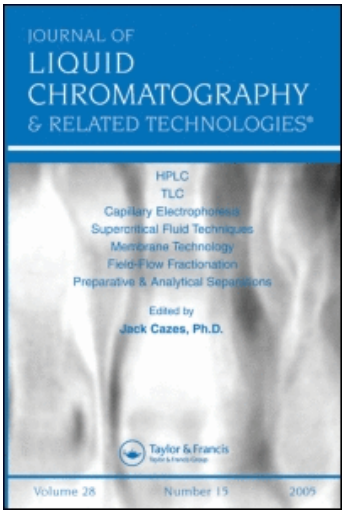

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Flow Injection Analysis of Mitomycin-C in Flacons

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Abstract: A precise and accurate flow injection analysis (FIA) method for the determination of mitomycin-C (MMC) in pharmaceutical flacons is described. Britton–Robinson (BR) buffer at pH 7.0 was used as a carrier solvent at a flow-rate of 1.0 mL min^{-1}. MMC signals were detected at 363 nm using a UV-spectrophotometric detector. The intra- and inter-assay precisions were less than 2.0%. The calibration curves were also linear in the concentration range of 2.0×10^{-6}–1.0×10^{-5} M for intra- and inter-day studies with high correlation coefficients. The limit of detection (LOD) and limit of quantification (LOQ) values were found to be 1.93×10^{-8} and 6.45×10^{-8} M, respectively. The results obtained with the application of the method were compared with those of high performance liquid chromatography (HPLC) and UV-spectrophotometry. It was found that there was insignificant difference between the methods.

Keywords: Mitomycin-C, flow injection analysis, pharmaceutical analysis, validation

INTRODUCTION

Mitomycin-C (MMC), [1a*S*-(1aα, 8β, 8aα, 8bα)]-6-amino-8-[[(aminocarbonyl) oxy] methyl]-1, 1a, 2, 8, 8a, 8b-hexa hydro-8a-methoxy-5-methyl azirino [2′,3′ : 3,4]pyrrolo[1,2-a]-indole-4,7-dione, is an anti-neoplastic drug widely used in clinical chemotherapy, especially in the treatment of breast,

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stomach, prostate, and colon cancers. It is known that the anti-cancer activity of MMC is based on its covalent binding to DNA, after MMC is chemically or enzymatically reduced.[1–4] The chemical structure of MMC is shown in Figure 1.

Certain analytical techniques have been used for the determination of MMC in pharmaceutical preparations and biological samples. In these studies, MMC was determined by liquid chromatography with UV-VIS detection,[5–12] differential pulse polarography,[13] dual electrode coulometry,[14] voltammetry,[15] and adsorptive stripping voltammetry.[3] The determination of MMC in aqueous solutions and in biological fluids have also been reviewed.[16] The proposed method in the USP XXIV pharmacopoeia is also a liquid chromatographic method with UV detection.[17] To the best of our knowledge, no flow injection method has been reported so far for the determination of MMC in pharmaceutical flacons.

Flow injection analysis (FIA), which is essentially based on the introduction of a sample into a solution stream continuously passing through a detector, is a very important methodological application in analytical chemistry, which is customarily characterized by simple chemical processes, low cost apparatus, easy manipulation, and ability to yield results that are usually of good quality. The advantages of short analysis times, allowing 60–120 injections per hour of sample and small injection volumes as low as 20 μL, make this method attractive for the determination of active substances in the pharmaceutical industry. There are several important points to consider for the application in pharmaceutical studies: adequate lower limit of quantification (LOQ), large dynamic range, high sample throughput, and small sample volume requirements for analysis. In the past, the use of FIA in pharmaceutical studies was reported to determine active substances.[18–23]

The aim of the study is to develop a new and validated analytical method for simple, accurate, cheap, and rapid determination of MMC in pharmaceutical flacons. It was applied to the pharmaceutical flacons of MMC and the results of the proposed method were compared with those of UV-spectrophotometry and high performance liquid chromatography (HPLC).

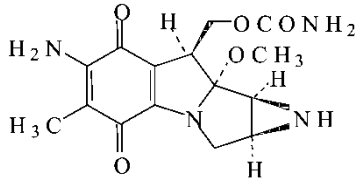

Figure 1. The chemical structure of MMC.

EXPERIMENTAL

Materials and Reagents

The standard of MMC is obtained from Sigma (St. Louis, MO, USA); the declared analysis was 2 mg MMC and 48 mg NaCl. The internal standard was p-aminoacetophenone (for HPLC) and was also supplied by Sigma. These chemicals were used without further purification. Potassium monohydrogen phosphate, sodium tetraborate, acetic acid, sodium hydroxide, and gradient grade methanol were of analytical grade and provided by Merck Co. (Darmstadt, Germany). Double distilled water and ethanol were produced in our laboratory using an all-glass apparatus.

Instrumentation

Common spectrophotometric studies were conducted using a UV-2401 PC spectrophotometer measuring the absorbance in 1 cm quartz cells.

The FIA determination was carried out employing a model LC-6A pump. Signals were detected by a model SPD-10A UV-visible detector, and data were processed by a model CR-7A integrator (all from Shimadzu, Japan). Standard solutions and samples were injected to a Rheodyne model with a 20 microliter loop injection port (Cotati, CA, USA) by a 22 gauge injection needle.

The HPLC determination was performed in a system which consisted of a Shimadzu series LC-10A solvent delivery pump, SPD-M10A diode array detector, and LC-Class 10 data processing software. MMC was separated on a 100 mm × 4.6 mm I.D., 3 μm particle size, Luna C_{18} analytical column (Phenomenex, Torrence, CA, USA). The absorbance of the effluent was monitored at 363 nm. The flow-rate was 0.8 mL min^{-1}. Carrier solvent was always filtered with a glass filter and sonicated with a model B-220 (Branson, CA, USA) sonicator.

All pH measurements were done by employing a model P 114 pH meter with glass electrode (Consort, Belgium).

Preparation of Solutions

The carrier solution used for the FIA study was the Britton–Robinson (BR) buffer at pH 7.0, which was prepared by mixing the solutions of orthophosphoric acid, acetic acid, and boric acid, 0.04 M each, and adjusted to the desired pH value with an appropriate volume of 0.2 M sodium hydroxide solution.

The standard MMC solution was prepared by dissolving it in BR buffer and made up to 25 mL. This stock solution was employed for the preparation of other dilutions.

The mobile phase for HPLC determination was monobasic potassium phosphate (pH 7.0, 10 mM)–methanol (75 : 25, v/v) and was adopted from a study.[12] *p*-Aminoacetophenone (internal standard, IS) was prepared in ethanol and it was always used in a final concentration of 4.3×10^{-5} M.

The commercial flacons of MMC were supplied from Kyowa Hakko Kogyo (Tokyo, Japan) and contained 2 mg of MMC and 48 mg sodium chloride. They were obtained from a local pharmacy, dissolved in 25 mL BR buffer, and dilutions were made from that solution.

RESULTS AND DISCUSSION

Optimization of the Method

The effect of pH and temperature on the solution stability of MMC was shown by examining its degradation at different time points and pH interval between 3 and 10; it was determined that MMC was unstable in acidic and basic conditions, especially in acid solutions.[24] Certain electrochemical studies have also been performed in the pH range of 5–8, because MMC is stable in that interval for 1 week in aqueous solutions.[3,4] Therefore, it was decided to conduct the experiments around pH 7 and a stock solution of MMC was prepared in BR buffer. Prior to conducting the FIA experiments, UV spectrum of MMC in BR buffer was recorded in the range of 200–400 nm to decide on the detection wavelength. There were two maxima in the UV spectrum at 216 and 363 nm. The detection was performed at 363 nm for all FIA experiments to avoid possible interferences. Certain wavelengths between 300 and 365 nm have already been used elsewhere.[11,24]

The effect of the pH of the carrier buffer solution on the peak morphology of MMC responses for the FIA optimization was investigated in the pH range of 5.0–8.0. The best peaks with sharp and morphologically symmetrical were obtained in BR buffer at pH 7.0 and that pH was used for the rest of the FIA experiments.

The effect of flow-rate on the peak area and peak height of MMC (1.0×10^{-5} M) was examined in the range of 0.1–2.5 mL min^{-1}. Bigger peak areas and skewed peaks appeared by pumping through with a flow-rate value lower than 0.6 mL min^{-1}. Asymmetric peaks with lower peak areas were observed with a flow-rate higher than 1.2 mL min^{-1}. Peak area response was plotted against flow-rate as shown in Figure 2. A flow-rate of 1.0 mL min^{-1} was chosen to conduct the rest of the FIA experiments.

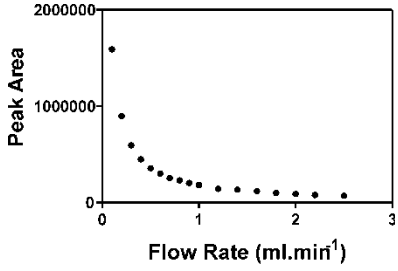

Figure 2. The variation of peak area of MMC against flow-rate in the range of 0.1–2.5 mL min^{-1} and at 363 nm detection wavelengths.

Validation of the Method

Repeatability

Repeatability and intermediate precision were tested using 1.0×10^{-5} M MMC solution in the BR buffer carrier system at a flow-rate of 1.0 mL min^{-1} and detection wavelength of 363 nm, in three operating days with eight samples. The repetitive injections into the flow system are demonstrated in Figure 3. The results were evaluated statistically relating to mean, standard deviation (SD), relative standard deviation (RSD %), and confidence limits (CL at $p < 0.05$) for the response of MMC involving peak area and

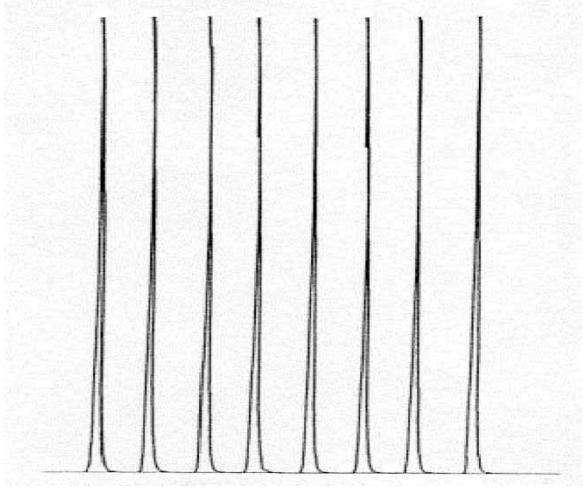

Figure 3. The absorbance signals obtained at 363 nm for MMC (1.0×10^{-5} M) in BR buffer carrier system at flow-rate of 1.0 mL min^{-1}.

peak height, as seen in Table 1. Variation coefficients lower than 2% of RSD were obtained showing the method is sufficiently precise. Although, the RSD % of peak height response of MMC is a little higher than those of peak area and can be equally usable for quantification, it was preferred to use peak area response for the further studies.

Linearity

Calibration equations were constructed by employing the peak area responses versus concentration range of 2.0×10^{-6}–1.0×10^{-5} M of MMC solution at three operating days. The peak area signals were obtained and processed by common statistics. The detailed statistical results are shown in Table 2. Statistically evaluated data show that an acceptable linearity is obtained with high regression coefficients and intercepts very close to the origin, in the studied range for FIA.

The detection limit (LOD) of the FIA method was found to be 1.93×10^{-8} M according to the criteria of signal-to-noise of $S/N = 3$, and the limit of quantification (LOQ) was calculated to be 6.45×10^{-8} M with the signal-to-noise of $S/N = 10$.

Application of the Method

Both the standard and the commercial forms of MMC flacons contain 2 mg MMC and 48 mg sodium chloride. The developed method for the determination of MMC was applied to the commercial flacons employing optimum FIA conditions, such as flow-rate of 1.0 mL min^{-1}, detection wavelength of 363 nm, and carrier solvent of BR buffer at pH 7.0.

The accuracy expresses the closeness of agreement between the value found and the value that is accepted as a reference value. It is not only accepted as the measurement of systematic errors but also of random errors in the whole analytical process. Two standard methods, UV-spectrophotometry and HPLC, were used to compare the accuracy of the FIA method.

UV-spectrophotometry was employed as the first comparison method. Good linear relations between absorbance and concentration of standard MMC was obtained in the range of 9.6×10^{-6}–3.36×10^{-5} M at 363 nm in BR buffer as a blank. It fits the equation of [$A = 19560\,C$ (M) + 0.018; $r = 0.9997$], where A is the absorbance of MMC solution at 363 nm and C is molar concentration of MMC.

HPLC determination was another comparison method adopted from a publication.[12] A 5.9×10^{-6} M of standard MMC solution was injected to the HPLC system as described in the experimental section, with the mobile phase consisting of monobasic potassium phosphate (pH 7.0, 10 mM)–methanol (75 : 25, v/v) at a flow-rate of 0.8 mL min^{-1} and detection at

Table 1. The Results of repeatability and intermediate precision tests of MMC (1.0×10^{-5} M) signals as peak area and peak height

	Repeatability						Intermediate precision ($n = 24$)	
	Day 1 ($n = 8$)		Day 2 ($n = 8$)		Day 3 ($n = 8$)			
	Area	Height	Area	Height	Area	Height	Area	Height
Mean	142,806	32,975	142,013	32,755	142,735	32,933	142,518	32,888
SD	860	564	1,475	623	1,080	562	1,173	566
RSD %	0.60	1.71	1.04	1.90	0.76	1.71	0.82	1.72
CL ($p < 0.05$)	±698	±470	±1,230	±520	±901	±469	±978	±472

Table 2. The linearity results of MMC peak area signals in the concentration range of 2×10^{-6}–1×10^{-5} M with 1.0 mL min^{-1} flow-rate and at 363 nm detection wavelengths

	Intra-day			Inter-day
	Day 1 ($n = 5$)	Day 2 ($n = 5$)	Day 3 ($n = 5$)	Whole days ($n = 15$)
A	1.40×10^{10}	1.39×10^{10}	1.41×10^{10}	1.40×10^{10}
B	783	1000	232	672
R	0.9998	0.9997	0.9997	0.9998
RSD % of A	1.97	2.26	2.41	2.31
CL of A ($p < 0.05$)	$\pm 2.64 \times 10^{8}$	$\pm 3.01 \times 10^{8}$	$\pm 3.23 \times 10^{8}$	$\pm 1.47 \times 10^{8}$

Note: A, slope; B, intercept; R, correlation coefficient; RSD % of A, % relative standard deviation of slope; CL of A, confidence limits of slope.

363 nm. Under these conditions, IS (*p*-aminoacetophenone) and MMC were baseline separated and showed symmetrical peaks. Retention times for IS and MMC were 4.6 min (1.16% RSD) and 5.1 min (1.47% RSD), respectively. A standard curve of corrected peak area ratio [calculated by $R =$ (peak area of MMC/retention time of MMC)/(peak area of IS/retention time of IS)] against MMC molar concentration was plotted and the equation of the line was computed using least-squares regression. The method was linear over the concentration range of 5.9×10^{-7}–5.9×10^{-6} M for standard MMC solution. The calibration equation was found to be [$R = 462416\ C\ (\mathrm{M}) - 2.75 \times 10^{-3}$; $r = 0.9998$] providing sufficient quantitation of MMC by HPLC.

The results of the commercial flacons assayed by FIA, UV-spectrophotometry, and HPLC, as described in the experimental section, are demonstrated in Table 3. The results of statistical analysis show no significant difference between the proposed method and standard methods according to

Table 3. The determination results of MMC in commercial flacons (declared MMC amounts in flacons = 2 mg)

	FIA	UV	HPLC
Mean ($n = 6$)	2.06	2.04	2.04
SD	0.032	0.036	0.041
RSD %	1.56	1.80	1.99
CL ($p < 0.05$)	2.06 ± 0.03	2.04 ± 0.04	2.04 ± 0.04
t-test ($p < 0.05$)	0.65	$t_{0.05} = 2.57$	$t_{0.05} = 2.57$
F-test ($p < 0.05$)	1.58	$F_{0.05} = 5.05$	$F_{0.05} = 5.05$

the results of *t*- and *F*-tests, including method accuracy and precision at the 95% probability level. Furthermore, ANOVA tests used to compare the results of three different series show no statistically significant difference among the methods ($F_{2,14} = 6.157$, $p < 0.05$).

The agreement of the results proves that no effect of ingredient is exhibited. Compared with the other analytical methods, FIA has advantages such as ease of automation, more sensitivity, high repeatability, and short analysis time. The developed FIA method has been compared with UV-spectrophotometry and HPLC, and it is concluded that the difference among the methods is not statistically significant at 95% probability level, and is in the limits of official requirements.[17]

In conclusion, the proposed method is simple, accurate, precise, and rapid. Therefore, it seems a promising method for the analysis of MMC flacons regarding the time of analysis, consumption of solvents, and size of the sample required for a routine analysis of MMC.

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