Cancer Chemother Pharmacol (1991) 28: 22–26
034457049100050X

Cancer Chemotherapy and Pharmacology

High-performance liquid chromatography assay of bleomycin in human plasma and rat hepatocytes in culture

Rachid Mahdadi[1], Abdelraouf Kenani[2], Nicole Pommery[1], Jean Pommery[1], Jean Pierre Hénichart[2], and Michel Lhermitte[1,2*]

[1] Laboratoire de Toxicologie, Faculté de Pharmacie de Lille II, 3 rue du Professeur Laguesse, F-59045, Lille Cedex, France
[2] Unité INSERM no. 16, Place de Verdun, F-59045, Lille Cedex, France

Summary. A sensitive and rapid linear-gradient, ion-paired, reversed-phase high-performance liquid chromatography technique using fluorescence detection was developed to quantify bleomycin (BLM) metabolites in the plasma of patients undergoing BLM therapy and in rat hepatocytes that had previously been incubated with 5×10^{-5} M BLM. We could detect about 70 ng/ml using this procedure. BLM metabolites were assayed in the supernatant fractions of precipitated human plasma and in pellets of rat hepatocytes. Metabolite concentrations were below the level of detection in human plasma samples. In hepatocyte pellets, metabolites such as deamido-BLM A_2 and deamido-BLM B_2 were detected, indicating that isolated rat hepatocytes in culture can metabolize BLM analogues to the corresponding deamido-BLMs. The high-performance liquid chromatography procedure developed during this work can be used to study the metabolism of BLM in cell-culture systems.

Introduction

Bleomycin (BLM), an antitumor antibiotic drug isolated from *Streptomyces verticillus* [32, 33], is effective against a variety of neoplasms, particularly squamous-cell carcinoma, lymphoma and testicular carcinoma [7]. Its antineoplastic activity is associated with side effects such as cutaneous reactions, neurologic and hematologic toxicities [15] and pulmonary fibrosis [10, 11], which represent the major drawback to its clinical use [5, 9]. Its antitumor and cytotoxic activities are believed to result from the effect of the drug on physical and functional DNA integrity [14, 31]. DNA binding and DNA cleaving are thought to be the two essential steps of the cytotoxic process [12, 13]. In vitro, the cytotoxicity of BLM depends on its inactivation and its uptake by the cell. The inactivation of BLM has been shown to be important in limiting its antitumor activity [1, 2, 23].

Umezawa et al. [24] have suggested the presence of an enzyme that inactivates BLM, based on the selective action of the drug against squamous-cell tumors. This enzyme, identified by these authors [35] as being aminopeptidase B-like and called BLM hydrolase, acts on the carboxamide group of the aminoalanine moiety of BLM, converting it to the corresponding deamido-BLM. Using an isocratic HPLC assay, Lazo et al. [20, 22] determined the concentrations of BLM and deamido-BLM in organs of the mouse and rabbit so as to measure the amount of BLM hydrolase as opposed to aminopeptidase B [27]. This method has been applied to the measurement of BLM and its metabolites without the determination of criteria for quality.

In the present study, we developed a procedure for the separation and quantitation of BLMs in human plasma and in rat hepatocytes and attempted to determine (a) whether BLM metabolites are present in the plasma of patients under therapeutic treatment with BLM and (b) whether isolated rat hepatocytes in culture can metabolize BLM in a manner similar to that displayed by the 105,000-*g* supernatant fractions of mouse liver and rabbit pulmonary-cell homogenates in the previous studies mentioned above.

Materials and methods

Drugs and chemicals. Blenoxane (Cu-free BLM) and copper-free peplomycin (Cu-free PEP) were obtained from Roger Bellon (Neuilly sur Seine, France). Cu-free BLM A_2 (Cu-fre BLM A_2) was supplied by the National Institute of Health of Japan. BLM B_2 (Cu-complex) and deamido-BLM B_2 were obtained from Nippon Kayaku Co, Ltd. (Japan). Dulbecco's minimum essential medium (DMEM) was used for all cell cultures; it was supplemented with 10% foetal calf serum (Gibco, BRL-France). Collagenase was obtained from Boehringer Mannheim (France) and trichloroacetic acid and methanol, from Merck (Darmstadt, FRG). Acetonitrile was of HPLC grade (Carlo-Erba, Italy). The sodium salt of pentanesulfonic acid was supplied by Sigma (St. Louis, Mo., USA). Water used for the mobile phase was purified by passage through a reversed-osmosis four-filter system (Millipore; Bedford, Mass., USA). Ammonia (28%), pure acetic acid and copper sulfate were purchased from Prolabo (Paris, France).

Offprint requests to: M. Lhermitte

Chromatographic conditions and HPLC system. The HPLC system consisted of a model 5000 liquid chromatograph (Varian, Orsay, France) equipped with a variable-volume injector, an automatic sampling system and a detector; a Jasco FP 210 spectrofluorometer was used throughout the procedure. Excitation and emission wavelengths were set at 297 and 355 nm, respectively. A 250-mm Lichrosorb RP-18 reversed-phase column (inside diameter, 10 mm; particle size, 7 µm; Merck) was used for separation. This column was protected by a 37 × 3.9-mm Corasil C18-filled guard column placed just after the injection port. Elution solvents were water (solvent A) and a mixture of methanol and acetonitrile (25/75, v/v; solvent B). Each contained 5 mM sodium pentanesulfonate as the paired ion [16] and 0.5% acetic acid. Solvent A was adjusted to pH 4.3 with ammonia (28%). The mobile phase was a linear gradient of 13%–33% solvent B in solvent A run for 20 min at a flow rate of 1.8 ml/min. The temperature of the column and the solvents was maintained at 35° C during separations.

Standard solutions in human plasma and aqueous hepatocyte suspensions. Aqueous suspensions of hepatocytes to be used as standards were prepared as follows: isolated rat hepatocytes were resuspended in water and supplemented with Cu-free BLM A_2 and Cu-complex BLM B_2. Aqueous stock solutions of Cu-free BLM A_2, BLM B_2, deamido-BLM B_2 and Cu-free PEP (internal standard) were prepared in polypropylene tubes at concentrations of 10 and 18 µg/ml, respectively. The internal-standard stock solution was diluted with water to give a final concentration of 3 µg/ml. All solutions were kept at –20° C until use.

Standard solutions of BLM A_2, BLM B_2 and deamido-BLM B_2 in human plasma and aqueous hepatocyte suspensions were prepared at concentrations of 0.125, 0.25, 0.5, 1, 2 and 3 µg/ml. The 3 µg/ml standard was prepared by the addition of 1.4 ml drug-free human plasma or drug-free aqueous hepatocyte suspension to 0.6 ml BLM stock solution (10 µg/ml). The remaining standards were prepared by stepwise dilution with drug-free human plasma or drug-free aqueous hepatocyte suspension. All calibration standards were deep-frozen and stored in small fractions at –20° C until analysis.

Pharmacokinetic study. Plasma concentrations of BLM were measured after i.v. infusion of 30 mg blenoxane over 48 h. Measurements were done at 5, 10, 30 and 60 min and at 6, 24 and 48 h. Blood samples were centrifuged at 2,000–3,000 rpm for 5 min. Aliquots of plasma were transferred to other tubes and stored at –20° C until analysis. For the assay, suitable plasma volumes were combined with the internal standard and sample preparation was carried out as indicated below.

Hepatocyte preparation. Hepatocytes were obtained from adult male Wistar rats (Iffacredo-Lyon, France) as described by Berry and Friend [6] and modified by Krack et al. [18, 19]. Rat livers were perfused in situ with 100 ml Ca-free Krebs Henseleit buffer containing 0.7 mg collagenase/ml; they were then removed from the abdomen, mechanically disrupted and centrifuged to yield intact hepatocytes. About $250–300 \times 10^6$ cells were suspended in DMEM supplemented with 10% foetal calf serum and were incubated at a final concentration of 5×10^6 cells/ml following dilution with the same medium.

Kinetic and metabolic studies. Cell cultures were performed in duplicate and incubated in medium supplemented with 5×10^{-5} M Cu-free BLM. Aliquots (1 ml) of cell suspensions were removed after 1.25, 2.5 and 3.75 h incubation. Each aliquot was centrifuged at 26 *g* and the supernatant was removed. The pellet was then rinsed with 1 ml physiological serum and recentrifuged at 26 *g*. The second supernatant was added to the first. The final pellet was resuspended in 200 µl water and stored separately with the supernatant at –20° C until analysis.

Sample preparation of human plasma and aqueous hepatocyte suspension. For sample preparation, 75 µl trichloroacetic acid (20% in water) containing 1 mM $CuSO_4$ to improve the HPLC peak resolution [24] was added to 0.3 ml of each standard of human plasma or aqueous hepatocyte suspension containing 0.9 µg internal standard. The tubes were vortexed for 1 min and centrifuged at 850 *g* for 10 min. The supernatants were transferred to another tube. The pellet was rewashed with 75 µl trichloroacetic acid and was again vortexed and centrifuged. The two supernatants were mixed, completed with water to the initial extracted volume and centrifuged; 100 µl was then injected twice into the liquid chromatograph.

Quantitation and recovery. Calibration standards in water, human plasma and aqueous hepatocyte suspension, covering the expected range of 0.125–3 µg/ml Cu-free BLM A_2, were processed. Peak area ratios of BLMs to the internal standard were calculated and the calibration curves were obtained using linear regression of the peak area ratios against the concentrations. This equation was then used to calculate the drug concentration in the unknown samples, which were combined with the internal standard (3 µg/ml) and prepared as described above. Relative recoveries were determined for two different concentrations by the comparison of preparations from human plasma and aqueous hepatocyte suspensions using a direct assay of the standards in water.

Results

High-performance liquid chromatography

Separation of three analogues, BLM A_2, BLM B_2 and PEP (Figs 1 A, 2 A), from human plasma and of two metabolites, deamido-BLM A_2 and deamido-BLM B_2, from isolated rat hepatocytes (Fig. 2 A) was achieved using a linear-gradient of 13%–33% solvent B in solvent A run over 20 min on a Lichrosorb RP18 column. No peak that could have interfered with the determination of drugs was seen (Figs. 1 B, 2 B).

Linearity, recovery and precision of the preparative procedure

All analytical criteria for the preparative procedure described above were established at two different concentrations (0.25 and 2 µg/ml) of BLM A_2 in human plasma and in aqueous hepatocyte suspension. Double precipitation of protein with trichloroacetic acid enabled purification of BLM analogues and PEP (internal standard) from each sample, resulting in a detection limit of 70 ng/ml and a good linear relationship between peak area ratios and the standard solutions. Correlation coefficients are shown in Table 1 for each compound in aqueous hepatocyte suspensions and human plasma samples. The relative recovery of BLM A_2 from each medium was about 100%, as indicated in Table 2. Within-run and day-to-day coefficients of variation for BLM A_2 were below 5.3% and 6.6% in aqueous hepatocyte suspensions and human plasma, respectively (Table 3).

Assay of BLM metabolites in hepatocyte cultures

BLM metabolites were assessed in 850-*g* supernatant fractions of precipitated pellets of isolated rat hepatocytes that had been incubated with 5×10^{-5} M Cu-free BLM for 3.75 h. Figure 2 A shows the chromatographic separation of BLM analogues and metabolites in an 850-*g* supernatant fraction of precipitated rat hepatocytes following incubation with 5×10^{-5} M Cu-free BLM for 1.25 h. At 3.75 h there was an increased concentration of metabolites (Fig. 3). Deamido-BLM A_2, BLM A_2, deamido-BLM B_2,

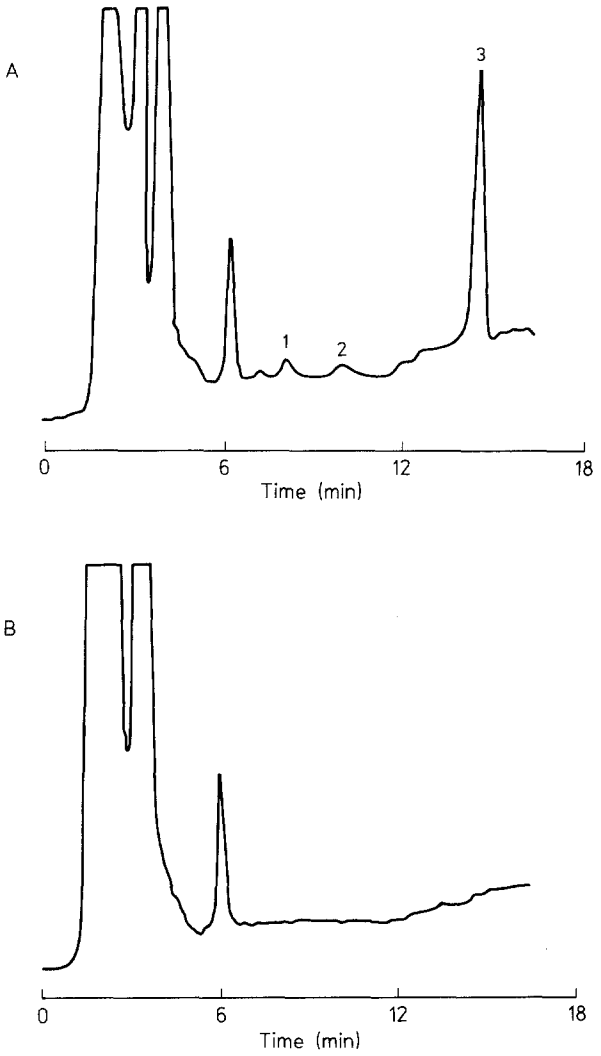

Fig. 1 A. Chromatogram of plasma (500 µl) from a patient who had received BLM 1 h previously. The plasma was combined with PEP (internal standard), mixed twice with trichloroacetic acid and centrifuged at 850 *g*. Supernatant fractions were mixed, completed to the initial volume with water and recentrifuged and 100 µl was then injected twice onto a Lichrosorb RP18 column. Chromatographic conditions are described in Materials and methods. *1*, BLM A_2 peak (0.161 µg/ml); *2*, BLM B_2 peak; *3*, PEP peak (internal standard, 3 µg/ml). **Fig. 1 B.** Chromatogram of BLM-free human plasma in which no interference can be seen

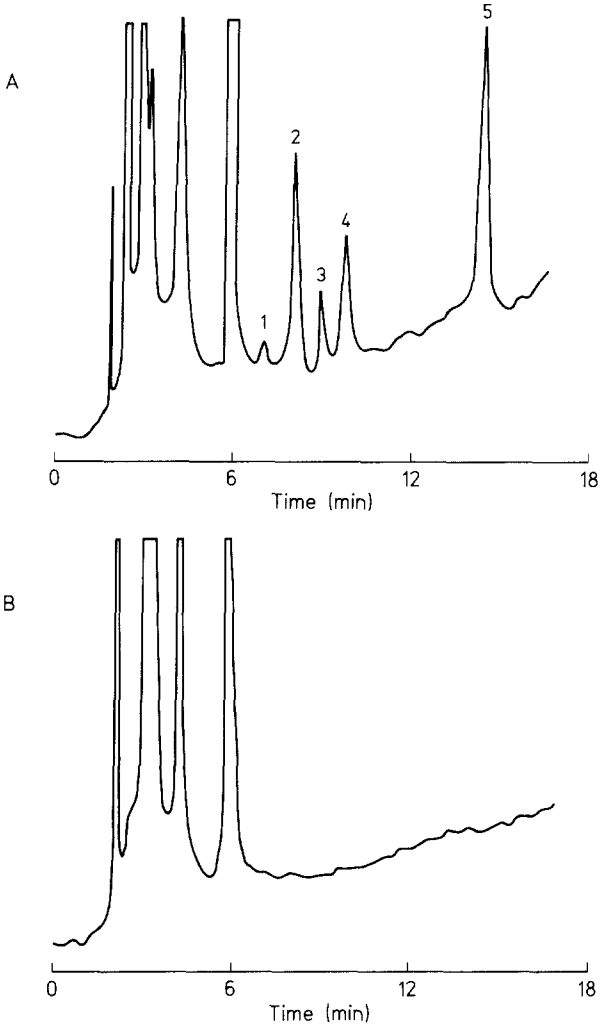

Fig. 2 A. Chromatogram of a pellet of hepatocytes that was previously incubated for 1.25 h with 5×10^{-5} M Cu-free BLM. The pellet was resuspended in water, combined with PEP (internal standard), mixed twice with trichloroacetic acid and centrifuged at 850 *g*. Supernatant fractions were mixed, completed to the initial volume with water and recentrifuged and 100 µl was then injected twice onto a Lichrosorb RP18 column. Chromatographic conditions are described in Materials and methods. *1*, Deamido-BLM A_2 peak (0.124 µg/mg); *2*, BLM A_2 peak (0.52 µg/mg); *3*, Deamido-BLM B_2 peak; *4*, BLM B_2 peak; *5* PEP peak (internal standard, 3 µg/ml). **Fig. 2 B.** Chromatogram of BLM-free hepatocytes in which no interference with co-extracted material can be seen

Table 1. Linear regression equations for BLM A_2, B_2 and deamido-BLM B_2

	BLM A_2	BLM B_2	Deamido-BLM B_2
Aqueous solution	$y = 0.36x - 0.013$ ($r = 0.999$)	$y = 2.16x + 0.043$ ($r = 0.998$)	$y = 5.99x + 0.014$ ($r = 0.999$)
Human plasma sample	$y = 0.44x - 0.010$ ($r = 0.999$)	$y = 2.60x + 0.012$ ($r = 0.997$)	$y = 7.06x + 0.042$ ($r = 0.998$)
Hepatocyte suspension sample	$y = 0.36x - 0.035$ ($r = 0.998$)	$y = 2.06x - 0.022$ ($r = 0.997$)	$y = 5.60x + 0.018$ ($r = 0.997$)

y, Peak area ratio of BLM to internal standard; x, BLM concentration

Table 2. Relative recoveries of BLM A_2

Sample	Concentration (μg/ml)	Relative recovery[a] (%)
Human plasma	0.25	96.8 ± 3.23
	2	101 ± 3.05
Hepatocyte suspension	0.25	106.4 ± 1.76
	2	99.1 ± 2.1

[a] Number of determinatons = 12

Table 3. Intra- and inter-assay coefficient of variation for BLM A_2

Sample	Concentration (μg/ml)	Within-run[a] (%)	Day-to-day[a] (%)
Human plasma	0.25	6.07	6.6
	2	5.5	4.6
Hepatocyte suspension	0.25	2.44	5.33
	2	3.18	3

[a] Number of determinations = 15

BLM B_2 and PEP were eluted at 7.1, 8.1, 9, 9.9 and 14.4 min, respectively.

HPLC assay of bleomycin A_2 and its metabolites in human plasma

Our work enables the establishment of quality-control criteria for the method and, using HPLC, we can measure the BLM A_2 level versus time in the plasma of patients undergoing i.v. treatment with blenoxane. Figure 4 shows the pattern obtained for up to 48 h following drug administration. As shown in Fig. 1, in the present experiments no peak that corresponded to BLM metabolites was seen at 1 h after drug administration.

Discussion

BLM analogues have been quantified in animal plasma [30], urine [29], organ homogenates [20–22] and aqueous solutions [26] by isocratic HPLC separation using a mobile phase containing acetonitrile with water and methanol. These procedures were time-consuming. Klett et al. [16, 17] separated BLM A_2 and BLM B_2 in aqueous solution within about 20 min using linear gradients of 28%–48% and 40%–50% solvent B (without acetonitrile) in solvent A run over 30 and 45 min through a μBondapack C18 column and a Novapack C18 column, respectively.

In the present HPLC procedure, PEP was eluted at 20 min when solvent B was used without acetonitrile; however, the addition of 75% acetonitrile in methanol enabled a rapid elution of material and all BLM analogues. BLM analogues and PEP (internal standard) from each sample were purified by a double precipitation of proteins with trichloroacetic acid [30]; this results in a detection limit of 70 ng/ml and a good linear relationship between peak area ratios and the standard solution. The detection limit reported by Shui et al. [29, 30] was 500 ng/ml for BLM B_2.

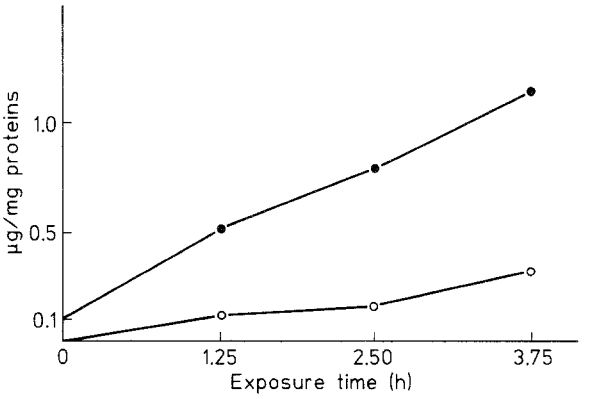

Fig. 3. Levels of BLM A_2 (—●—) and deamido-BLM A_2 (—○—) obtained after incubation of isolated rat hepatocytes with Cu-free BLM. The cells were exposed to 5×10^{-5} M Cu-free BLM for 3.75 h and samples were removed every 1.25 h. Extraction, separation and quantitation procedures are described in Materials and methods

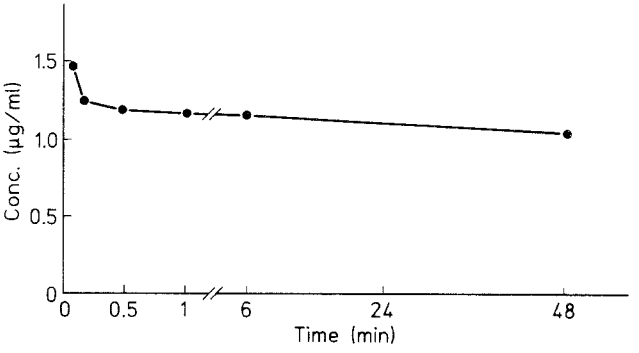

Fig. 4. BLM A_2 concentrations (*Conc.*) versus time in plasma obtained from a patient who had been treated with 30 mg blenoxane

Our procedure enables the quantitation of BLM analogues and their metabolites in rat hepatocytes. Like mouse liver [34] and rabbit pulmonary cells in culture [35], rat hepatocytes could metabolize BLM A_2 and BLM B_2 to the corresponding deamido-BLMs, indicating the likely presence of BLM hydrolase which is in agreement with the hypothesis of Umezawa et al. [34, 35]. The rate of metabolism of Cu-free BLM A_2 to deamido-BLM A_2 appeared to be proportional to the duration of exposure of cells to the drug. The concentration of deamido-BLM A_2 in the cells was enhanced with time, as was the level of Cu-fre BLM A_2.

The present method also enables the detection of BLM A_2 and its metabolite in human plasma. All quantitative analyses of BLM analogues in the plasma of adults or children have been done using radioimmunoassay [3, 8, 28, 36], a technique that cannot detect BLM analogues or metabolites with a sufficient degree of specificity. HPLC is used particularly for the assay of BLM analogues in pharmaceutical preparations [4, 17]. All techniques applied in these determinations were time-consuming and used an ultraviolet detection method that was not sensitive and not specific; moreover, quality-control criteria were not reported.

For our technique, we chose fluorescence detection, which is sufficiently sensitive and specific. We used PEP as the internal standard. This BLM analogue is not present in the pharmaceutical preparation and possesses a structure and absorption spectrum that is close to those of other BLM analogues. Shui et al. [30] and Shui and Goehl [29] have used BLM B_2, which is present in the pharmaceutical preparation, as the internal standard for the quantitation of BLM A_2 in the plasma and urine of rabbits. We could measure the level of BLM A_2 versus time in the plasma of patients receiving intravenous infusions of blenoxane; our results agreed with those obtained by Paladine et al. [25] and Alberts et al. [3]. No peak corresponding to BLM metabolites was seen at 1 h after drug administration.

The presence of such metabolites is likely, since liver, spleen, kidneys and pulmonary tissues from patients have been shown to metabolize this antibiotic in vitro [27]. However, it may be preferable to look for BLM metabolites in urine rather than in plasma, because the amount of drug in the urine may increase with time. More adequate extraction and separation procedures should be developed for the detection of these metabolites.

References

1. Akiyama SI, Kuwano M (1981) Isolation and preliminary characterization of bleomycin-resistant mutants from Chinese hamster ovary cells. J Cell Physiol 107: 147
2. Akiyama SI, Ikezaki K, Kuramochi H, Takahashi K, Kuwano M (1981) Bleomycin-resistant cells contain increased bleomycin-hydrolase activities. Biochem Biophys Res Commun 101: 55
3. Alberts DS, Chen HSG, Mayersohn M, Perrier D, Moon TE, Gross JF (1979) Bleomycin pharmacokinetics in man: II. intracavitary administration. Cancer Chemother Pharmacol 2: 127
4. Aszalos A, Crawford J, Vollmer P, Kantor N, Alexander T (1981) High-performance liquid chromatographic determination of components of bleomycin. J Pharm Sci 70: 878
5. Bedrossian CWM, Luna MA, Mackay B, Lichtiger B (1973) Ultrastructure of bleomycin toxicity. Cancer 32: 44
6. Berry MN, Friend DS (1969) High-yield preparation of isolated rat liver parenchymal cells. A biochemical and fine structure study. J Cell Biol 43: 506
7. Blum RH, Carter SK, Agre K (1973) A clinical review of bleomycin: a new antineoplastic agent. Cancer 31: 903
8. Broughton A, Strong JE (1976) Radioimmunoassay of bleomycin. Cancer Res 36: 1418
9. Comis RL, Kuppinger MS, Ginsberg SJ, Crooke ST, Gilbert R, Auchincloss JH, Prestayko AW (1979) Role of single-breath carbon monoxide-diffusing capacity in monitoring the pulmonary effects of bleomycin in germ cell tumor patients. Cancer Res 39: 5076
10. Crooke ST, Bradner WT (1977) Bleomycin, a review. J Med 7: 373
11. De Lena M, Guzzon A, Monfardini S, Bonadonna G (1972) Clinical radiological and histopathological studies on pulmonary toxicity induced by treatment with bleomycin (NSC-125 066). Cancer Treat Rep 56: 343
12. Henichart JP, Bernier JL, Helbecque N, Houssin R (1985) Is the bithiazole moiety of bleomycin a classical intercalator? Nucleic Acids Res 13: 6703
13. Henichart JP, Bernier JL, Houssin R, Lohez M, Kenani A, Catteau JP (1985) Copper(II)- and iron(II)-complexes of methyl 2-(2-aminoethyl)-aminomethyl-pyridine-6-carboxyl histidinate (AMP His), a peptide mimicking the metal-chelating moiety of bleomycin. An ESR investigation. Biochem Biophys Res Commun 126: 1036
14. Iqbal ZM, Kohn KW, Ewig RAG, Fornace AJ (1976) Single strand scission and repair of DNA in mammalian cells by bleomycin. Cancer Res 36: 3834
15. Israel L, Breau JL, Morere JF, Aquielera J, Khon M (1986) Induction de réponses objectives par une chimiothérapie anticancéreuse fondée sur l'association prolongée de bléomycine et de cisplatine. Presse Med 25: 1183
16. Klett RP, Chovan JP (1985) Modification of a new high-performance liquid chromatography method for bleomycin to separate epi-, iso-, desamido and unmodified analogues. J Chromatogr 337: 182
17. Klett RP, Chovan JP, Raisfeld-Danse IH (1984) Reversed-phase paired-ion high-performance liquid chromatographic method for the separation and quantification of multiple bleomycin congeners. J Chromatogr 310: 361
18. Krack G, Goethals F, Deboyser D, Roberfroid M (1980) Interference of chemicals with glycogen metabolism in isolated hepatocytes. Toxicology 18: 213
19. Krack G, Gravier O, Roberfroid M, Mercier M (1980) Subcellular fractionation of isolated rat hepatocytes. A comparison with liver homogenate. Biochim Biophys Acta 632: 619
20. Lazo JS, Humphreys CJ (1983) Lack of metabolism as the biochemical basis of bleomycin-induced pulmonary toxicity. Proc Natl Acad Sci USA 80: 3064
21. Lazo JS, Boland CJ, Schwartz PE (1982) Bleomycin hydrolase activity and cytotoxicity in human tumors. Cancer Res 42: 4026
22. Lazo JS, Merill WW, Pham ET, Lynch TJ, McCallister JD, Ingbar DH (1984) Bleomycin hydrolase activity in pulmonary cells. J Pharmacol Exp Ther 231: 583
23. Miyaki M, Ono T, Hori S, Umezawa H (1975) Binding of bleomycin to DNA in bleomycin-sensitive and bleomycin-resistant rat ascites hepatoma cells. Cancer Res 35: 2015
24. Muraoka Y (1979) Liquid chromatography of bleomycin. In: Hecht SM (ed) Bleomycin: chemical, biochemical and biological aspects. Springer, New York Berlin Heidelberg, p 92
25. Paladine W, Cunningham TJ, Sponzo R, Donavan M, Olson K, Horton S (1976) Intracavitary bleomycin in the management of malignant effusion. Cancer 38: 1903
26. Sakai TT (1978) Paired-ion high-performance liquid chromatography of bleomycins. J Chromatogr 161: 389
27. Sebti SM, Lazo JS (1987) Separation of the protective enzyme bleomycin hydrolase from rabbit pulmonary aminopeptidases. Biochemistry 26: 432
28. Seki T, Muraoka Y, Takahashi K, Horinishi H, Umezawa H (1985) Radioimmunoassay of bleomycins. J Antibiot 38: 1251
29. Shiu GK, Goehl TJ (1980) High-performance paired-ion liquid chromatography determination of bleomycin A_2 in urine. J Chromatogr 181: 127
30. Shiu GK, Goehl TJ, Pitlick WH (1979) Rapid high-performance liquid chromatographic determination of bleomycin A_2 in plasma. J Pharm Sci 68: 232
31. Suzuki H, Nagai K, Yamaki H, Tanaka N, Umezawa H (1969) On the mechanism of action of bleomycin-scission of DNA strands in vitro and in vivo. J Antibiot 22: 446
32. Umezawa H, Maeda K, Takeuchi T, Okami Y (1966) New antibiotics, bleomycins A and B. J Antibiot 19: 200
33. Umezawa H, Suhara Y, Takita T, Maeda K (1966) Purification of bleomycins. J Antibiot 19: 210
34. Umezawa H, Takeuchi T, Hori S, Sawa T, Ishizuka M, Ichikawa T, Komai T (1972) Studies on the mechanism of the anti-tumor effect of bleomycin on squamous cell carcinoma. J Antibiot 25: 409
35. Umezawa H, Hori S, Sawa T, Yoshioka T, Takeuchi T (1974) A bleomycin-inactivating enzyme in mouse liver. J Antibiot 27: 419
36. Yee GC, Crom WR, Brodeur GM, Champion JE, Gutierrez ML, Lee FH, Evans WE (1983) Bleomycin disposition in children with cancer. Clin Pharmacol Ther 33: 668