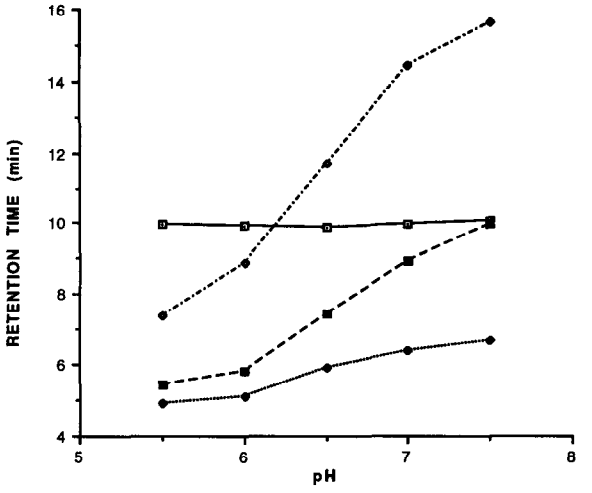

Fig. 3. Effect of pH on the retention time of mitomycin C and its three primary mitosene metabolites (see Fig. 1). Chromatographic conditions as in Experimental except that the ionic strength of sodium phosphate was 66.6 m*M* and adjustments to pH were performed with 1 *M* sodium hydroxide and 1 *M* orthophosphoric acid. (———) MMC; (—·—·—) 2,7-DM; (·····) trans-hydro; (----) cis-hydro.

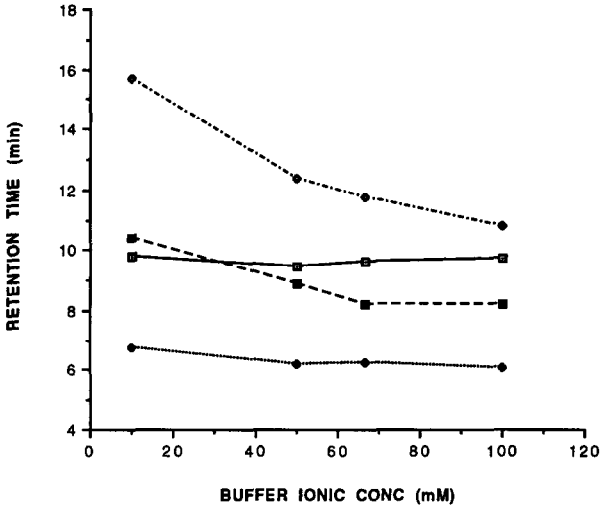

Fig. 4. Effect of buffer ionic strength on the retention time of mitomycin C and its metabolites (see Fig. 1). Chromatographic conditions as in Experimental except that the pH of sodium phosphate was 6.5 and its ionic strength was varied. (———) MMC; (—·—·—) 2,7-DM; (·····) trans-hydro; (-----) cis-hydro.

(see Fig. 4). The proportion of the organic modifier in the mobile phase was less critical, affecting all components equally and to a lesser extent so was the choice of stationary phase. pH had the greatest effect on 2,7-DM, increasing its retention time by almost 6 min from pH 6 to 7; cis-hydro was also more affected than trans-hydro, and MMC remained unaltered (Fig. 3). The same pattern was observed with buffer ionic strength, but here an increase in salt concentration resulted in a reduction in retention time (Fig. 4).

In previous HPLC studies on the *in vitro* metabolism of MMC, gradient elution was normally employed to fully resolve the large number of metabolites under investigation [5,7,20,21]. Although impressive results were achieved high flow-rates were necessary (3 ml/min) and non-standard stationary phases were employed (radial compression modules). Reducing flow-rates lead to longer gradient runs and marked peak tailing of 2,7-DM as it eluted at the top of the gradient together with possible interferences [8]. Shorter isocratic runs resolved considerably fewer peaks [9]. By carefully adjusting the ionic strength and pH of the mobile phase high efficiency along with good resolution and a short run time (15 min) have been achieved with an isocratic method.

Sample preparation technique

The efficiency of the liquid–liquid extraction method described is shown in Table II. Each entry represents a separate experiment. Control extractions validating the sample preparation technique were not performed with mixtures of MMC and its metabolites because of the distinct possibility that one component (*e.g.* MMC) could artefactually degrade into another during sample preparation (*i.e.* cis- and trans-hydro). A close examination of the traces from these studies showed that this, in fact, was not the case: no evidence of degradation was detected. Also, when tumour homogenates were spiked with mixtures of MMC, its three metabolites and PM the recoveries recorded were consistent with the data contained in Table II. Over a wide range of concentrations the extraction efficiency of MMC

TABLE II

EFFICIENCY OF EXTRACTION OF MITOMYCIN C AND ITS METABOOLITES FROM SPIKED TUMOUR HOMOGENATES (33%, w/v, IN 154 m*M* POTASSIUM CHLORIDE)

Amount per ml homogenate (μg)	Extraction efficiency (mean ± S.D.)[a] (%)				
	MMC	2,7-DM	Trans-hydro	Cis-hydro	PM
100	70 ± 7	–	–	–	–
50	57 ± 7	–	–	–	–
10	72 ± 7	52 ± 2	62 ± 8	58 ± 7	–
5	81 ± 6	46 ± 11	–	–	–
1	83 ± 6	51 ± 9	103 ± 14	65 ± 10	–
0.5	91 ± 10	49 ± 10	–	33 ± 8	66 ± 14
0.1	58 ± 12	62 ± 16	–	–	–
0.05	63 ± 9	50 ± 20	–	–	105 ± 21

[a] $n = 10$ for each component at each concentration.

remained high (mean ± S.D. of means 71.9 ± 12.4%). Values dropped to around 50% for 2,7-DM (mean ± S.D. of means 51.7 ± 5.4%) and cis-hydro (mean of means 52%) but were more variable for trans-hydro. The recovery of the internal standard, PM, tended to be higher than that of MMC and was significantly higher than those of the three mitosenes. Therefore, the external standard method is recommended for quantitation. The sample preparation method did not extract any endogenous substances from tumour tissue which could potentially interfere with the identification of MMC and its three primary metabolites (see Fig. 5).

The data presented in Table II represent one of a few limited reports, and possibly the first, to describe the behaviour of primary mitosene metabolites of MMC in a tissue sample preparation technique. Most HPLC studies on MMC metabolism to date have been performed *in vitro* where no sample clean-up was necessary before direct injection on to the HPLC column [5,7–9].

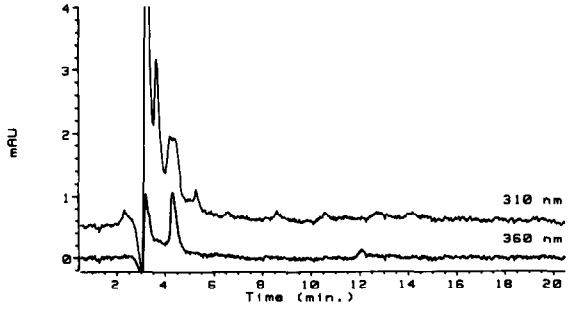

Fig. 5. HPLC analysis of a non-drug-treated Sp 107 rat mammary carcinoma tumour specimen which had been put through the liquid–liquid extraction sample preparation technique described in Experimental. Chromatograms were monitored at two wavelengths: 310 and 360 nm.

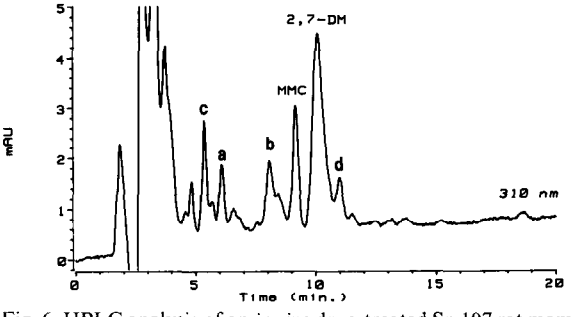

Fig. 6. HPLC analysis of an *in vivo* drug-treated Sp 107 rat mammary carcinoma specimen collected 1 h after intra-tumoural administration of 450 μg of MMC. Sample preparation and HPLC as described in Experimental. The chromatogram was monitored at 310 nm and five putative drug metabolite peaks were detected (peaks a–d and 2,7-DM) along with MMC. MMC (t_R 9.16 min) is mitomycin C (2.63 μg/g of tissue). 2,7-DM (t_R 10.06 min) is believed to be 2,7-diaminomitosene (1.04 μg/g) and its UV–VIS spectrum is compared to a standard in Fig. 7. Peak a (t_R = 6.07 min, 0.20 μg/g) co-chromatographed with 1,2-*trans*-1-hydroxy-2,7-diaminomitosene and its UV spectrum is compared to a standard in Fig. 8. Peak b (t_R = 8.06 min, 0.20 μg/g) co-chromatographed with 1,2-*cis*-1-hydroxy-2,7-diaminomitosene and its spectrum is compared to a standard also in Fig. 8. Peak c (t_R = 5.36 min) and peak d (t_R = 10.97 min) are mentioned in Results and discussion.

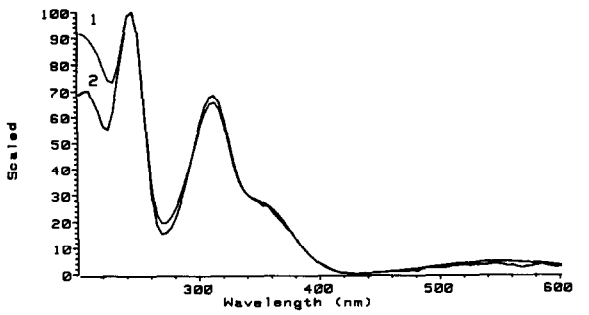

Fig. 7. UV–VIS absorption spectrum of the peak identified as 2,7-DM in Fig. 6 compared to a standard of 2,7-diaminomitosene. Both spectra have been normalised and superimposed. Spectrum 1, 2,7-DM from the tumour specimen; spectrum 2, 2,7-diaminomitosene standard.

Analysis of in vivo drug-treated tumour specimens and spectral analysis of the MMC-related metabolite peaks detected

Fig. 6 shows a typical profile of the putative metabolite peaks detected in a tumour specimen collected 1 h after i.t. administration of 450 μg of MMC. This chromatogram was monitored at 310 nm. Five peaks associated with MMC were identified (2,7-DM and peaks a–d). One peak labelled as 2,7-DM had a retention time (t_R) of 10.06 min compared to 10.09 min for a purified standard of 2,7-DM (see Fig. 2). Its UV–VIS spectrum is shown in Fig. 7 superimposed over the spectrum of a pure standard of 2,7-DM. The overlay displayed almost perfect coincidence and the three main absorption maxima were completely intact at 244, 313 and 558 nm. This peak continued to co-chromatograph with 2,7-DM when both a different mobile phase was employed (66.6 m*M* sodium phosphate pH 6.3 in 30% methanol) and a different stationary phase tested (LiChrosorb RP-18). We therefore feel confident that this peak genuinely represents 2,7-DM. Peak a (t_R 6.07) and peak b (t_R 8.06) in Fig. 6 co-chromatographed with standards of trans-hydro (t_R 6.08) and cis-hydro (t_R 8.01), respectively. UV–VIS absorption spectra of standards of cis- and trans-hydro together with peaks a and b from Fig. 6 are shown overlain collectively in Fig. 8. The spectrum of peak b (spectrum 3, Fig. 8) was in good agreement with cis-hydro (spectrum 1, Fig. 8) exhibiting the correct maxima at 250, 308 and 550 nm [21]. However, the spectrum of peak a (spectrum 4, Fig. 8) displayed an exaggerated shoulder at around 350 nm and significant attenuation of the maxima at 250, 308 and 550 nm indicative of a co-eluting MMC metabolite with significant absorption at 350 nm. Peak c (Fig. 6) did not have the characteristic UV–VIS spectrum of a 7-diaminomitosene product (data not shown) but may be related to the series of secondary metabolites identified *in vitro* in enzyme incubations with xanthine oxidase and cytochrome P-450 reductase [5]. It has recently been demonstrated that the Sp 107 tumour contains significant cytochrome P-450 reductase activity [22]. Peak d (Fig. 6) had a UV–VIS absorption spectrum identical to that of a 1-hydroxymitosene with λ_{max} at 250, 309 and 550 nm and may be related to decarbamoyl-1,2-*cis*- and -*trans*-1-hydroxy-2,7-diaminomitosene (most probably the *cis* isomer) which retain these maxima [20]. This product appeared in tumour specimens at the earlier time points studied (5 min) at a level greater than cis- and trans-hydro but never in excess of 2,7-DM. In order to further characterise its structure this peak is currently being purified. If it does turn out to be a decarbamoyl-1-hydroxymitosene then this would provide circumstantial evidence that bi-functional activation of MMC occurs *in vivo* in tumour tissue.

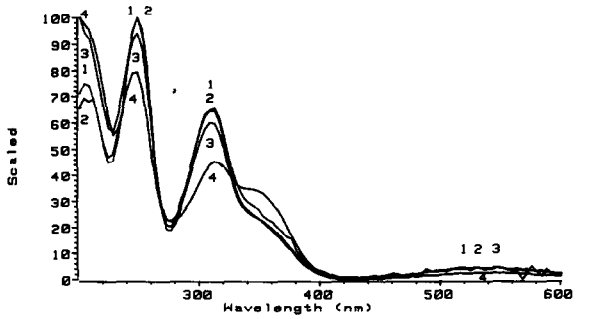

Fig. 8. UV–VIS spectra of peaks a and b identified in Fig. 6 compared to a standard of cis- and trans-hydro. All spectra have been normalised and superimposed. Spectrum 1, cis-hydro standard; spectrum 2, trans-hydro standard; spectrum 3, peak b from tumour specimen; spectrum 4, peak a from tumour specimen.

The above data appear to support the contention that MMC is being converted into its three

primary metabolites in the Sp 107 rat mammary carcinoma. Confirmation of this fact must await the purification of proposed metabolite peaks and elucidation of structure, which is presently ongoing. At this stage the main chromatographic issue is whether the three peaks putatively identified as 2,7-DM, cis-hydro and trans-hydro represent pure peaks free from co-eluting impurities derived from other metabolites of MMC. Pan *et al.* [5] have shown in elegant studies that, even in relatively simple enzyme incubations, a large number of MMC metabolites are evolved. *In vivo*, an even more complex picture might be expected. However, on closer inspection of the chromatographic data of Pan *et al.* [5], it is clear that the three primary metabolites were the most abundant species and a number of peaks were related to reaction of MMC intermediates with incubation buffers. Additionally, these adduct peaks eluted much earlier than the metabolite peaks and are unlikely to interfere with an isocratic HPLC separation. Apart from 10-decarbamoyl-1,2-*cis*- and -*trans*-1-hydroxy-2,7-diaminomitosene, the secondary metabolites of MMC have markedly different UV–VIS spectra from intact 7-diaminomitosenes. Their presence would be identified by the diode-array detector [20]. Therefore, we feel secure that the major metabolite peaks identified in this work do represent true single component peaks eluting largely uncontaminated with the possible exception of low levels of trans-hydro. Failure to identify these three peaks in previous *in vivo* studies may be due to the fact that they represent intermediates in a chain of events. They are proposed to have relatively short half-lives [5,17]. Nevertheless, occasional reports appear where HPLC peaks are identified with the characteristics of one of the three primary metabolites [14].

Cis- and trans-hydro derivatives of MMC can also be formed by acid-catalysed hydrolysis whereas 2,7-DM is normally only found after quinone reduction [17,23]. Acid hydrolysis heavily favours the cis-hydro isomer [23], whereas bioreduction favours the trans-hydro isomer. Their molar ratio can be used to give an indication on the nature of their formation. Similarly, after quinone reduction, a more acidic pH favours the formation of 2,7-DM over the cis- and trans-hydro products. At neutral pH a ratio of 1–2 has been reported for 2,7-DM/cis- + trans-hydro [5,17]. The profile of the three primary mitosene products detected in tumour specimens (see Fig. 6) yields a ratio close to unity for cis-hydro/trans-hydro and a value of 2 for 2,7-DM/cis- + trans-hydro, consistent with their formation being due to bioreduction as opposed to acid hydrolysis or drug degradation.

A greater generation of 2,7-DM has recently been linked to increased expression of DT-diaphorase, increased drug cytotoxicity and production of longer lived reactive intermediates capable of alkylating DNA [8,24]. From this point of view it is interesting that the Sp 107 tumour contains high levels of both cytosolic DT-diaphorase (94.3 nmol/min/mg protein) and significant levels of microsomal DT-diaphorase (28 nmol/min/mg) [22]. At more neutral pH these reactive intermediates appear to preferentially alkylate the protein itself rather than DNA, resulting in enzyme inactivation. In summary, in this work we have developed a rapid, isocratic HPLC separation and tissue sample preparation technique to determine *in vivo* MMC and its three primary mitosene metabolites in tumour tissue. At present this methodology is being used to perform full pharmacokinetic studies of MMC in the SP 107 tumour.

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