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CHROMATOGRAPHIC STUDIES OF MITOMYCIN C DEGRADATION IN ALBUMIN MICROSPHERES

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SUMMARY

Serum albumins and polylactic acid (PLA) have been used as bioerodable polymers in the preparation of drug-containing microspheres for parenteral drug delivery. The albumin microsphere may be prepared via either chemical cross-linking or heat denaturation of the protein. Heat-denatured albumin microspheres containing mitomycin C (MMC) have been used in pre-clinical and clinical investigations. Due to the high reactivity of MMC as a bifunctional alkylating agent, a study on the stability of MMC in the albumin and PLA microspheres has been carried out using a high-performance liquid chromatographic (HPLC) method. Human serum albumin (HSA) microspheres were prepared using an emulsion method via either heat denaturation at 120 or 170°C or the use of 0.5 *M* biacetyl as a cross-linking agent. The PLA microspheres were prepared by an emulsion method at 55°C. HPLC analysis of the HSA microspheres showed that about 37% of MMC was converted to 2,7-diaminomitosene derivatives in microspheres prepared by heat denaturation at 120°C. The degradation increased to 82% when the microspheres were prepared with a denaturation temperature of 170°C. The use of biacetyl as a cross-linking agent in the preparation of HSA microspheres resulted in a complete degradation of the incorporated MMC. Biacetyl was found to interact with MMC leading to the formation of 7-aminomitosene derivatives. In contrast to the albumin system, MMC may be incorporated into PLA microspheres without degradation.

INTRODUCTION

Interest in drug delivery mediated via carriers has increased considerably in the past few years due to its potential to alter the site of drug deposition and

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control the rate of drug release. Several systems for controlled delivery of drugs have been proposed, including liposomes [1], resealed erythrocytes [2], biodegradable nanoparticles [3] and microspheres [4]. Anticancer drugs are promising candidates for controlled drug delivery due to their high systemic toxicity, poor stability and short biological half-life [5,6]. Some of the anticancer agents incorporated into polymeric microspheres for therapeutic chemoembolization include adriamycin [7], 5-fluorouracil [8], 6-mercaptopurine and mitomycin C [9,10].

Biodegradable albumin microspheres have received increasing attention as carriers in drug delivery in past decade. Although originally developed for radiological studies [11], the emulsion–stabilization method is widely used for preparation of drug-containing microspheres [4,6,7,9,12]. Such microspheres are prepared by heating the drug–albumin solution to temperatures exceeding 100 °C or by treatment with highly reactive chemical cross-linking agents such as biacetyl or glutaraldehyde. Adriamycin-containing albumin microspheres were examined for chemical integrity of the incorporated drug by Cummings and Willmott [13]. No degradation of adriamycin was found to occur when microspheres were made at 120–160 °C in the presence of glutaraldehyde. While little has been shown regarding the effects of microencapsulation processes on the stability of mitomycin C (MMC), heat-denatured albumin–mitomycin C microspheres have been used in laboratory animals [12] and patients with inoperable hepatic cancer [14].

MMC, a potent anticancer antibiotic, is known to undergo biological reductive activation and cross-link DNA molecules through bifunctional alkylation [15,16]. The molecular mechanism of action has been shown to involve a one- or two-electron reduction of MMC to the hydroquinone form, followed by the spontaneous elimination of methanol and the generation of the C-1 and C-11 reactive centers [16,17]. Covalent bonding of the activated drug to DNA through linkages to guanine residues of adjacent strands has been demonstrated [18]. The alkylating function of MMC can also be activated by chemical reducing agents [19] and by mild acidic conditions [20] via the opening of its aziridine ring. A sum-

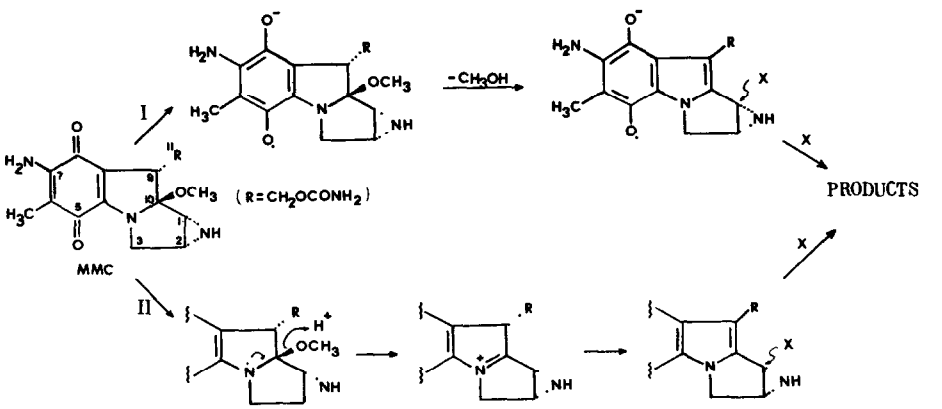

Fig. 1. Mechanism for the molecular activation of MMC via (I) the reductive pathway and (II) the acidic pathway. X indicates either a nucleophilic or a hydrogen abstraction reaction.

mary of the MMC activation processes is shown in Fig. 1. In light of the reactivity of MMC and its potential interactions with other macromolecules, studies were carried out to examine MMC degradation in human serum albumin (HSA) and polylactic acid (PLA) microspheres. PLA is a bioerodable polymer which has been used for the preparation of MMC microcapsules in this laboratory [10].

EXPERIMENTAL

Preparation of mitomycin C–albumin microspheres

Heat-denatured HSA microspheres were prepared by modification of the method of Zolle et al. [11]. An aqueous solution containing 2.5% MMC (kindly supplied by Bristol Labs., Syracuse, NY, U.S.A.) and 25% HSA (United States Biochemical Corp., Cleveland, OH, U.S.A.) was emulsified in light mineral oil (Fisher Scientific, Fairlawn, NJ, U.S.A.) and the temperature was increased to 120 or 170°C and maintained for 20 min followed by gradual cooling to room temperature.

Biacetyl (2,3-butadione, United States Biochemical Corp.) cross-linked microspheres were prepared by the following method: an 0.5-ml aqueous solution containing 25% albumin and 2.5% MMC was emulsified in 25 ml light mineral oil, and the desired amount of biacetyl was added. After the required stirring period (4 h) the partially solidified microspheres in suspension were transferred to 125 ml light mineral oil at 50°C and stirred under vacuum for 2.5 h to completely remove the residual biacetyl and water.

The solidified microspheres were collected by filtration and washed three times with *n*-heptane (Fisher Scientific) and stored for 24 h in *n*-heptane to remove any adhering mineral oil. Microspheres were then air-dried and stored at −4°C.

Preparation of mitomycin C–polylactic acid microspheres

PLA microspheres were prepared by an emulsion–solvent evaporation method, modified from Tsai et al. [10]. A solution containing 5% poly-*d,l*-lactic acid (Polyscience, Warrington, PA, U.S.A.) and 0.5% MMC in a mixture of acetonitrile and methylene chloride (3:1) maintained at 55°C was emulsified into light mineral oil at 55°C under constant stirring. Vacuum was applied for 20 min to remove the solvents. The solidified microspheres were filtered and washed three times with and stored in *n*-heptane for 24 h. The air-dried microspheres were stored at −4°C.

Analysis of mitomycin C and its degradation products

A known amount of HSA microspheres was homogenized in distilled water using Kontes tissue grinder (Kontes Glass, Vineland, NJ, U.S.A.) to artificially release the incorporated drug. The supernatant was diluted to contain 25 μg/ml total MMC and analysed for degradation of MMC.

PLA microspheres were dissolved in a solvent system containing acetonitrile and methylene chloride. The drug content was then extracted into water with ten-fold concentration dilution for further measurements.

Quantitative determination of the degradation of MMC was carried out using

a high-performance liquid chromatographic (HPLC) system equipped with a Model 6000A solvent delivery system and a Model U6K sample injector (both from Waters Assoc., Milford, MA, U.S.A.). A Model HP8450A diode array spectrophotometer, controlled by a Model HP85B personal computer (Hewlett-Packard, Palo Alto, CA, U.S.A.) was used as a detector. The separation was achieved using a 150 mm × 3.9 mm μBondapak C_{18} reverse d-phase column (Phenomenex, Rancho Palos Verdes, CA, U.S.A.) and a mobile phase of 20% methanol in 0.05 *M* phosphate buffer (pH 7.4) delivered at a flow-rate of 1.5 ml/min. Benzocaine (20 μg/ml) was used as an internal standard. The samples (30 μl) were injected and monitored by UV absorption at 254 and 362 nm. Simultaneously, complete UV–visible absorbance spectra of each peak were obtained. To provide positive identification of the degradation products, chemically synthesized derivatives of MMC of known structures were used as standards. *cis*- and *trans*-2,7-diamino-1-hydroxymitosene (*cis*-2d and *trans*-2d), the principal degradation products of MMC, were prepared according to the procedure of Beijnen and Underberg [21] whereas 2,7-diaminomitosene was prepared by the method of Tomasz and Lipman [22].

For the study of the effect of high temperature on the stability of the drug, aqueous solutions of MMC were heated to 120°C for 20 min in an autoclave (American Sterilizer, Erie, PA, U.S.A.) in the presence or absence of HSA. The solutions were then centrifuged and analysed for degradation of MMC by UV–visible spectrophotometry and HPLC.

Similarly an aqueous solution of MMC (pH 6.8, 0.05 *M* Tris buffer) was treated with biacetyl (0.5 *M*) at room temperature to study the effects of the cross-linking agent on the stability of mitomycin C. Samples were taken at various time intervals and evaporated until the volume reduced to half. The samples were then reconstituted and analysed for degradation of MMC.

RESULTS AND DISCUSSION

Fig. 2 shows the UV–visible absorption characteristics of drug content extracted from HSA microspheres prepared either by heat denaturation (spectrum c) or the use of biacetyl (spectrum d) as a cross-linking agent. Both extracts exhibit absorption maxima at 250, 310 and 540 nm that are clearly different from those of MMC (spectrum a), but are characteristic to those of the 7-aminomitosene derivatives [20,23]. For comparison, the spectrum of *cis*- or *trans*-2d (which are identical) is shown as spectrum b. These results indicate that significant degradation of MMC via the opening of the aziridine ring occurred during the preparation of the albumin microspheres. Drug extract from the PLA microspheres showed identical spectrum with that of MMC.

Fig. 3 shows the HPLC analysis of the drug contents extracted from the HSA and PLA microspheres. Under the chromatographic conditions, MMC, which can be detected by UV absorption at both 254 and 362 nm, exhibits a retention time of 7.2 min. The extract from the PLA microspheres shows an identical chromatogram (A) to that of the standard MMC, indicating that the drug did not interact with the polymer and the stability of MMC was not affected by the

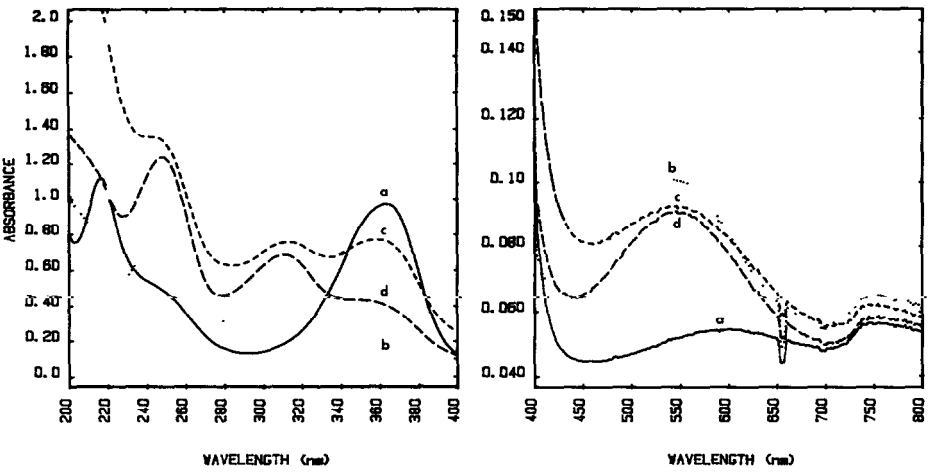

Fig. 2. Absorption spectra of (a) MMC, (b) *cis*- or *trans*-2d, (c) extract from HSA microspheres prepared via heat denaturation and (d) extract from HSA microspheres prepared via chemical cross-linking. All samples contained a drug concentration of $7.18 \cdot 10^{-5} M$ MMC.

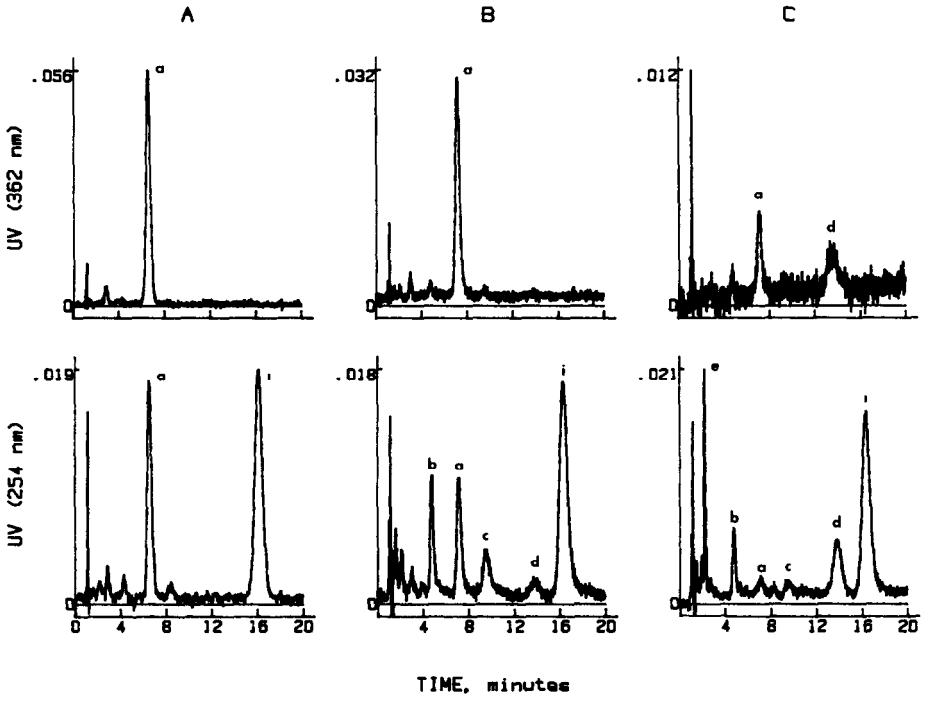

Fig. 3. HPLC analysis of the degradation products of MMC extracted from (A) PLA microspheres, (B) HSA microspheres prepared by heat denaturation and (C) HSA microspheres prepared via chemical cross-linking. Peaks a, b, c and d were identified to be MMC, *trans*-2d, *cis*-2d and 2,7-diaminomitosene, respectively. Compound e is unknown. Benzocaine (peak i) was used as an internal standard.

microencapsulation process. Several degradation products, however, were found in the extracts of the HSA microspheres. The extract from the heat-stabilized microspheres was found to contain three degradation products which are shown as peaks b, c and d in chromatogram B. Peak a, with a retention time of 7.2 min, represents undegraded MMC. The degradation products b, c and d, which do not show absorption at 362 nm but exhibit the 2,7-diaminomitosene chromophor as analyzed by spectrophotometry, have retention times identical to those of *trans*-2d (4.8 min), *cis*-2d (9.7 min) and 2,7-diaminomitosene (14.0 min), respectively. The major degradation products are the *cis*- and *trans*-isomers of 2,7-diamino-1-hydroxymitosene with a 1:2 ratio of product formation. The HPLC identification of the products is consistent with the results of Pan et al. [17]. They showed that the *cis*-isomer was less hydrophilic than either *trans*-2d or MMC and appeared at longer retention time than MMC in reversed-phase chromatography. Chromatogram C shows the degradation products of MMC from HSA microspheres prepared via the use of biacetyl. Here, the results show an extensive degradation of the encapsulated MMC and the formation of 2,7-diaminomitosene (peak d) and an unknown product (peak e) with a retention time of 2.2 min. While the identity of compound e has not been confirmed, it exhibited a UV–visible absorption typical of compounds containing the 7-hydroxymitosene chromophore [23]. The extract was found to contain very low content of *cis*- and *trans*-2d, suggesting that compound e may be a further degradation product of *cis*- and *trans*-2d. Indeed, our studies show that compound e can be produced via extensive hydrolysis of MMC in 0.1 *M* hydrochloric acid.

The *cis*- and *trans*-isomers of 2,7-diamino-1-hydroxymitosene and 2,7-diaminomitosene have been shown to be the primary metabolites of MMC in the NADPH cytochrome P-450 reductase and xanthine oxidase systems [17]. They attributed the production of these metabolites to the reductive activation of MMC to an active intermediate characterized by its possession of an aromatic indole moiety and the hydroquinone radical anion (Fig. 1). While nucleophilic attack on the free radical intermediate by water generated *cis*- and *trans*-2d, a competing pathway involving hydrogen abstraction by the intermediate was responsible for the formation of 2,7-diaminomitosene. The identification of the same products in the albumin microspheres, particularly 2,7-diaminomitosene, suggests that the denatured HSA and biacetyl also activate MMC leading to the opening of the aziridine ring. On further examination of the heat denaturation process, solutions containing MMC in the absence and presence of HSA were heated to 120°C for 20 min and analyzed using HPLC and spectrophotometric methods. Fig. 4 shows the absorption spectra of the heated MMC solutions. In the absence of HSA, the solution shows and UV absorption (spectrum a) similar to that of MMC. Further analysis by HPLC confirmed that the drug did not undergo degradation under this condition. The absorption spectrum b for the solution containing HSA, however, shows the formation of 7-aminomitosene derivatives. The degradation products were further identified by HPLC to be *cis*- and *trans*-2d. At room temperature the protein did not facilitate the degradation of MMC.

Although the degradation of MMC in protein solutions occurred only at high temperature, the conversion of MMC to *cis*- and *trans*-2d, which requires either

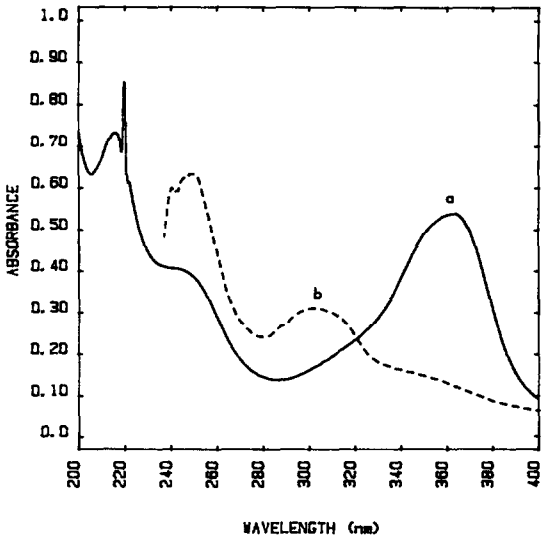

Fig. 4. Absorption spectra of $7.18 \cdot 10^{-5} M$ MMC solution in the absence (a) and presence (b) of 10 mg/ml HSA after heating at 120°C for 20 min.

a reductive [16,17] or an acidic [20] activation of the molecule, is, at least in part, due to the interaction of MMC with HSA. Our preliminary data showed that MMC indeed binds to HSA. In fact, the protein binding was found to inhibit the acidic activation of MMC at pH 4.0 [24]. The mechanism for the acidic activation of MMC involves the proton-catalyzed methanol elimination from the C-10 position of the MMC molecule (Fig. 1) [20]. This suggests that the protein interaction involves binding the drug at the active center which prevents the methanol elimination reaction. The protein-facilitated MMC degradation observed at high temperature may be attributed to the activation of MMC by the denatured protein, followed by nucleophilic attack of the aziridine ring by water. In this case, the major degradation products would be *cis*- and *trans*-2d.

MMC was found to undergo degradation at neutral pH and room temperature in solutions containing biacetyl either in the absence or in the presence of HSA. Fig. 5 shows the HPLC analysis of MMC degradation in solutions containing 0.5 *M* biacetyl. The chromatogram, recorded 2 h after sample preparation, shows that the interaction of MMC with biacetyl results in the formation of primarily *trans*- and *cis*-2d (peaks b and c), 2,7-diaminomitosene and compound e (peaks d and e) being the minor products. Fig. 6 shows the kinetic degradation of MMC in solutions containing 0.5 *M* biacetyl at neutral pH. Here it can be seen that MMC is stoichiometrically converted to *cis*- and *trans*-2d as a function of time. The formation of the *cis*- and *trans*-isomers is detected to be in a ratio of about 1:2, which is in agreement with the results of Pan et al. [17]. These results show that biacetyl, a photochemical sensitizer, also activates MMC leading to the formation of an active intermediate as the ones shown in Fig. 1. The increased production of 2,7-diaminomitosene observed in the extract of biacetyl cross-linked HSA microspheres may be attributed to hydrogen abstraction by the active interme-

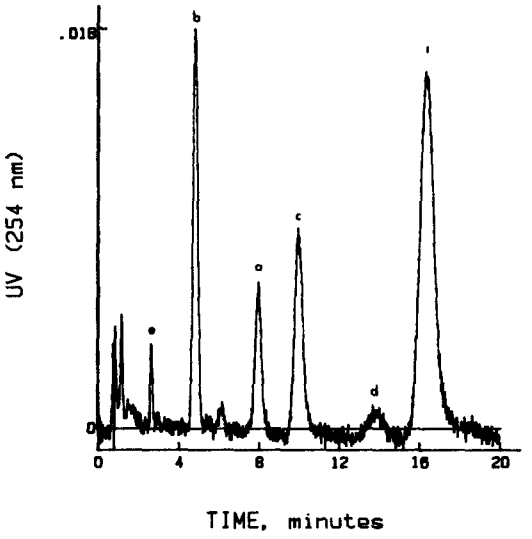

Fig. 5. HPLC analysis of the degradation of MMC in solutions containing 0.5 *M* biacetyl. The chromatogram was recorded 2 h after the sample preparation. Peaks a, b, c, d and e were identified to be the same as given in Fig. 3.

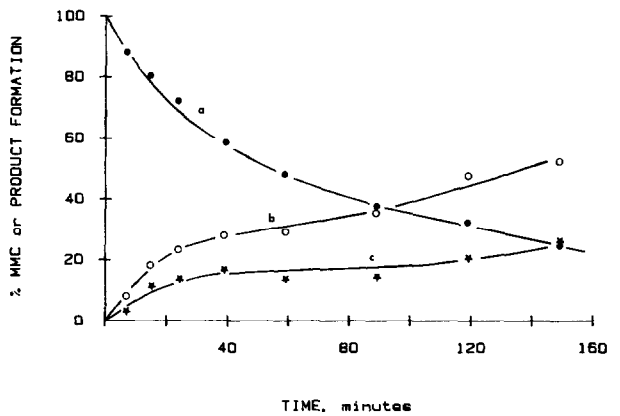

Fig. 6. Time dependency for the degradation of $7.18 \cdot 10^{-5} M$ MMC (a) and the production of *trans*-2d (b) and *cis*-2d (c) in a solution containing 0.5 *M* biacetyl.

diate, which has been shown to occur at conditions where hydrogen may be abstracted from suitable sources such as protein, lipids and water [17]. The presence of HSA facilitated the hydrogen abstraction reaction.

The present investigation shows that MMC is extremely unstable to the processes used in the preparation of albumin microspheres. Interaction of MMC with either the protein at high temperature or the cross-linking agent lead to the activation of the drug and the formation of inactive but potentially toxic products. The results on the quantitative determination of MMC in various microspheres show that in the heat-denatured HSA microspheres prepared at 120 and 170 °C, the degradations are 37 and 82%, respectively. The use of biacetyl at concentrations of 0.5 and 1.0 *M* resulted in a complete degradation of the incorporated

MMC. The PLA microspheres, on the other hand, gave nearly 100% recovery of the encapsulated MMC. PLA is non-toxic and bioerodable and hence provides a suitale alternative to serum albumins for the delivery of MMC via chemoembolization.

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