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Sodium hyaluronate as a vehicle for an improved tolerance of 5-fluorouracil administered subconjunctivally to rabbits

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Abstract

5-Fluorouracil (5-FU) is gaining clinical interest as an adjunct treatment for patients at high risk of failure after glaucoma filtration surgery. The purpose of this study is to compare the clinically used commercial solution of 5-FU with a suspension of 5-FU in a gel of 1% sodium hyaluronate (SH) for the subconjunctival injection of a 10 mg dose in healthy rabbits. The commercial solution has a pH of 9.0, which is irritant for the eye, and the gel of sodium hyaluronate has a pH of 7.4. An HPLC method for the quantitation of 5-FU in the aqueous humor has been developed to evaluate the bioavailability of 5-FU. No purification of the samples was required before chromatography. Both vehicles led to the same mean concentrations of 5-FU in the aqueous humor at each observation time as confirmed by a t-test ($p > 0.05$ for all observation times). The bleb formed by the subconjunctival injection remained longer in SH-5-FU treated eyes (> 24 h) than in eyes having received the commercial 5-FU formulation (4 h). Eyes receiving the commercial formulation were more inflamed compared to those receiving 5-FU in SH. Although sodium hyaluronate gives the same bioavailability as that obtained with the commercial solution of 5-FU, it seems to be more suitable for the subconjunctival administration of 5-FU because this formulation is less inflammatory due to its physiological pH. Therefore, the SH-5-FU suspension could gain clinical attention in selected cases of glaucoma filtration surgery.

Key words: 5-Fluorouracil; HPLC; Sodium hyaluronate; Bioavailability; Tolerance; Rabbit; Ophthalmics; Glaucoma

5-Fluorouracil (5-FU) has been widely investigated for its beneficial effects on glaucoma filtration surgery in patients with a high risk of failure (Parrish, 1992). The wound healing mechanisms involved in glaucoma surgery and the pharmacological options available to increase the success rate have been reviewed (Skuta and Parrish, 1987; Joseph et al., 1988; Tahery and Lee, 1989). Various studies confirm that 5-FU is helpful to maintain a functional filtering bleb, but secondary effects such as conjunctival wound leaks and corneal epithelial defects can be a serious problem (The Fluorouracil Filtering Surgery Study Group, 1993).

The available commercial formulation currently used in human glaucoma surgery is a solution intended for intravenous use, and consists of 5-FU in water with a pH adjusted with sodium hydroxide to approx. 9.2. In fact, increasing the

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pH value allows one to increase the solubility of 5-FU. This solubility in neutral distilled water is approx. 10 mg/ml whereas the aqueous commercial solution at pH 9.2 contains 50 mg/ml of 5-FU. It seems logical to assume that in the case of repeated subconjunctival injections of 5-FU, this alkaline solution will be deleterious to the conjunctival and corneal epithelia. We therefore formulated a suspension of 5-FU, using as a vehicle a gel of sodium hyaluronate 1% intended for intraocular use and having a pH of 7.4.

This article introduces an HPLC method for the quantitation of 5-FU in aqueous humor. We compared the amount of 5-FU found in the aqueous humor of healthy rabbits 2, 4, 6, and 24 h after the subconjunctival injection of 10 mg of 5-FU dissolved in water or suspended in a 1% sodium hyaluronate gel. Clinical observations were performed to compare the duration of the injection bleb and the tolerance to the injected formulations.

Reagents: 5-FU for intravenous perfusion (50 mg/ml in water, pH 9.0) was purchased from Hoffmann-La Roche (Switzerland) and used as obtained. A sterile gel of 1% sodium hyaluronate in phosphate buffer solution (Healon®) was purchased from Kabi Pharmacia (Sweden). 5-FU and 5-CU (reagent grade) were purchased from Sigma (U.S.A.), and the rabbit aqueous humor used for the preparation of the standards was obtained from a local slaughterhouse. A weighed amount of 5-FU was sterilized with γ-irradiation and then mixed with SH immediately before use. The preparation, having a final concentration of 50 mg 5-FU/ml of SH, was transferred into a syringe. Ammonium dihydrogen phosphate and diammonium hydrogen phosphate were purchased from Fluka AG (Switzerland) and used to prepare a 0.05 M phosphate buffer with a pH of 6.8. Methanol for HPLC was obtained from Reactolab SA (Switzerland).

5-FU administration to rabbits and aqueous humor sampling: New Zealand albino rabbits were used, and the protocol has been approved by the ethical committee of the State of Geneva, Switzerland. The rabbits received local anesthesia with one drop of oxybuprocaine hydrochloride 0.4%, followed by one drop of the same agent at a concentration of 1%. The subconjunctival injection was performed nasally to the superior rectus muscle insertion while the rabbits were in restraining boxes. One eye per animal was used. Rabbits received 200 μl of either the commercial solution with a 25 G needle or the mixture of SH + 5-FU with a 20 G needle. The total dose injected was 10 mg of 5-FU. For both treatments, aqueous humor was collected by paracentesis through the superior peripheral cornea under local anesthesia 2, 4, 6, and 24 h after the injection. Each time point represents the average of six or seven values. Clinical observations were made before each sampling. The conjunctival inflammation was graded on a 0 to 3 + scale (0 = no inflammation, 1 = slight inflammation, 2 = moderate inflammation, 3 = severe inflammation). The persistence of the injection bleb was noted.

Quantitation of 5-FU by HPLC: 5-FU (Sigma and Hoffmann-La Roche) stock solutions (1.0 mg/ml in methanol), and a 5-CU solution (1.0 mg/ml in methanol) were prepared and stored at −20°C. The 5-FU stock solution was diluted with water to obtain standards of 0.5, 1, 2, 5, 10, 20, 50, and 100 μg/ml for the calibration curve in water (StdW). In parallel, the 5-FU stock solution was diluted with pooled rabbit aqueous humor to obtain standards of 1, 2, 5, 10, 20, 50, and 100 μg/ml for the calibration curve in aqueous humor (StdAH). The 5-CU stock solution was diluted 1:10 with water to obtain a 100 μg/ml 5-CU solution. Each final standard (StdW and StdAH) was obtained by mixing 200 μl of the standard solution with 20 μl of 5-CU 100 μg/ml. The experimental samples were later submitted to the same dilution. A high performance liquid chromatograph (Waters 600E System Controller) was equipped with a variable wavelength spectrophotometer (Waters Lambda-Max model 481) set at 264 nm and connected to a computer (ALR 386/220) with the software Maxima 820 (Waters). The column used was a reverse-phase μBondapak C18 (3.9 × 300 mm, 10 μm). The mobile phase was a 98:2 mixture of 0.05 M ammonium phosphate buffer (pH 6.8) and methanol. The flow rate was set at 1.0 ml/min. Similar conditions were established (Stetson et al., 1985) for

the quantitation of 5-FU in plasma. The volume of injection was 20 μl. Under these conditions, the retention times of 5-FU and 5-CU were 5.7 and 9.3 min, respectively. A standard curve in aqueous humor was run with each experiment and used to quantify the experimental samples, and a standard curve in water was obtained to compare the sensitivity of the assay in water versus aqueous humor. Two standard curves were obtained with standards prepared with the commercial solution of 5-FU (in water and in aqueous humor) to validate the chromatographic response (equivalent with the two sources of 5-FU). For each aqueous humor sample, 5 μl of 5-CU 100 μg/ml were added to 50 μl of sample and 20 μl of the resulting solution was injected for chromatography. The concentration of 5-FU was calculated from the measured peak area (MPA) with the use of the linear equation related to the calibration curve obtained on the same day. Calibration curves in aqueous humor and water were obtained for each experiment ($n = 6$). Preliminary studies gave three more calibration curves of each kind. The response obtained when plotting the ratio MPA 5-FU/MPA 5-CU against the concentration of 5-FU was linear in all cases and gave correlation coefficients > 0.995 for curves in aqueous humor. Calibration curves in water always had correlation values higher than those of the calibration curves in aqueous humor obtained on the same day. The reproducibility between injections was evaluated by injecting four times standards of three different concentrations (1, 10, and 100 μg/ml), both in water and in aqueous humor. The coefficient of variation between injections averaged 3% for standards prepared in water and 9% for standards prepared in aqueous humor. All these assays were performed with freshly prepared solutions. To evaluate their stability, the same solutions were kept for various periods of time at 4 and -20°C and were reanalysed. Aqueous humor standards are stable for 3 days at 4°C and degrade to a limited extent after freeze-thawing. Fresh aqueous humor standards were therefore prepared for each calibration curve. Standards prepared in water can be frozen in aliquots and used within at least 1 month. Stock solutions stored at -20°C can be used up to 6 months. The commercial solution of 5-FU remains stable for at least 3 months after the opening of a vial when it is kept at room temperature in a tight container protected from light.

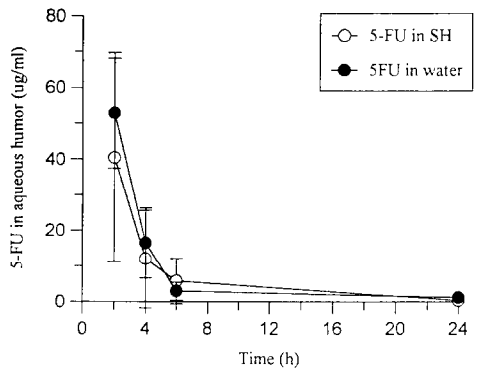

Fig. 1. Concentration of 5-FU in rabbit aqueous humor as a function of time after the subconjunctival injection of a 10 mg dose.

Clinical observations showed that the bleb formed by the subconjunctival injection remained longer in the SH-5-FU treated eyes than in those having received the commercial 5-FU solution (> 24 h vs 4 h). The conjunctiva overlaying the SH-5-FU bleb remained uninflamed (grade 0) throughout the study except for one eye graded 1+, whereas an inflammation grade from 1 to 3+ (hyperemia) was observed up to 24 h in all eyes treated with the commercial solution.

Fig. 1 shows the amount of 5-FU found in the rabbit aqueous humor after the subconjunctival injection of the commercial solution or the suspension in a gel of sodium hyaluronate (SH-5-FU). The two aqueous humor concentration vs time curves are essentially similar. No statistical difference between the two formulations was found at any observation time (t-test, $p > 0.05$ in all cases).

Fig. 2 illustrates typical chromatograms for (a) control aqueous humor (no internal standard added), (b) standard in aqueous humor containing 20 μg/ml of 5-FU, (c) experimental sample of aqueous humor containing 18 μg/ml of 5-FU, and (d) standard in water containing 20 μg/ml of 5-FU. The retention times of aqueous humor

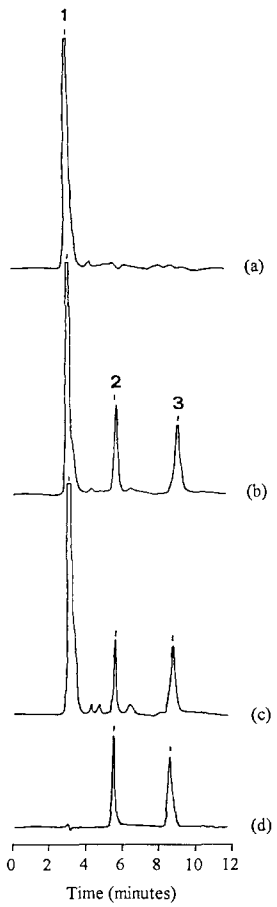

Fig. 2. Typical chromatograms obtained for control aqueous humor (no internal standard added) (a), standard in aqueous humor containing 20 μg/ml of 5-FU (b), experimental sample of aqueous humor containing 18 μg/ml of 5-FU (c), and standard in water containing 20 μg/ml of 5-FU (d). Peak numbering: 1, aqueous humor proteins; 2, 5-fluorouracil; 3, 5-chlorouracil.

proteins, 5-FU, and 5-CU were 3.1, 5.7, and 9.3 min, respectively. Various publications have described the quantitation of 5-FU by HPLC in plasma and/or blood samples (Heggie et al., 1987; Rustum and Hoffman, 1988; Chung et al., 1991) and in other biological samples (Sommadossi et al., 1982; Celio et al., 1983; Del Nozal et al., 1992). The analysis of aqueous humor by HPLC using previously published chromatographic conditions is complicated by components interfering with the 5-FU peak. We adapted the protocol reported by Stetson et al. (1985) and obtained a good separation of the 5-FU peak from the main aqueous humor peak as shown in Fig. 2. No purification of the biological samples is necessary and the analysis can be performed with volumes as small as 50 μl.

This study was undertaken to evaluate the potential of sodium hyaluronate as a vehicle for the subconjunctival delivery of 5-FU. The undesirable secondary effects reported up to now in relation with the use of this drug have led to numerous investigations aiming at optimizing its delivery, mainly by reducing the injected dose and the number of injections. Another option is to develop a controlled drug delivery device in order to achieve a slow release of 5-FU over time instead of performing repeated administrations (Kay et al., 1986; Lee et al., 1988; Hasty et al., 1990; Herschler, 1992).

While working on the controlled drug release approach, we decided to use as a control a commercial solution of 5-FU such as reported in most published human clinical trials, namely, 5-fluorouracil Hoffmann-La Roche (50 mg/ml) for intravenous perfusion. We measured the pH of this solution and found a value of 9.0. We reviewed 35 published clinical trials performed in humans and their respective protocols in order to obtain information on the pH of the injected solution. The preparations injected most often consisted in diluting the commercial solution to 10 mg/ml with a balanced salt solution. We performed all the described dilutions with NaCl 0.9% and obtained in all cases solutions having a pH ranging from 8.8 to 9.0. None of the 35 publications mentioned the pH of the solution that was injected subconjunctivally to patients. In nine animal studies reviewed, six did not mention the pH and three explained that trimethamine or NaOH was used to bring the pH up to 8.4 in order to obtain the desired solubility for 5-FU.

The fact that the commercial 5-FU solution has a pH of 9.0 is due to the poor solubility of 5-FU in water at a lower pH. This poor solubility has been reported recently (Ando et al., 1992). In order to prepare a solution with a concentration of 5-FU higher than 10 mg/ml, it is therefore necessary to increase the pH. The elevated pH is

not known to be a problem for intravenous perfusions because the administration into the venous circulation is typically performed in a total perfusion volume of 250 ml administered over several hours. However, in the case of repeated subconjunctival injections of 5-FU, this alkaline solution may be deleterious to the conjunctival and corneal epithelia. We question whether the conjunctival wound leaks observed as a side effect in patients subjected to this repeated therapy may possibly be due to the alkalinity of the subconjunctivally injected solution. These leaks may also trigger the epithelial defects frequently seen in these patients. Ando et al. (1992) reported that 5-FU did not affect healthy corneal epithelium in rabbits. However, patients submitted to glaucoma surgery often have a long history of topical medication(s) that can modify the integrity of their conjunctival epithelium (Sherwood et al., 1989), and their corneal surface may be more fragile to the 5-FU preparation. The surgery may also irritate the conjunctival epithelium.

Since SH has been widely used in intraocular surgery for over a decade, and more recently for ocular drug delivery (Bernatchez et al., 1993), this substance seemed appropriate for the design of a 5-FU formulation intended for subconjunctival use. Moreover, SH is recognized as being non-inflammatory (McKnight et al., 1987). Our clinical observations showed that the injected bleb remained longer, and that the inflammation was avoided in SH treated eyes compared with the eyes having received the commercial formulation. Fig. 1 illustrates the aqueous humor concentration versus time curves for the tested formulations. These curves are essentially similar. On the one hand, the commercial solution is more irritant due to its pH, and the induced irritation should lead to increased penetration. On the other, the sodium hyaluronate preparation contains a suspension of 5-FU which must first dissolve before diffusing through the ocular tissues and reaching the aqueous humor. Theoretically, this formulation should slow down the penetration of the drug into the aqueous humor. However, no statistical difference was observed at any time point between the two treatments. It is assumed that the SH containing formulation may compensate for the possibly increased irritation-induced penetration obtained with the commercial solution, and explain the equivalence in bioavailability of the two formulations.

To summarise, our results indicate that a sodium hyaluronate gel can be used to formulate a suspension of 5-FU intended for subconjunctival injections. This formulation provides the same bioavailability to the aqueous humor in healthy rabbits than the currently available solution, without irritating ocular tissues. The quantitation of 5-FU in aqueous humor can be achieved by HPLC (Fig. 2). Human clinical studies are required to evaluate this 5-FU formulation designed for ocular use.

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