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Note

High-performance paired-ion liquid chromatographic determination of bleomycin A_2 in urine

GERALD K. SHIU* and THOMAS J. GOEHL**

Department of Medicinal Chemistry, University of Pittsburgh, Pittsburgh, Pa. 15261 (U.S.A.)

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The antineoplastic antibiotic agent bleomycin was isolated from fermentation products of *Streptomyces verticillus* [1]. It has been shown to be effective against a variety of human neoplasms, particularly squamous cell carcinoma, lymphoma and testicular carcinoma [2—5]. The most significant value for this agent over other antineoplastic agents is a lack of bone marrow toxicity [6—8].

Various analytical methods have been used to assay a bleomycin mixture in biological fluids [2, 9—14]. However, all of these methods do not distinguish between the various components of the bleomycin mixture or their metabolites.

We recently reported a rapid method for the specific determination of bleomycin A_2 in plasma, using high-performance liquid chromatography (HPLC) with the paired-ion chromatographic technique [15]. In that method, trichloroacetic acid (TCA) solution was added to precipitate the plasma proteins prior to employing the chromatographic system. Since the strong interfering constituents present in urine are not protein in nature, the TCA precipitation is neither needed nor effective. A different pre-assay purification procedure was necessary and was developed. The method utilized a cartridge with reversed-phase packing as used in the analytical column to separate bleomycin from urine before chromatography.

* To whom correspondence should be addressed.
** Present address: Division of Biopharmaceutics, Food and Drug Administration, Washington, D.C. 20204, U.S.A.

EXPERIMENTAL

Reagents and materials

Bleomycin A_2, bleomycin B_2-Cu (internal standard) and bleomycin mixture (Blenoxane®) were generously supplied by Bristol Laboratories (Syracuse, N.Y., U.S.A.). The sodium heptanesulfonate was obtained from Eastman Kodak Company (Rochester, N.Y., U.S.A.). Solvents were of HPLC grade. All other materials were reagent grade.

Stock solutions of bleomycin A_2 and bleomycin B_2-Cu were prepared by dissolving 10 mg of samples in 10 ml of deionized water. Urine standards were prepared by spiking control urine with appropriate small volumes of stock solutions.

Apparatus

A modular high-performance liquid chromatograph consisting of a constant-flow pump (Model M-6000A), a loop-type injector (Model U6K), a stainless-steel reversed-phase column (μBondapak C_{18}, 30 cm × 3.9 mm I.D., 10 μm particle size) and UV detector (Model 440) was purchased from Waters Assoc. (Milford, Mass., U.S.A.). The output from the detector was connected to a strip-chart recorder (Model 255, Linear Instruments, Irvine, Calif., U.S.A.). A guard column (3.9 mm × 6 cm) filled with pellicular reversed-phase packing (CO:Pell ODS) was obtained from Whatman (Clifton, N.J., U.S.A.). The cartridge (SEP-PAK C_{18} TM) which was used to separate bleomycin from urine was supplied by Waters Assoc.

Chromatographic conditions

The HPLC mobile phase was methanol—acetonitrile—0.0085 *M* sodium heptanesulfonate—acetic acid (30:10:59:1). A flow-rate of 2.0 ml/min was established. The resulting pressure was approximately 2500 p.s.i.

Assay procedure

To 1.0 ml of urine in a 12-ml conical centrifuge tube were added 40 μg of internal standard (40 μl of a 1.0 mg/ml aqueous bleomycin B_2-Cu solution). The samples were mixed and then 0.2 ml of the mixture was passed through a SEP-PAK C_{18} cartridge, which was prewashed with methanol and water. After washing with 2 ml of each of water, acetone, water and methanol, 2 ml of 0.02 *M* sodium heptanesulfonate in methanol solution were used to elute the cartridge. The effluent was collected and vortexed. Fifty microliters of the effluent were injected into the column.

The concentrations of bleomycin A_2 were determined from standard curves prepared by plotting the peak height ratios of bleomycin A_2 and internal standard against the spiked concentrations.

Recovery

The recovery of bleomycin from urine was determined by comparing the peak heights resulting from spiked urine samples and the peak heights obtained from direct injection of same amount of drug in aqueous solution.

Validation of the assay method

Aliquots of control urine were spiked with known amounts of bleomycin A_2 to give several concentrations. The samples were assayed in triplicate as previously described. The coefficient of variation (C.V.) and relative error for the assay were calculated.

Rabbit urine sample

Four female New Zealand White rabbits received 1—2 mg of bleomycin A_2 per kg in the marginal ear vein. After medication, the rabbits were placed in separate metabolic cages which were located in a quiet place. A regular diet and drinking water were supplied. The urine was collected through a funnel into a 250-ml Erlenmeyer flask which was surrounded with dry ice in an insulated foam box. After collecting the urine for 24 h, it was thawed at room temperature and then filtered. The filtrate volume was determined and mixed well. Aliquots of the 24-h urine were assayed as previously described. The percentage of bleomycin A_2 excreted unchanged in 24-h urine was calculated.

RESULTS AND DISCUSSION

In previous reported work on the HPLC determination of bleomycin A_2 in plasma, the interfering plasma proteins were removed by TCA precipitation prior to chromatography [15]. Since the strongly interfering constituents present in urine are not protein in nature, the TCA step was found not to be effective. In addition, because of its extreme hydrophilic property, bleomycin could rarely be extracted from urine. The use of the cartridge which was packed with similar packing materials to that present in the analytical column was found to be the most appropriate approach to separating bleomycin before assay.

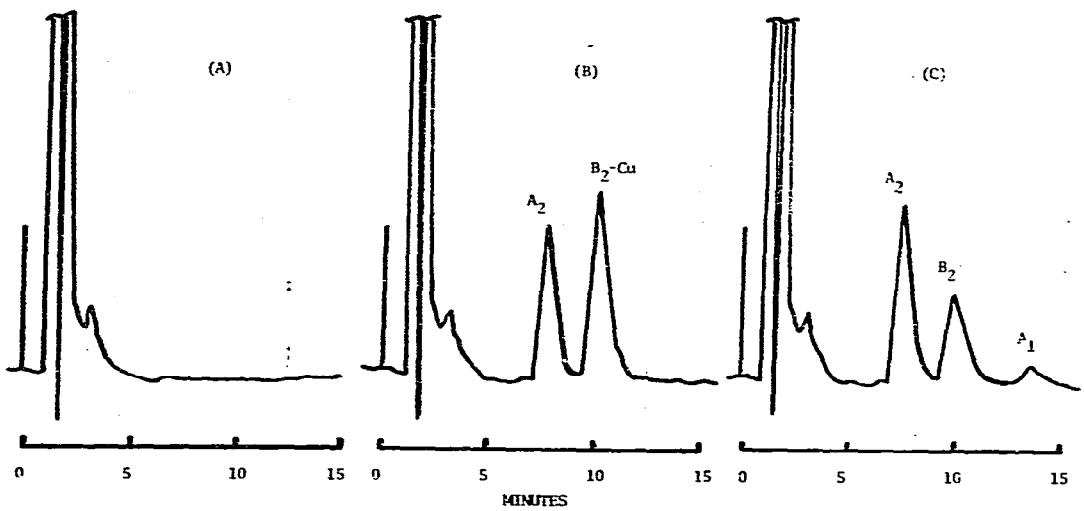

Fig. 1. Chromatograms obtained from HP-PIC assay of (A) control rabbit urine, (B) urine from a rabbit 24 h after receiving a single dose of bleomycin A_2. Peak for bleomycin A_2 was found to correspond to a concentration of 30 μg/ml. Peak for internal standard bleomycin B_2-Cu was 40 μg/ml. (C) Urine from a rabbit 24 h after receiving a single intravenous dose of bleomycin mixture 4 units/kg.

TABLE I

PRECISION AND ACCURACY FOR ASSAY OF BLEOMYCIN A_2 IN URINE

Actual concentration (μg/ml)	Experimental concentration [μg/ml; mean* (range)]	Coefficient of variation	Relative error (%)
Control	–	–	–
8.00	8.10 (7.19—9.54)	15.5	12.0
16.0	15.4 (14.1—17.1)	10.0	7.10
28.0	29.5 (28.3—31.0)	4.70	3.70
40.0	40.5 (40.0—41.0)	1.30	1.00
50.0	49.0 (46.7—52.0)	5.60	4.10
	Overall	(7.40)	(5.60)

* $n = 4$.

In a preliminary study, bleomycin was found to be adsorbed onto the cartridge from aqueous solution and could not be eluted from the cartridge by using either water or organic solvents such as methanol, acetonitrile, acetone or ethyl acetate. However, it could be easily eluted by small volumes of 0.02 *M* sodium heptanesulfonate in methanol solution. Though there is no definitive explanation to this adsorption phenomenon, most of the bleomycin was retained and all of the interfering substances were removed with the described procedures.

Chromatograms obtained from the analysis of control rabbit urine and urine containing bleomycin A_2 and bleomycin mixture are shown in Fig. 1. The chromatogram of control urine showed no interfering peaks. Under the assay conditions, bleomycin A_2 and bleomycin B_2-Cu had retention times of 7.8 and 10.2 min, respectively. The retention time of bleomycin B_2 is found to be identical to that of bleomycin B_2-Cu.

Excellent linearity (r>0.990) was observed for the standard curve over the 5—50 μg/ml range. The precision and accuracy for the assay of bleomycin A_2 are reported in Table I. The coefficient of variation (C.V.) ranged from 1.3 to 15.5%, with an average of 7.4%. The mean relative error was 5.6%.

The recovery of bleomycin A_2 from urine after a primary separation using new cartridges was found to be 60%. However, the recovery could be improved

TABLE II

PERCENTAGE OF BLEOMYCIN A_2 EXCRETED UNCHANGED IN RABBIT URINE 24 h AFTER SINGLE-DOSE INTRAVENOUS ADMINISTRATION OF BLEOMYCIN A_2

Rabbit No.	Dose (mg/kg)	Percentage of bleomycin A_2 excreted unchanged
1	1.0	50.5
2	1.0	54.8
3	2.0	56.6
4	2.0	67.0
	Mean ± S.D.	57.2 ± 7.0

by conditioning the cartridges with an ion-pairing reagent, 0.02 *M* sodium heptanesulfonate in methanol. In attempting to condition the cartridge, 2 ml of aliquots were passed through the cartridge followed by washing with 10 ml of methanol and 10 ml of water. This procedure was undertaken repeatedly. The recovery was found to be as high as 85% after the cartridge was conditioned with more than 60 ml of the methanolic ion-pairing reagent.

To demonstrate the utility of this method as an appropriate procedure for the determination of bleomycin A_2 in urine, urine obtained from rabbits that had received a single intravenous dose of the drug were assayed (Table II). The percentage of the bleomycin A_2 excreted unchanged in 24-h urine was calculated.

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