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Short Communication

Determination of mitomycin C in human aqueous humor and serum by high-performance liquid chromatography

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ABSTRACT

Mitomycin C (MMC) is used in the treatment of disseminated adenocarcinoma of the stomach and pancreas and is used in ophthalmology as adjunctive therapy in trabeculectomy. Since only small volumes of aqueous humor are available for analysis, a sensitive method requiring limited sample preparation was developed. An internal standard, 4-aminoacetophenone, was added to aqueous humor specimens, and the solution was directly injected into the high-performance liquid chromatographic (HPLC) column. The use of a short 50-mm C_{18} reversed-phase column gave adequate resolution of peaks with improved sensitivity. The method was applicable for determination of MMC in serum, although solid-phase extraction for sample clean-up was required prior to injection into the HPLC column, and analytical columns of 150–250 mm were necessary for adequate resolution of peaks. The method has been validated and is linear from 6.25 to 50 ng/ml in aqueous humor and from 10 to 500 ng/ml in serum.

INTRODUCTION

Mitomycin C (MMC) is one of several antibiotic substances produced by *Streptomyces caespitosus* possessing antineoplastic (antimetabolite) activity. It is currently used in the palliative treatment of disseminated adenocarcinoma of the stomach and pancreas that is unresponsive to other treatment modalities [1]. In ophthalmology, MMC has been used as adjunctive therapy in trabeculectomy in glaucoma patients [2–4]. Its use in filtration surgery has shown enhanced success rate resulting in lower intraocular pressure (IOP). The safety of MMC in filtration surgery

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has not been clearly established since MMC aqueous humor concentrations following various modes of administration have not been determined.

MMC has previously been determined in biological samples using several methods [5]. Of these, HPLC appeared to be most suitable for pharmacokinetic studies and monitoring of MMC levels. Previously reported methods, however, either did not utilize an internal standard [6–8] or used a relatively uncommon material, porfiromycin, as the internal standard [9,10]. Futhermore, very low MMC aqueous humor levels were predicted following the mode of application in trabeculectomy, and only a limited volume of aqueous humor (50–100 μl) could be obtained during the surgical procedure. This necessitated the development of a specific, sensitive and reliable method for monitoring MMC concentrations in aqueous humor. This paper describes an HPLC method for the determination of MMC in aqueous humor as well as in serum using 4-aminoacetophenone as an internal standard.

EXPERIMENTAL

Materials and reagents

MMC and human serum were purchased from Sigma (St. Louis, MO, USA). The internal standard, 4-aminoacetophenone, was obtained from Aldrich (Milwaukee, WI, USA). Reversed-phase solid-phase extraction columns (C_{18}, 500 mg) were obtained from Baxter (Muskegon, MI, USA). Ammonium phosphate, monobasic, was obtained from Mallinckrodt (Paris, KY, USA) and HPLC-grade methanol from EM Science (Gibbstown, NJ, USA).

Instrumentation

The HPLC system consisted of a Perkin Elmer Series 410 LC solvent delivery pump, ISS-100 autosampler, LC 90 UV spectrophotometric detector, and LCI-100 laboratory computing integrator. MMC from aqueous humor was separated on a 50 mm × 4.6 mm I.D., 3 μm particle size, C_{18} analytical column (Short-One, Microsorb C_{18}, Rainin, Emeryville, CA, USA), preceded by a C_{18} guard column (Microsorb 80-200-G3, Rainin). MMC from serum specimens was determined using a 150 mm × 4.6 mm I.D., 5 μm particle size, C_{18} analytical column preceded by a Microsorb C_{18} guard column (Rainin). The absorbance of the effluent was monitored using a variable-wavelength UV detector at 365 nm.

Mobile phase

The mobile phase consisted of methanol–water (28:72) containing 10 m*M* monobasic ammonium phosphate adjusted to pH 7.0 with dilute ammonium hydroxide. The mobile phase was filtered through a Nylon 66 membrane filter (Sartorius, Gottingen, Germany) and deaerated with helium gas. Samples were eluted isocratically at a mobile phase flow-rate of 1.5 ml/min and a pressure of 190 bar.

Preparation of standard solutions

A stock solution of MMC was prepared by dissolving an accurately weighed amount of MMC in methanol. Appropriate dilutions of this solution were made with methanol to give a final concentration of 12.5 μg/ml. This solution was stored at −70°C. Standard solutions of MMC were prepared by dilution of the stock solution with water to give final MMC concentrations of 5.0, 10.0, 25.0 and 50.0 ng/ml. In addition, standard solutions of MMC in human serum were prepared by spiking serum with the appropriate volume of stock solution giving final MMC serum concentrations of 10.0, 25.0, 50.0, 125.0, 250.0 and 500.0 ng/ml. The internal standard solution was prepared by dissolving 10 mg of 4-aminoacetophenone in 10 ml of methanol. This solution was diluted with water to give a final internal standard solution concentration of 5 μg/ml.

Sample preparation

Aqueous humor samples and MMC standard solutions in water. To 100 μl of aqueous humor or MMC standard solutions in water (5.0, 10.0, 25.0, 50.0 ng/ml) were added 25 μl of internal standard solution (4-aminoacetophenone, 5 μg/

ml). The solution was vortex-mixed, then 100 μl of solution were injected into the HPLC column.

Serum specimens and MMC standards in serum. To 500 μl of serum were added 100 μl of internal standard solution. The solution was mixed, then passed through a preconditioned C_{18} reversed-phase solid-phase extraction column. The extraction columns were preconditioned by passing through one cartridge volume of methanol followed by two cartridge volumes of water. After the serum sample was passed through the column, the column was washed with two 0.5-ml volumes of water. MMC and internal standard were eluted from the column using 3 × 0.5 ml of methanol. The three methanol eluents were combined and evaporated to dryness in a water bath (40–50°C) under a stream of dry, filtered air. The residue was reconstituted in 150 μl of water, centrifuged at 13 700 *g* for 5 min, and 50 μl of the supernatent was injected into the HPLC column.

Determination of standard curve and assay validation

A standard curve of the peak-height ratio of MMC to internal standard *versus* MMC concentration was plotted, and the equation of the line was determined using least-squares regression. Assay reproducibility was determined at four different concentrations in water and five concentrations in serum measured on five consecutive days. Minimum assay sensitivity is defined as the amount of MMC giving a peak height greater than four times the noise level. Between-run and within-run precision were determined at three MMC serum concentrations (25.0, 250.0 and 500.0 ng/ml).

Recovery and stability of MMC

Solid-phase extraction efficiency was determined by comparing the peak heights before and after passage through the extraction column of six standards ranging in concentration from 10.0 to 500.0 ng/ml MMC and containing 833 ng/ml 4-aminoacetophenone. The stability of aqueous solutions of MMC at concentrations ranging from 50.0 to 500.0 ng/ml was determined at −20°C, 20°C, and 50°C to provide information regarding conditions for storage of standard solutions and biological specimens.

RESULTS AND DISCUSSION

Fig. 1A and B show a typical HPLC pattern of MMC and internal standard from aqueous humor and from serum containing 50 ng/ml MMC. The retention times for MMC and internal standard from aqueous humor were 2.7 and 2.3 min, respectively, using a 50-mm column, and from serum 7.5 and 9.0 min, respectively, using a 150-mm column.

Previously reported HPLC methods for determination of MMC from biological specimens using liquid- or solid-phase extraction did not show interfering peaks at an analytical wavelength of 365 nm [5]. These methods did not utilize an in-

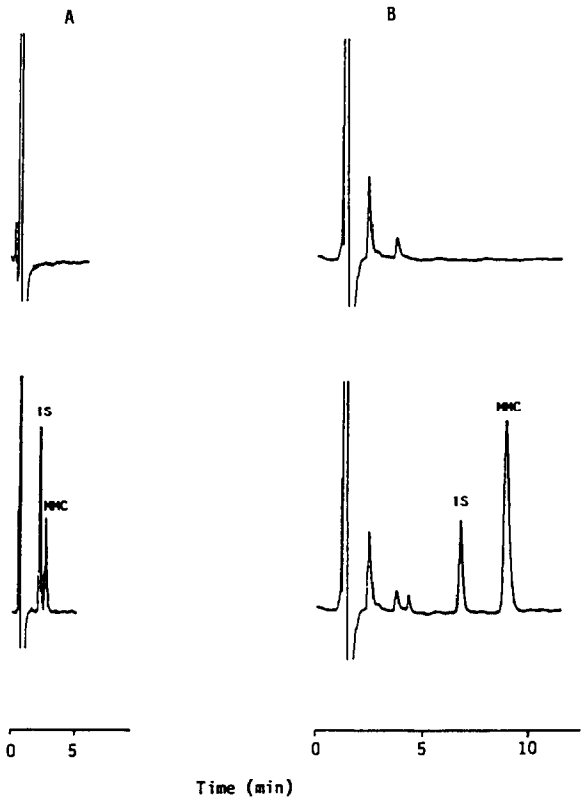

Fig. 1. Typical HPLC profile of mitomycin C in (A) aqueous humor spiked with 50 ng/ml MMC and 1.25 μg/ml internal standard (IS) (lower), compared to blank aqueous humor (upper), and (B) human serum spiked with 250 ng/ml MMC and 1.0 μg/ml IS (lower), compared to blank human serum (upper).

ternal standard. Other methods have used porfiromycin as an internal standard but it is not a commonly available substance. As an alternative, 4-aminoacetophenone was found to be a suitable internal standard, although its wavelength of maximum absorbance occurs at 310 nm.

Satisfactory resolution of peaks could be obtained using C_{18} columns ranging in length from 50 to 250 mm. Shorter columns gave improved peak shape with a reduction in retention time. Since only a limited volume (50–200 μl) of aqueous humor can be obtained during surgical procedures for analysis of MMC, methods requiring a minimum of sample preparation were developed. Blank aqueous humor showed no interference in the region of the MMC and internal standard peaks when injected directly into the HPLC column (Fig. 1A). Thus, aqueous humor containing MMC and internal standard could be injected directly into the HPLC system without pretreatment resulting in complete resolution of the peaks. The use of a short 50-mm column resulted in improved assay sensitivity, with a minimum detectable amount of MMC of 0.5 ng, whereas a 250-mm column resulted in a minimum detectable amount of 5 ng, primarily due to peak broadening. Total assay cycle time per injection, including a pre-injection washout, was only 5 min.

Serum samples, however, required a clean-up procedure using solid-phase extraction and concentration prior to injection into the HPLC column. A 150-mm C_{18} column gave satisfactory resolution of MMC and internal standard from interference due to matrix effects. The minimum detectable MMC concentration in serum was 10 ng/ml. Total assay cycle time per injection was 15–18 min.

Assay variability was determined by assaying four MMC concentrations in water and six in serum on five separate days. Results of this study are presented in Table I. Because of the limited availability of aqueous humor for the preparation of standard curves, solutions of MMC in water were used to prepare a standard curve for the determination of MMC in aqueous humor. Table II shows a comparison of peak-height ratios of MMC at 10 and 50 ng/ml in water and in aqueous humor. The slopes and y-intercepts for MMC in water and aqueous humor did not differ substantially. Therefore, a standard curve generated from standard solutions of MMC in water could be used for the determination of MMC in aqueous humor. The standard curve for MMC in water was linear from 6.25 to 50.0 ng/ml with a slope of $9.88 \cdot 10^{-3}$, a y-intercept of $3.00 \cdot 10^{-3}$, and a correlation coefficient of 0.999. The standard curve for MMC in serum was linear from 10 to 500 ng/ml with a slope of $9.51 \cdot 10^{-3}$, a y-

TABLE I

DAY-TO-DAY VARIABILITY OF THE MITOMYCIN C ASSAY AT FOUR CONCENTRATIONS MEASURED ON FIVE SEPARATE DAYS

Mitomycin C concentration (ng/ml)		C.V. (%)
Actual	Found	
Water		
6.25	6.0 ± 0.5	8.1
12.5	12.2 ± 0.8	6.1
25.0	25.7 ± 1.4	5.3
50.0	49.5 ± 0.7	1.4
Serum		
10.0	10.7 ± 0.8	7.7
25.0	25.3 ± 0.7	2.6
50.0	50.4 ± 1.5	3.0
125.0	127.7 ± 4.3	3.3
250.0	249.0 ± 6.4	2.5
500.0	502.3 ± 8.3	1.6

TABLE II

COMPARISON OF PEAK-HEIGHT RATIOS OF MITOMYCIN C IN AQUEOUS HUMOR AND WATER

MMC concentration (ng/ml)	Peak-height ratio	
	Aqueous humor	Water
10	0.105	0.103
50	0.501	0.498
Slope	$9.90 \cdot 10^{-3}$	$9.88 \cdot 10^{-3}$
y-Intercept	$6.0 \cdot 10^{-3}$	$4.25 \cdot 10^{-3}$

TABLE III

DETERMINATION OF BETWEEN-RUN PRECISION FOR MITOMYCIN C ASSAY IN SERUM

Run No.	Concentration (ng/ml)		
	25.0 ng/ml	250.0 ng/ml	500.0 ng/ml
1	26.3	258.0	535.1
2	26.7	247.0	508.2
3	22.6	265.1	490.0
4	25.3	248.0	521.2
5	28.6	263.0	508.7
Mean ± S.D.	25.9 ± 2.2	256.2 ± 8.4	512.6 ± 16.8
C.V. (%)	8.5	3.3	3.3
Error (%)	3.6	2.5	2.5

intercept of $1.21 \cdot 10^{-3}$, and a correlation coefficient of 1.000.

Between-run and within-run precision were determined at three selected serum concentrations, 25.0, 250.0 and 500.0 ng/ml, reflecting typical low, medium and high concentrations for the assay. The results of the between-run and within-run validations are shown in Tables III and IV. The mean (± S.D.) found concentration of five assays for the 25.0 ng/ml sample was 25.9 ± 2.2 ng/ml, with a C.V. of 8.5% and an error of 3.6%. A mean (± S.D.) found concentration of 256.2 ± 8.4 ng/ml was obtained for the 250.0 ng/ml sample, with a C.V. of 3.3% and an error of 2.5%; for the 500.0 ng/ml sample, the mean (± S.D.) found concentration was 512.6 ± 16.8 ng/ml, with a C.V. of 3.3% and an error of 2.5%.

TABLE IV

DETERMINATION OF WITHIN-RUN PRECISION FOR MITOMYCIN C ASSAY IN SERUM

Sample No.	Concentration (ng/ml)		
	25.0 ng/ml	250.0 ng/ml	500.0 ng/ml
1	27.2	243.5	504.3
2	25.5	253.7	536.2
3	27.1	247.4	514.8
4	25.7	247.4	479.8
5	25.9	236.9	533.9
Mean ± S.D.	26.3 ± 0.8	245.8 ± 6.2	513.8 ± 23.2
C.V. (%)	3.1	2.5	4.5
Error (%)	5.2	1.7	2.8

Similarly, the within-run precision was determined at MMC concentrations of 25.0, 250.0 and 500.0 ng/ml. The mean (± S.D.) found concentrations for the within-run validation were 26.3 ± 0.8, 245.8 ± 6.2 and 513.8 ± 23.5 ng/ml, respectively, with C.V. values below 4.5% and errors under 5.2%. The accuracy of the method was very good even at low concentrations.

The mean extraction efficiency using C_{18} solid-phase extraction columns was determined at six different MMC concentrations, ranging from 10.0 to 500.0 ng/ml, and with a concentration of 4-aminoacetophenone of 833 ng/ml. The mean (± S.D.) recovery for MMC for all concentrations tested was 73.7 ± 4.9% (range 71.9–76.1%), and for the internal standard 74.2 ± 2.5% (range 71.4–77.8%). In comparison, a recovery of 68–71% was previously reported using solvent extraction [9]. Although recovery was equally consistent using liquid-phase extraction, solid-phase extraction proved to be more efficient and rapid.

The stability of aqueous solutions of MMC at concentrations of 50 and 500 ng/ml was determined at −20°C, 20°C, and 50°C. Over a six-day period, MMC concentrations decreased 5 and 35% in samples stored at 20 and 50°C, respectively. However, no significant change in concentration was observed in samples stored at −20°C. In methanolic solution, no degradation was observed over a three-month period in solutions stored at −70°C. These findings suggest that specimens should be assayed as rapidly as possible after collection, and aqueous standards should be freshly prepared.

Twenty-eight patient aqueous humor specimens were assayed for MMC. Of these, fifteen specimens contained MMC levels above the minimum detectable level for the method. One of the specimens had an MMC level of 120 ng/ml, and the remaining fourteen specimens ranged from 66.1 to 4.9 ng/ml.

The method proved to be highly sensitive and reliable, and required a minimum of sample handling. The method is applicable for the determination of MMC in aqueous humor or serum.

REFERENCES

1 S. T. Crooke and W. T. Bradner, *Cancer Treat. Rev.*, 3 (1976) 121–139.
2 S. S. Palmer, *Ophthalmology*, 98 (1991) 317–321.
3 M. R. Wilson, D. A. Lee and R. S. Baker, *J. Ocul. Pharmacol.*, 7 (1991) 1–8.
4 K. Kawase, H. Matsushita, T. Yamamoto and Y. Kitazawa, *Ophthalmology*, 99 (1992) 203–207.
5 J. H. Beijnen, *Anal. Profiles Drug Subst.*, 16 (1987) 361–401.
6 K. Kono, Y. Hara, S. Eguchi and M. Tanaka, *J. Chromatogr.*, 164 (1979) 404–406.
7 R. G. Buice, P. Sidhu, B. J. Gurley and H. B. Niell, *Ther. Drug Monit.*, 6 (1984) 113–115.
8 S. C. Hopkins, R. G. Buice, R. Matheny and M. S. Soloway, *Cancer*, 53 (1984) 2063–2068.
9 A. K. Matra and A. T. Mailloux, *Int. J. Pharm.*, 32 (1986) 133–141.
10 K. E. Choi, J. A. Sinkule, W. R. Crom, E. I. Thompson and W. E. Evans, *J. Chromatogr.*, 345 (1985) 197–202.