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Quantitation of mitomycin C in human ocular tissues by high-performance liquid chromatography–photo-diode array detection

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Abstract

A chromatographic method, which can quantitate mitomycin C (MMC) along with two antiglaucoma drugs, is described. The separation of MMC, alphagan and timolol was performed on a reversed-phase C_{18} column with water–methanol–trifluoroacetic acid (65:35:0.01, v/v) as the mobile phase. By monitoring at 360, 248 and 296 nm, the lower limits of detection for MMC, alphagan and timolol are, respectively, 1.0, 2.0 and 5.0 ng (injection amount) at three-time S/N ratio. The dynamic ranges of quantitation for the three drugs are, respectively, 1.0 ng–10.0 μg, 2.0 ng–10.0 μg and 5.0 ng–10.0 μg with linearity being larger than 0.9960. This method was applied to the determination of MMC levels in Tenon's and trabeculum tissues of 10 glaucoma patients. MMC levels in these tissues, which were obtained from glaucoma filtering surgery, were determined following a multiple extraction with methanol. The recovery of MMC for a two-batch extraction was better than 91.2%. The reproducibility of measurement for the MMC levels in these tissues is 2.5–6.0% RSD for triplicate injections. The intra-day variation of retention times for the MMC peaks was less than 1.6% RSD ($n=3$). The inter-day variation of retention times for the MMC peaks was less than 4.8% RSD ($n=3$). MMC was detectable in three trabeculum tissues out of 10 cases (ranging from 0.8 to 25.5 ng/mg specimen), while MMC was detected in nine Tenon's tissues out of 10 cases (ranging from 0.3 to 21.1 ng/mg specimen). The results obtained show that the method is sensitive and selective for the quantitation of MMC.

Keywords: Mitomycin C; Alphagan; Timolol

1. Introduction

Mitomycin C (MMC) is an antiproliferative and anticancer drug. It has been widely used as chemotherapy for the treatment of breast and prostate cancer, and is the drug of first choice for local intravesical administration in superficial bladder cancer [1]. In ophthalmology, MMC has been used as adjunctive therapy in trabeculectomy and glaucoma filtering surgery [2,3]. The use of MMC in glaucoma filtration surgery increases the surgical success rates in terms of preventing excessive scarring in the subconjunctiva space; however, its use increases the risk of cataract and long-term complications. Severe toxicity has been reported in human ocular tissues after intraocular instillation/application of MMC [4,5]. Especially, MMC may induce Tenon's fibroblast apoptosis [6] and cause ciliary

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body damage [7] if it is not restricted in the appropriate sites during the surgery. Too high or too low amounts of MMC remaining in the application site after surgery would compromise the wound healing process. The determination of the optimal concentration of MMC application during filtering surgery is still an open question up to now [8]. As a result, proper control of the MMC levels in the concerned tissues in glaucoma filtering surgery is one of the critical steps during surgery. To enhance the long-term success rates of the surgery, knowledge of the MMC levels remaining in the concerned tissues after surgery is important for surgeons to optimize the MMC application procedure. Unfortunately, little information on MMC levels in human ocular tissues obtained from glaucoma filtering surgery is available in the literature.

The quantitation of MMC levels in biological/clinical specimens has been reported. The majority of the MMC assays are based on microbiological [9], immunological [10], and chromatographic methods [11–13]. Because of its versatility and flexibility, high-performance liquid chromatography (HPLC) is the main technique that has been widely used in analysis of MMC in various samples. There are several reports on the quantitation of MMC in human aqueous humor (intraocular fluid) by using reversed-phase C_{18} columns for separation and UV spectrophotometry for detection [14–16]. Few papers report on the determination of MMC in human ocular tissues. Kawase et al. used a silica gel column to separate the MMC and UV detection to quantitate the MMC levels in animal conjunctiva and human sclera tissues [17]. However, no potential interference of pre-operative medications on the separation results was taken into account in their report. That method could not be used in the case where the glaucoma patients have undergone long-term pre-operative medications.

Timolol and alphagan are common antiglaucoma drugs used topically in the management of glaucoma patients for the purpose of lowering intraocular pressure (IOP). When these drugs do not effectively reduce IOP, surgical methods are sought to manage the disease. Due to long-term use of these drugs for the patients, accumulation of these drugs in intraocular [18] or periocular tissues [19] is inevitably remarkable. The existence of these drug residues in Tenon's and trabeculum tissues would interfere with the quantitation of MMC in the concerned tissues obtained from the glaucoma filtering surgery. To avoid interference from these drug residues, development of a chromatographic method that can detect MMC in the presence of these common antiglaucoma drugs is very important.

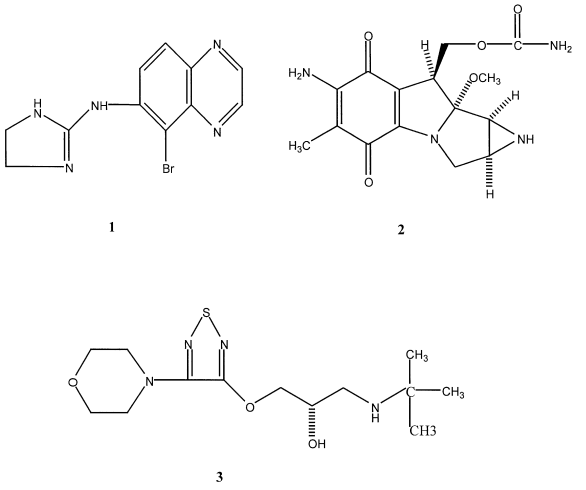

Fig. 1. Structures of the drugs. 1, Alphagan; 2, MMC; 3, timolol.

The purpose of this work is to develop a new chromatographic method to quantitate MMC levels in trabeculum and Tenon's tissues obtained from glaucoma filtering surgery in the presence of pre-operative medications (alphagan and timolol antiglaucoma drugs). Fig. 1 illustrates the structures of these drugs. Based on the HPLC technique coupled with photo-diode array detection (PDA), it is hoped that this method could be transferred to a routine laboratory and meets the requirements of clinical assay of the MMC levels in ocular tissues.

2. Experimental

2.1. Chemicals and standard solutions

All organic solvents were HPLC grade obtained from Fisher Scientific (Leicestershire, UK). Water was purified by a Milli-Q system (Millipore, Bedford, MA, USA). MMC (2 mg potency for injection per bottle) was purchased from Kyowa Hakko Kgyo (Tokyo, Japan). Alphagan (brimonidine tartrate)

0.2% solution (5 ml per bottle) was obtained from Allergan (Irvine, CA, USA). Timolol (maleate salt) 0.5% solution (5 ml per bottle) was obtained from Chauvin (Montpellier, France). Spectrophotometric-grade (99+%) trifluoroacetic acid (TFA) was purchased from Sigma (St. Louis, MO, USA). The standard solutions of MMC were prepared by dissolving a known amount of MMC in methanol, and then diluted successively with the mobile phase. The standard solutions of the other two drugs were prepared directly by diluting a known volume of each drug with the mobile phase. The standards were stored at 4°C and free from repeated cycles of freezing and thawing.

2.2. Apparatus

Chromatographic analysis was carried out on a Delta PAK C_{18} column (Waters, Milford, MA, USA), 3.9×300 mm, 100 Å, 15 μm, which was connected to two Waters pumps (Model 515), a PDA system (Model 996), and an injector (Model 7725). The operation of the whole system was controlled by Millennium version 3.05.01 (Waters). Sonication of the tissues was performed on a Microson Ultrasonic cell disruptor (Misonix, Farmingdale, NY, USA). Evaporation of the tissue extracts was conducted in a Freezone4.5 freeze–dry system (Labconco, Kansas City, MO, USA).

2.3. Surgical procedure and collection of samples

Standard trabeculecutomy was performed on 10 glaucoma patients. A limbal-based conjunctival flap was raised in the superior temporal or superior nasal quadrant 8–10 mm from the limbus. Hemostasis was secured and an approximately 5×3 mm partial thickness scleral flap was raised. Dry surgical sponge measured 1×4×1 mm was fully saturated in a solution of 0.4 mg/ml MMC (dissolved in sterile distilled water) and applied to the episcleral bed of the scleral flap. The overlying Tenon and conjunctiva was draped over the upper surface of the soaked sponge. After 5 min, the sponge was removed and the surgical site was irrigated with two aliquots of 20 ml of normal saline solution. The first aliquot was sprayed via a Gauge 23 cannula connected to a 20-ml syringe, and the second aliquot was applied without the cannula.

The trabeculectomy was completed with excision of a piece of trabeculum tissue (2–6 mm^2). A small piece of Tenon's tissue (4–12 mm^2) over the scleral flap was also excised before the conjunctiva was closed with interrupted sutures using 10Φ nylon. The trabeculum and Tenon's tissues were weighed immediately after the surgery. They were stored in a freezer at −80°C until analysis.

2.4. Extraction of MMC from human ocular tissues

Trabeculum specimens (wet mass less than 3 mg) obtained from the glaucoma filtering surgery were extracted with 200 μl of methanol by vortexing for 3 min. The extract was recovered by centrifugation at 3000 *g* for 10 min. A 35-μl volume of the extract was mixed with 65 μl of 0.01% TFA aqueous solution. The entire mixture was injected into the Delta PAK C_{18} column. The column was eluted by 35% methanol (with 0.01% TFA additive) at 1 ml/min, monitored by PDA at 360 nm.

Each Tenon's specimen (wet mass less than 25 mg) was suspended in 200 μl of methanol and mixed by vortexing for 3 min. The insoluble tissue was removed after centrifugation at 3000 *g* for 10 min. A 35-μl volume of the extract was mixed with 65 μl of 0.01% TFA aqueous solution. The extract was analyzed using the same procedure as described above.

3. Results and discussion

3.1. Chromatographic analysis

With 35% methanol eluent (isocratic) at 1 ml/min, all the drugs as shown in Fig. 1 could be eluted out from the C_{18} column within 20 min. Nevertheless, the peak shapes for these drugs were not symmetrical except the peak corresponding to MMC. A broad peak of alphagan heavily overlapped with the MMC peak. This made the quantitation of MMC inaccurate when peak area or height was used to quantify the

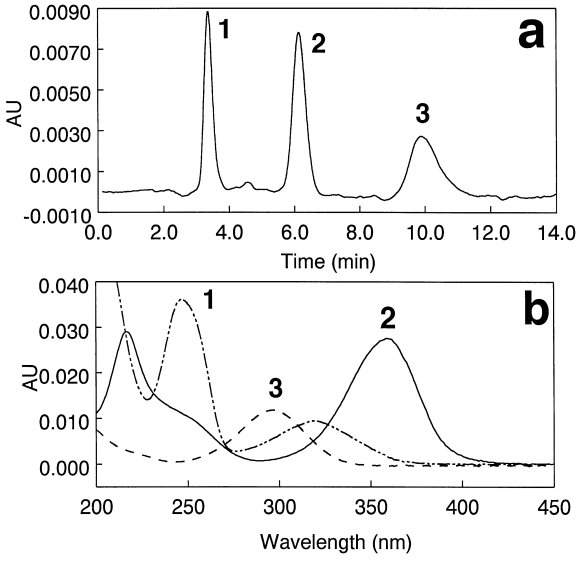

Fig. 2. (a) Chromatogram of alphagan, MMC and timolol under the optimal conditions. Column: C_{18}; mobile phase: 35% methanol with 0.01% TFA additive; flow-rate: 1 ml/min (isocratic); injection volume: 10 μl (1, alphagan, 20 μg/ml; 2, MMC, 20 μg/ml; 3, timolol, 50 μg/ml); detection: 325 nm. (b) UV spectra of the three drugs in the range of 200–450 nm.

MMC level. To increase the resolution of separation for these peaks, 0.01% TFA was added to the mobile phase. Under this condition, all of the three drugs were baseline separated and showed symmetrical peak shapes. No significant change in peak shapes for these drugs had been observed when the TFA concentration was increased to 0.05% in the mobile phase. Fig. 2a shows the separation result of the three drugs under the optimal conditions. Fig. 2b illustrates the UV spectra of the drugs.

3.2. Validation of the method

3.2.1. Limit of detection and dynamic range of quantitation

The determination of the detection limit for the three drugs was performed under the conditions as given in Fig. 2a. A three-time signal-to-noise ratio (S/N) criterion was used to evaluate the limit of detection. Because the characteristic wavelengths for the three drugs are different as shown in Fig. 2b, the quantitation of them was carried out at the wavelengths corresponding to their maximal absorption. Table 1 gives the validation results of the method for the three drugs. External standard method was used to correlate the concentration of the drugs with peak areas observed on the chromatogram. From the linearity and detection limit, we see that the present method has a wide dynamic range and high sensitivity for the quantification of MMC in the presence of the two antiglaucoma drugs.

3.2.2. Extraction of MMC from trabeculum and Tenon's tissues

To extract MMC from the tissues completely, a multiple extraction method was used throughout the experiments. Generally, MMC could be extracted into methanol nearly completely after two-batch extraction with 100 μl of methanol in each time when the surgical specimen mass was less than 10 mg. If the specimen was heavier than 25 mg, the above extraction procedure could not recover MMC from the tissues completely. This observation was confirmed by sonication of the tissue using a cell disruptor at 10% output power for a short period of time (about 30 s) after it was extracted for two times

Table 1
Summary of the validation results of the present method

Drug	Detection wavelength (nm)	Limit of detection[a] (ng)	Dynamic range[a]	Linearity[e]
Mitomycin C	360	1.0[b]	1.0 ng–10.0 μg	0.9968
Alphagan	248	2.0[c]	2.0 ng–10.0 μg	0.9960
Timolol	296	5.0[d]	5.0 ng–10.0 μg	0.9997

[a] Injection amount with reproducibility ranging from 2.0 to 3.0% RSD for triplicate injections.
[b] The detection limit amounts to injection of 100 μl of 10 ng/ml MMC onto the column.
[c] The detection limit amounts to injection of 100 μl of 20 ng/ml alphagan onto the column.
[d] The detection limit amounts to injection of 100 μl of 50 ng/ml timolol onto the column.
[e] Square of correlation coefficients (r^2) of the calibration lines.

with 100 μl of methanol. About 25% of the total MMC amount in the surgical specimen could be further recovered after the sonication. Owing to the uncontrolled evaporation of methanol during sonication, this step was omitted in subsequent experiments. For simplicity, the methanol volume was increased to 200 μl in each extraction. When the surgical specimen was lighter than 25 mg, the first extraction would recover 91.2–97.5% of the MMC residue in the tissues. As a result, two extractions, each time with 200 μl of methanol, were carried out for all the specimens as listed in Table 2. If the MMC levels were still high after two extractions, the specimen was extracted for the third time. To minimize the back-adsorption of the extracted MMC onto the tissue, the tissue extract was isolated from the tissue using filter centrifugation after each batch extraction. The total amount of MMC in the tissues was the summation of each extraction.

3.3. MMC levels in trabeculum and Tenon's tissues

Ten patients with different types of glaucoma were chosen for the present study under the informed consent. Table 2 gives the diagnostic results of the patients along with the MMC levels in trabeculum and Tenon's tissues determined by the above method. There was no detectable amount of MMC in seven out of 10 cases (mass ranged from 0.3 to 2.1 mg). Among the three detectable cases, one case was barely detectable (case 3 in Table 2); the other two cases were significantly detectable (cases 1 and 2 in Table 2). Fig. 3a shows the typical chromatogram and the UV spectrum of the significantly detectable cases. Fig. 3b illustrates the chromatogram and the UV spectrum of one of the undetectable cases. From the UV spectra obtained, it was certain that MMC levels in these tissues were much lower than the detection limit of the method. However, it was uncertain whether trace amounts of MMC were still present in the tissues. To confirm the existence of trace amounts of MMC in these clinical specimens, 100 μl of the methanol extracts from each of the undetectable cases was combined and concentrated by lyophilizing them to dryness in a freeze dry system. The concentrated extracts were then redissolved in 100 μl of the mobile phase and injected into the separation column. Fig. 3c shows the chromatogram and the UV spectrum of the combined

Table 2
MMC levels in trabeculum and Tenon's tissues of 10 glaucoma patients

Patient No.[a]	Type of glaucoma[b]	Trabeculum			Tenon		
		Wet mass (mg)	MMC in tissue[e]		Wet mass (mg)	MMC in tissue[e]	
			Total (ng)	ng/mg specimen (wet mass)		Total (ng)	ng/mg specimen (wet mass)
1	CAAG	0.6	15.3	25.5	0.7	8.2	11.7
2	POAG	0.7	1.8	2.6	6.6	70.3	10.7
3	POAG	0.4	0.3[c]	0.8	0.7	0.9[c]	1.3
4	POAG	0.8	<[d]	–	5.7	120.1	21.1
5	POAG	0.3	<[d]	–	3.1	3.7	1.2
6	POAG	2.1	<[d]	–	0.5	5.4	10.7
7	CACG	0.3	<[d]	–	24.0	121.7	5.1
8	CACG	0.6	<[d]	–	4.1	7.6	1.9
9	CACG	1.2	<[d]	–	1.8	<[d]	–
10	CACG	0.8	<[d]	–	7.6	2.3[c]	0.3

[a] Alphagan and timolo antiglaucoma drugs are individually or commonly used by the patients as pre-operative medications.
[b] CAAG, Chronic acute-angle glaucoma; POAG, primary open-angle glaucoma; CACG, chronic angle-closure glaucoma.
[c] The MMC injection amount of the tissue extracts is lower than the detection limit of the method. Its existence was confirmed by the UV spectrum of MMC. The results are estimations of the MMC peaks superposed on the noisy baseline.
[d] The MMC injection amount is lower than the detection limit. Its existence could not be confirmed by the UV spectrum of MMC.
[e] The reproducibility of measurement ranges from 2.5 to 6.0% RSD for triplicate injections.

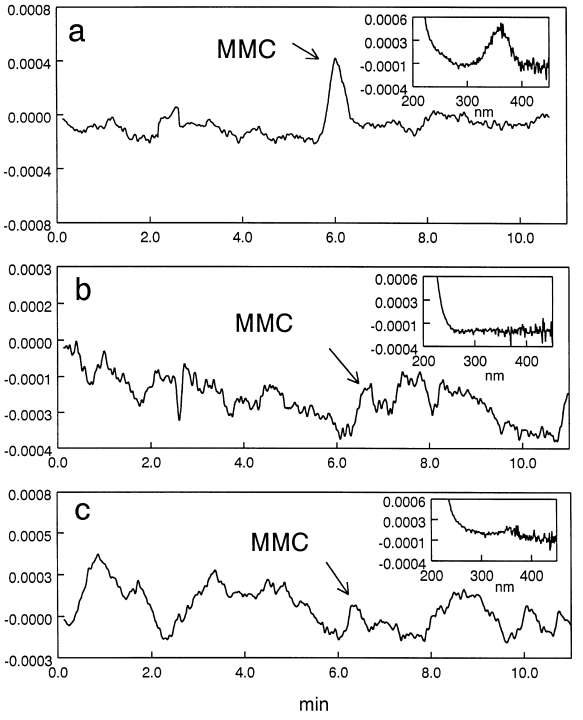

Fig. 3. Chromatogram of trabeculum extracts under the conditions as described in Fig. 2. (a) Chromatogram for the case in which MMC was significantly detected in the trabeculum extract. (b) Typical chromatogram for the case in which MMC could not be detected. (c) Chromatogram of combined trabeculum extracts in which MMC was not detectable with individual injection of the extract. Inset: the UV spectra of MMC peaks at the range from 200 nm to 450 nm. Here all *y*-axes are expressed in absorption units (AU).

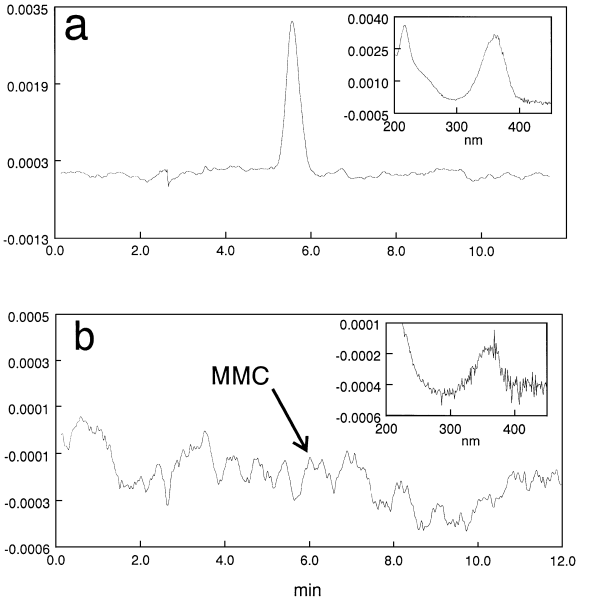

Fig. 4. Chromatogram of Tenon's tissue extracts under the conditions described in Fig. 2. (a) Chromatogram for the case in which MMC was significantly detected. (b) Chromatogram for the case in which MMC was barely detected. Inset: the UV spectra of MMC peaks at the range between 200 nm and 450 nm. Here all *y*-axes are expressed in absorption units (AU).

extracts. Because the peak corresponding to MMC superposes on the background noise, it is difficult to identify the MMC peak from the chromatogram. But the characteristic absorption of MMC at 360 nm, as illustrated in the inset of Fig. 3c, clearly indicates that a trace amount of MMC do exist in the combined extracts. The total amount of MMC in the combined extracts was estimated to be 0.2 ng.

On the other hand, MMC was detectable in nine out of 10 Tenon's tissues (mass ranged from 0.5 to 24.0 mg as listed in Table 2). A broad range of MMC concentration in these tissues was observed. The MMC amount in these clinical specimens ranged from 0.3 ng/mg specimen (wet mass) to 21.1 ng/mg specimen (wet mass). Fig. 4a illustrates the chromatogram and the UV spectrum of case 4 in Table 2, in which MMC was significantly detected. Fig. 4b shows the chromatogram and the UV spectrum of case 3 in which MMC was only barely detectable. The intra-day variation of retention times for the MMC peaks was less than 1.6% RSD ($n=3$). The inter-day variation of retention times for the MMC peaks was less than 4.8% RSD ($n=3$).

From Table 2, we see that no significant amount of MMC could be detected in most trabeculum tissues. This indicates that no excess amount of MMC diffused into the intraocular tissues during the surgery. The potential side effect caused by MMC is minimized for most of the patients. However, three cases (case 1 to 3) in Table 2 show detectable amount of MMC in trabeculum tissues. Where are the sources of the MMC residue for those tissues is not clear at this moment. It probably resulted from contamination via uncontrollable procedures during surgery. Special attention would be required to investigate the clinical factor or surgical procedure

that accounted for the cases in which MMC was detectable and the long-term outcome of these patients.

On the other hand, the majority of the 10 patients in Table 2 show detectable amount of MMC in Tenon's tissues. They varied in a wide range from 0.3 ng/mg specimen (wet mass) to 21.1 ng/mg specimen (wet mass), although the application approach of MMC was the same for all the patients. The large difference in MMC levels may attribute to the uncontrolled process during the surgery or to the difference in mechanisms of MMC binding on the tissues [20]. In addition, the difference in pre-operative medications for all the patients may play a role for this observation. It is interesting to correlate the MMC levels remained on the tissues with the long-term success rates of the surgery.

It should be noted that alphagan and timolol were not detected in the tissues. Although some peaks having the same retention times as those of the drugs observed on the chromatograms for the tissue extracts, their UV spectra were different from those illustrated in Fig. 2b. These peaks might be the metabolites of the antiglaucoma drugs extracted together with MMC. What those metabolites are remains to be further studied. Nevertheless, these peaks were baseline separated from the MMC peak in question; therefore, no interference from these peaks on the quantitation of MMC has been observed in all the tissue extracts.

The present method provides direct information on MMC levels in the trabeculum and Tenon's tissues. This information may help surgeons to optimize the application procedure of MMC and properly control the MMC levels on the concerned tissues during glaucoma filtering surgery. As a result, the wound healing process would be modulated, and the surgery success rates would be enhanced. By quantifying the MMC levels remained in the Tenon's tissue, we can correlate the MMC levels with the fibroblast death and understand the mechanism that regulates this biological process. Thereby new therapeutic strategies to modulate the wound healing process in glaucoma filtering surgery would be developed.

In conclusion, the present method is sensitive and selective in the quantitation of MMC in human ocular tissues. It has a wide dynamic range for MMC determination in the presence of alphagan and timolol antiglaucoma drugs. It could be used in a clinical laboratory for analysis of MMC.

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References

[1] J. Verweij, H.M. Pinedo, in: H.M. Pinedo, B.A. Chabner, D.L. Longo (Eds.), Cancer Chemotherapy and Biological Modifiers, Annual II, Elsevier, Amsterdam, 1990, p. 67, Chapter 5.
[2] A.M. Francoeur, A. Assalian, M.R. Lesk, I. Morin, F. Tétreault, K. Calleja, A. Guttman, M. Rauth, S. Pan, J. Glaucoma 8 (1999) 242.
[3] S.S. Palmer, Ophthalmology 98 (1991) 317.
[4] O. Oram, R.L. Gross, K.R. Wilhelmus, J.A. Hoover, Arch. Ophthalmol. 113 (1995) 19.
[5] A. Fregona, D. Violato, E. Midena, Invest. Ophthalmol. Vis. Sci. 40 (1999) S625, Abstract No. 3289.
[6] J.G. Cronston, A.N. Akbar, P.H. Constable, L. Chang, J.T. Daniels, P.T. Khaw, Invest. Ophthalmol. Vis. Sci. 40 (1999) S968, Abstract No. 5101.
[7] H. Mietz, K. Addicks, M. Diestelhorst, G.K. Krieglstein, Int. Ophthalmol. 19 (1995) 89.
[8] J.C. Cheung, M.M. Wright, S. Muradi, J.E. Pederson, Ophthamology 104 (1997) 143.
[9] H. Fujita, Jpn. J. Clin. Oncol. 12 (1971) 151.
[10] K. Fujiwara, H. Saikusa, M. Yasunom, T. Kitagawa, Cancer Res. 42 (1982) 1487.
[11] R. Paroni, C. Arcelloni, E. De Vecchi, I. Fermo, D. Mauri, R. Colombo, Clin. Chem. 43 (1997) 615.
[12] Z.D. Zhang, G. Guetens, G. De Boeck, K. Van Cauwenberghe, R.A.A. Maes, C. Ardiet, A.T. van Oosterom, M. Highley, E.A. de Bruijn, U.R. Tjaden, J. Chromatogr. B 739 (2000) 281.
[13] G. Joseph, W. Biederbickw, U. Woschee, M. Theisohn, W. Klaus, J. Chromatogr. B 698 (1997) 261.
[14] W.Y. Li, S.K. Seah, R.T. Koda, J. Chromatogr. 619 (1993) 148.
[15] A.F. Rump, W. Biederbick, S. Botuinik-Helling, M. Stemmler, J. Schierhotz, S. Conrady et al., Arzneim.-Forsch. 45 (1995) 1329.
[16] S.K.L. Seah, J.A. Prata Jr., D.S. Minckler, R.T. Koda, G. Baerveldt, P.P. Lee, D.K. Heuer, Eye 7 (1993) 652.

[17] K. Kawase, H. Matsushita, T. Yamamoto, Y. Kitazawa, Ophthalmology 99 (1992) 203.
[18] C. Baudouin, P.J. Pisella, K. Fillacier, M. Goldschild, F. Becquet, M.D.S. Jean, A. Bechetoille, Ophthalmology 106 (1999) 556.
[19] W.E. Sponsel, S. Terry, H.D. Khun, K.W. Lam, H. Frenzel, Survey Ophthalmol. (Suppl.) 43 (1999) S210.
[20] C. Vass, M. Georgopoulos, I.E. Menyaw, S. Rodda, P. Nimmerrichter, Invest. Ophthalmol. Vis. Sci. 41 (2000) S580, Abstract No. 3084.