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Journal of Pharmaceutical and Biomedical Analysis 40 (2006) 100–104
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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LC-ESI-MSD fast determination of residual mitomycin C in hen aqueous humour after corneal refractive surgery

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Received 8 February 2005; received in revised form 13 June 2005; accepted 13 June 2005
Available online 2 August 2005

Abstract

A simple, fast and reliable method has been developed for the assay of traces of mitomycin C (MMC) in hen aqueous humour samples. The determination was carried out by high-performance liquid chromatography with electrospray ionization mass spectrometric detection. In isocratic elution analysis, the mobile phase was a mixture of water–acetonitrile (78:22, v/v) and the chromatographic column was C_{18} at 35 °C.

The method has been validated over a range from 0.1 to 250 μg L^{-1} in hen aqueous humour with correlation coefficients higher than 0.999. Limit of detection and limit of quantification for MMC based in signal to noise ratio of 3 and 10, respectively, were 20 and 71 ng L^{-1}. The developed method allows the analysis of MMC in hen aqueous humour samples obtained at different times and conditions in order to evaluate and compare the efficacy of the drug administration.

Keywords: Aqueous humour; Mitomycin C; LC-ESI-MSD

1. Introduction

The introduction of the laser excimer technique of the cornea in the 1980s allowed to correct the refraction index with a high level of security, efficacy and predictability [1]. For this reason, a lot of people have been operated with this technique. As with any surgical technique, secondary problems can arise, one of them is the corneal opacity called "haze". To avoid this problem, pharmacological modulation with some agents, such as corticoids, artificial tears and inmunosupressors like mitomycin C (MMC) have been tested [2,3]. These therapeutical corrections have been established in an empirical way and their adverse effects are not still well known, mainly long-term problems, since the drug can pass through the cornea and affect the corneal endothelium, which is the inner surface of the cornea and cannot be regenerated.

Mitomycin C is an antibiotic with anti-neoplastic effect, widely used in clinical chemotherapy [3,4] and it is considered as the prototype of bioreductive agents. It acts as an alkylation agent that cross-links DNA [5] by covalent binding, avoiding its replication and producing cellular death. To a lesser extent, it also reduces the proliferation of fibroblastes, which diminish the "haze" post refractive surgery. The drug binds with proteins, so this could promote long-term toxicity effects because its elimination is difficult. In other ocular applications, the above-mentioned toxicity has been reported [6–9].

Taking into account the importance of the treatments with this drug, there are many studies devoted to the elucidation of its redox behaviour, mainly by electrochemical techniques [10–13]. Its determination is also carried out using high-performance liquid chromatography on C_{18} columns with UV detection. The determination of the antibiotic in aqueous solutions and in biological fluids, mainly plasma and urine, has been subject to special interest in several papers [14,15], in many instances for pharmacokinetic studies that

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doi:10.1016/j.jpba.2005.06.024

consider very low concentrations, which imply measuring at trace levels. For this reason, it is very common to use some preconcentration steps, preferably by solid phase extraction, although other options have also been proposed, most of them including a step of protein elimination [16–19]. In ophthalmology, MMC has been used as a complementary therapy in trabeculectomy and glaucoma filtering surgery [6,7] and that is the reason why there are some papers devoted to drug determination in ocular tissues and fluids [20–23] trying to establish the optimal concentration in MMC application. Those papers, in general, lack analytical considerations because in some instances, the calibration is made on aqueous standards, and therefore, interferences are not usually considered, identification is carried out only on the basis of retention time and protein effect is ignored.

To test the security of the topical treatment at ocular level with this drug and its possible secondary effects on other ocular structures, it is necessary to measure exactly its concentration in the intraocular compartments, mainly in the anterior chamber of the eye, which is the part close to corneal endothelium. For this reason, in this paper, we present a method that allows the correct determination of trace quantities of this drug in aqueous humour samples.

2. Experimental

2.1. Chemicals and standards

HPLC grade acetonitrile was obtained from Rathburn Chemicals Ltd. (Walkerburn, Scotland) and HPLC grade methanol was supplied by Lab-Scan Ltd. (Dublin, Ireland). Ultrapure water was obtained in a Mili-RO plus system together with a Mili-Q system from Milipore (Bedford, MA, USA). MMC was obtained from Sigma–Aldrich Chemie Gbmh (Steinheim, Germany). The standard solution of MMC was prepared in methanol at a concentration of 200 mg L^{-1}. Working solutions of the compound were made daily by an appropriate dilution with a mixture of water/methanol (1:1, v/v). All standards and stock solutions were stored at +4 °C.

2.2. Instrumentation

An Agilent Technologies (Palo Alto, CA, USA) 1100 series LC-MSD system consisting of a vacuum degasser, an autosampler, a quaternary solvent pump, an autosampler with a column oven and a single quadropole MSD analyzer with an electrospray (ESI) interface, all controlled by a Chemstation software.

A Synergi 4 μm Hydro-RP 80 A (50 mm × 4.60 mm i.d.) from Phenomenex (Torrance, CA, USA) was used as analytical column for LC separation and was protected by a guard column Security guard Max-RP from Phenomenex (Torrance, CA, USA).

2.3. Chromatographic conditions

The mobile phase was a mixture of water–acetonitrile (78:22, v/v) at a flow rate of 0.6 mL min^{-1}, pH 7. Column temperature was set at 35 °C. The injection volume was 70 μL.

Operating conditions of the ESI interface in positive mode were: drying gas (N_2) temperature of 350 °C, 12 L min^{-1} drying gas flow (N_2), 60 psi nebulizer gas (N_2) pressure and 3500 V of capillary voltage. The fragmentor voltage was set at 200 V.

Full-scan LC-MS spectra were obtained by scanning *m*/*z* from 100 to 1000.

SIM mode of the most abundant ion of the compound was used for quantitation.

2.4. Surgical procedure and collection of samples

The animals were kept and handled in accordance with the Association for Research in Vision and Ophthalmology Resolution on the use of animals in research.

The aqueous humour samples were obtained from the eyes of adult hens, according to the following procedure. First of all, the hen was anaesthetized; after that, the epithelia was eliminated, then MMC was applied with the help of a sponge, and afterwards, a physiologic solution was also applied to the eye of the hen. Before the extraction of the aqueous humour, there should be a waiting period, when it had finished, the hen was anaesthetized again, the humour was aspired with a needle (small volume, 0.15 mL), and then, it was transferred into an eppendorf tube. The same process was repeated for the other eye. The samples were stored at +4 °C.

2.5. Sample treatment

A sample preparation step was necessary because the direct injection of the hen aqueous humour led to overpressure. To solve this problem, the aqueous humour should be diluted before the injection. Some solvents were tested for this purpose and the best results were obtained using water. Some experiments were made to set the suitable volume of water; 100 μL of non-treated aqueous humour were diluted with different volumes of water, it was found that 300 μL of water was enough to avoid the overpressure.

3. Results and discussion

3.1. Organic modifier percentage

Experiments were made with water/methanol and water–acetonitrile mixtures. Acetonitrile was chosen instead of methanol because it enhanced notably the peak symmetry. In addition, ionization problems for high percentages of acetonitrile in the mobile phase were not observed. Table 1 shows the variation of the retention times and peak symmetry when

Table 1
Retention time and peak symmetry at different percentages of acetonitrile, pH and temperatures

	% ACN					pH				Temperature (°C)				
	15	18	22	26	30	5	6	7	8	15	25	35	45	55
Retention time (min)	1.84	1.76	1.71	1.65	1.59	1.68	1.70	1.71	1.73	1.81	1.77	1.72	1.66	1.60
Peak symmetry	0.70	0.81	0.97	1.15	1.32	0.91	0.90	0.91	0.91	0.81	0.83	0.96	1.24	1.56

the percentage of acetonitrile increased from 15 to 30%. As it can also be observed, the retention time decreases when the percentage of acetonitrile is higher but for very high percentages of organic modifier the peak symmetry decreases. The best results were obtained in isocratic mode with a mixture of water–acetonitrile (78:22, v/v) as mobile phase.

3.2. Influence of the pH

Considering the poor stability of MMC outside the pH range 5–8, tests were made between these pH values. Table 1 shows the values of retention time and peak symmetry at different pH. The modification of the pH values in this interval did not affect significantly the retention time, as it can be seen in Table 1. So, pH 7 was selected.

3.3. Temperature

The variation of the retention time and the peak symmetry according to the column temperature can be seen in Table 1. As it was expected, the increase in the temperature decreased slightly the retention times. A loss of symmetry was observed at low and high temperatures. Because of that, a temperature of 35 °C was chosen.

3.4. Injection volume

On account of the low MMC concentrations expected in aqueous humour, the possibility of enhancing the detection limits by using shorter columns and injecting high sample volumes was considered. Once the column had been selected, in the established conditions, volumes from 50 to 100 μL of a standard solution of 10 μg L^{-1} were injected. The obtained results are shown in Table 2. An increase in the peak areas was observed in the injection up to 70 μL without any loss of symmetry or overload of the column. For higher injection volumes, the chromatographic peaks began to be somewhat deformed.

This fact indicated that the linear capacity of the column was surpassed, and in consequence, an injection volume of 70 μL was adopted as optimum.

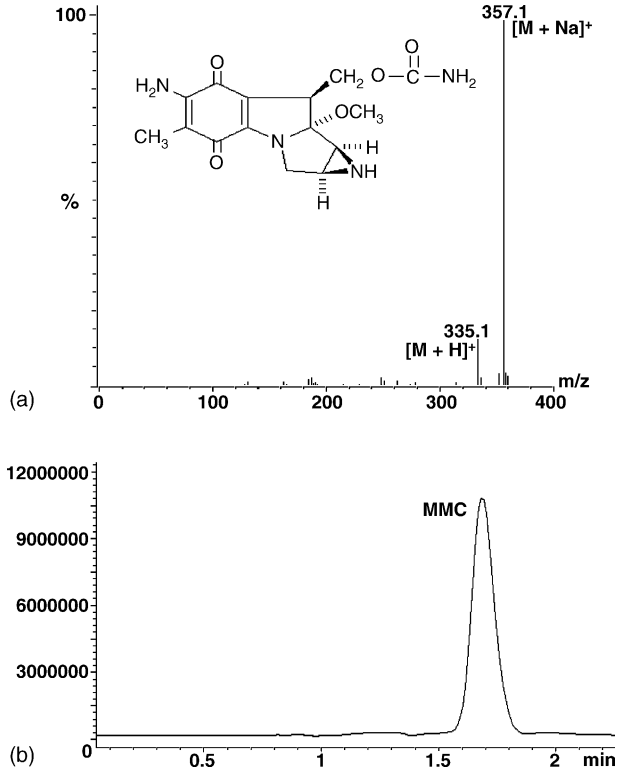

Fig. 1. (a) Mass spectra of MMC and (b) LC-ESI-MSD (in the SIM mode) chromatogram of a standard solution (1 mg L^{-1}) of MMC.

3.5. Mass spectrometry

The first LC-MS experiments to select the optimum MS parameters and the appropriate ions were carried out by flow injection analysis (FIA) of the individual solutions of the standards. Best sensitivity was obtained using the conditions reported in Section 2.2.

Selected ion monitoring (SIM) mode was used to obtain the maximum sensitivity for quantitative analysis.

To summarize, the HPLC separation was carried out using a C_{18} column and the mobile phase was an isocratic mixture of water–acetonitrile (78:22, v/v) at pH 7, that allowed us to obtain the highest separation efficiency,

Table 2
Area and peak symmetry at different injection volumes

	Injection volume (μL)					
	50	60	70	80	90	100
Area	456789	565342	674040	798765	907867	1023456
Peak symmetry	0.94	0.93	0.97	1.15	1.26	1.40

at a column temperature of 35 °C, an injection volume of 70 μL and a flow rate of 0.6 mL min^{-1}. The following mass-to-charge (m/z) ratios were chosen: 357.1 $[M+Na]^+$ for quantitation and 335.1 $[M+H]^+$ for confirmation to MMC, mass spectra and chromatogram of MMC are shown in Fig. 1.

3.6. Validation of the procedure for LC-ESI-MSD

Validation was carried out following the ICH guidelines [24] and IUPAC technical report of 2002 [25], determining selectivity, limits of detection and quantitation, linearity, precision and accuracy for MSD.

To check the selectivity, extracts of non-spiked aqueous humour samples were injected. From Fig. 2, it can be deduced that there are not interferences in the extracts of hen aqueous humour.

The detection limit (LOD) and quantitation limit (LOQ) were determined injecting a number of extracts of non-spiked hen aqueous humour samples ($n = 5$) and measuring the magnitude of the background analytical response. LOQ should be identifiable, discrete and reproducible with a precision corresponding to a maximum 20% R.S.D. We estimated the LOD and LOQ as 3 or 10 times the signal-to-noise ratio. The values obtained were 20 and 71 ng L^{-1}, respectively.

With the aim of estimating the linearity of the calibration graph, we used a matrix-standard calibration: aqueous humour samples were spiked with variable amounts of MMC, from 0.1 to 250 μg L^{-1} and by triplicate. Plotting the peak areas versus six concentrations, a graph which was a straight line of intercept not significantly ($p < 0.05$) different from zero was obtained. This confirmed the linearity through the range studied and the lack of bias with a correlation coefficient $r^2 = 0.9990$. The results of the statistical treatment of the calibration data were "a" (slope) = 67.46×10^3; $s_a = 0.86 \times 10^3$; "b" (y-intercept) = 25,229; $s_b = 7738$; $s_{y/x} = 16{,}503$.

The precision was evaluated by the same analyst; the results of repeatability and intermediate precision experiments are shown in Table 3. The method was found to be precise as the R.S.D. values for repeatability and intermediate precision studies were <1 and <3%, respectively.

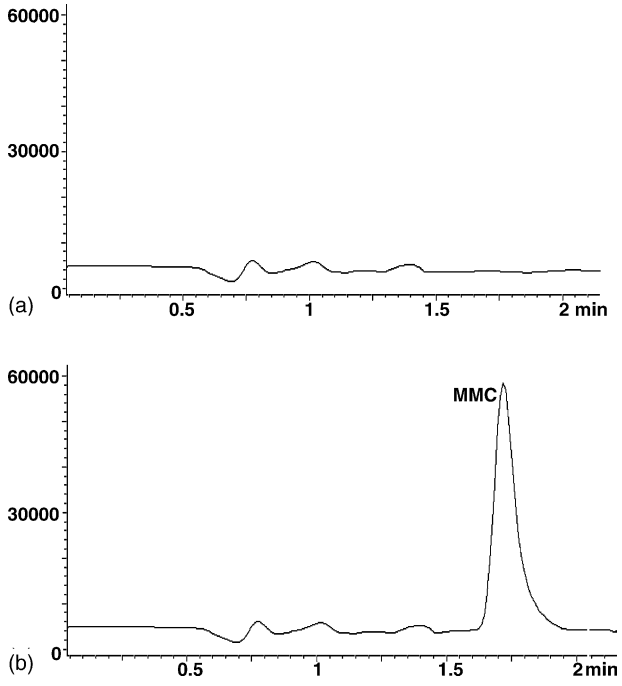

Fig. 2. (a) LC-ESI-MSD chromatograms obtained from a blank of hen aqueous humour and (b) a hen aqueous humour sample spiked with 5 μg L^{-1} of mitomycin C (MMC).

The MS area responses for accuracy determination are depicted in Table 4. Good recoveries (94.3–102.1%) of the spiked samples were obtained at each antibiotic added concentration, indicating that the method was accurate.

3.7. Application of the method

The developed method has been applied to the analysis of MMC in hen aqueous humour samples obtained at different times and conditions, in order to evaluate and compare the efficacy of the drug administration.

Two groups of samples have been studied. With the first group, called A, the epithelia was eliminated and the eye of

Table 3
Precision studies

Spiked concentration (μg L^{-1})	Measured concentration ± S.D. (μg L^{-1}), R.S.D.	
	Repeatability ($n = 6$)	Intermediate precision ($n = 3$)
1	1.013 ± 0.010, 0.98	0.987 ± 0.027, 2.73
50	50.574 ± 0.334, 0.66	51.034 ± 1.233, 2.41
150	152.356 ± 0.856, 0.56	151.309 ± 2.436, 1.60

Table 4
Accuracy studies ($n = 3$)

Spiked concentration (μg L^{-1})	Measured concentration ± S.D. (μg L^{-1}), R.S.D. (%)	Recovery (%)
1	1.023 ± 0.012, 1.17	98.7
50	50.734 ± 0.433, 0.85	94.3
150	152.516 ± 1.036, 0.68	102.1

Table 5
Concentrations of MMC in hen aqueous humour samples from groups A and B obtained at different extraction times

Time	Group A ($\mu g\,L^{-1}$)	Group B ($\mu g\,L^{-1}$)
10 min	193	93
	190	87
30 min	112	47
	105	45
	108	49
	117	44
1 h	55	15
	59	25
	76	24
	65	29
6 h	*X*[a]	*X*
	17	*X*
	20	*X*
	X	*X*
12 h	*X*	*X*
	X	*X*
	X	*X*
	X	*X*

[a] *X*: <LOQ.

the hen suffered a stromal ablation with six diopters laser (67 μm) and after that, MMC was applied for 2 min.

In the second group, called B, MMC was applied for 2 min over the intact epithelia (without laser ablation).

For each group, they were five extraction times of the aqueous humour after a waiting period of: 10, 30 min, 1, 6 and 12 h.

The hen aqueous humour samples were diluted with water and analyzed with the validated method. The results are listed in Table 5. As it can be seen, significant differences (<0.005) between groups A and B were found for extraction times of 10, 30 min and 1 h. There were not differences between both groups for extraction times of 6 and 12 h.

4. Conclusions

A fast and sensitive method for the determination of residual MMC in hen aqueous humour samples has been developed. The use of short columns and the dilution of the samples with water (1:3, v/v) was enough to avoid overpressure. A mass spectrometric detection in electrospray positive ionization mode allowed the determination of the analyte. By using the selected chromatographic conditions, the total run time was reduced to 2.5 min for each analysis.

The application of the method has been successfully demonstrated by the measurement of MMC concentrations in hen aqueous humour samples.

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