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Determination of 5-fluorouracil in vitreous gel and liquid by high-performance liquid chromatography

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ABSTRACT

A high-performance liquid chromatographic method was developed for the determination of 5-fluorouracil (5-Fu) in vitreous gel and liquid. After addition of an internal standard (5-fluorocytosine), the vitreous humour samples were extracted with *n*-propanol–diethyl ether (16:84, v/v) with average recoveries of 95%. The extract was evaporated to dryness and the residue was reconstituted in 1 ml of mobile phase (0.05 *M* phosphate buffer, pH 3.5) and chromatographed isocratically on a C_{18} reversed-phase column coupled to an ultraviolet detector set at 254 nm. This method was applied to the pharmacokinetic study of 5-Fu in the vitreous gel and liquid of rabbit eyes after the injection of the drug.

INTRODUCTION

Over the last few years, 5-fluorouracil (5-Fu) has shown promise in the pharmacological treatment of proliferative vitreoretinopathy (PVR). The use of 5-Fu, however, is associated with a risk of retinal toxicity, which may be a consequence of high local drug concentrations. In order to avoid these effects, doses of 5-Fu must be fitted and the pharmacokinetics of the intravitreal injection of this drug must be studied correctly. Therefore, it is necessary to have an exact method in order to measure adequately the concentrations of 5-Fu in the vitreous cavity [1].

No methods have been described for measuring 5-Fu in vitreous gel and liquid. Nevertheless, different methods for determining this drug in other biological fluids such as plasma and urine have been developed and several procedures for extraction and determination have been proposed. Usually a previous deproteinization step, using different methods [2–5] is required, followed by extraction of the drug using, *e.g.*, ethyl acetate or *n*-propanol–diethyl ether mixtures [4].

The first reported methods for the determination of 5-Fu, based on the use of nuclear magnetic resonance [6,7] and radionuclides [8], showed poor sensitivity and resolution, so chromatographic methods were tried. Gas chromatography is not recommended because of the instability of the drug and also a laborious extraction procedure is required [4,9]. Currently most use high-performance liquid chromatography (HPLC) with fluorescence or UV detection [2,4,10–12]. Usually reversed-phase columns are employed [5,13] with phosphate mobile phases at different pH values [2–5,9,11,13]. We initially applied these HPLC methods to vitreous gel and liquid samples spiked with 5-Fu but adequate results were not obtained. However, this previous experience provided useful information for developing a satisfactory method for vitreous gel and liquid samples.

In this work, different methods of extraction and chromatographic columns were studied in order to

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establish a simple HPLC procedure for determining the concentration of 5-Fu in the vitreous gel and liquid in rabbit eyes after an initial dose of the drug.

EXPERIMENTAL

Chemicals

5-Fluorouracil (5-Fu) and 5-fluorocytosine (5-Fc) were obtained from Sigma (St. Louis, MO, USA). Monobasic ammonium phosphate and all other chemicals used for the preparation of buffers were of analytical-reagent grade (Merck, Darmstad, Germany). The water used was purified by passage through a Nanopure II system (Barnstead, Newton, MA, USA). Diethyl ether and *n*-propanol were obtained from SDS (Peypin, France).

Instrumentation and chromatographic conditions

The chromatographic set-up consisted of a CD4000 multiple solvent partitioning pump, a SM4000 UV–VIS variable-wavelength detector and a CI4000 integrator, all from LDC Analytical (Riviera Beach, FL, USA).

The columns adopted were Spherisorb 5 ODS 2 (25 × 0.46 cm I.D.), Ultramex C_{18} (25 × 0.2 cm I.D.) and Spherisorb SAX (15 × 0.4 cm I.D.) from Phenomenex (Torrance, CA, USA), Vydac C_{18} (25 × 0.46 cm I.D.) from The Separations Group (Hesperia, CA, USA) and Pinkerton GFF II (25 × 0.46 cm I.D.) from Regis Chemical (Morton Grove, IL, USA); all the packings were of 5-μm particle size. The mobile phase was 0.05 *M* phosphate buffer (pH 3.5) and was pumped at a flow-rate of 1.0 ml/min. Samples were injected by means of a Rheodyne (Berkeley, CA, USA) Model 7125 injector with a fixed-volume loop of 20 μl.

Animal study

The animals used were cared for and handled according to the ARVO Resolution on Use of Animals in Research. A total of 21 albino rabbits weighing between 2.0 and 3.0 kg were used.

All surgical procedures were performed under a combination of intramuscular ketamine (100 mg/kg) and diazepam (0.5/kg) anaesthesia using topical 1% cyclopentolate and 10% phenylephrine for mydriasis.

One eye of each animal was injected with 1 mg of commercial 5-Fu (Hoffmann la Roche, Basle, Switzerland) in a total volume of 0.05 ml using a tuberculin syringe and a 30-gauge needle. The needle was introduced through the proptosed globe at the equator under ophthalmoscopic control, taking great care to avoid damage to the lens, ciliary body and adjacent retina. The jet stream of fluid was directed towards the mid-vitreous gel and liquid. The intraocular pressure was allowed to equilibrate, the needle withdrawn and the perforation site covered for 30 s in order to avoid the reflux of the drug. Following drug administration samples were obtained at 0.1, 2, 6, 12, 24, 48 and 72 h. At each of the seven times three animals were killed and the treated eyes were immediately proptosed and enucleased, the adherent episclera being dissected. The vitreous gel and liquid was expressed, disrupted with a sonicator and the microtip was used for 30 s, centrifuged at 1000 *g* for 5 min and frozen.

Sample extraction

The extraction procedures using solid–liquid and liquid–liquid extraction were compared.

In the solid–liquid extraction, sample clean-up was done using solid-phase extraction (SPE) columns (Bond Elut, Analytichem, Harbor City, CA, USA). The cartridges were preconditioned by flushing with the appropriate solvent. The samples were loaded on the cartridges and the eluate was filtered on 0.2-μm filters (MFS, Dublin, CA, USA) and injected into the chromatographic system.

In the liquid–liquid extraction ethyl acetate or *n*-propanol–diethyl ether mixtures were added to samples of vitreous gel and liquid (0.2 ml), raw samples and samples spiked with 5-Fu, placed into separating funnels and shaken for 10 min. The organic phase was then separated and dried in a rotary evaporator from Büchi (Flawil, Switzerland). The residues obtained were dissolved in 1 ml of mobile phase and injected into the chromatographic system.

Internal standard calibration graph

Stock solutions of 5-Fu and 5-Fc were prepared in Nanopure water at a concentration of 100 μg/ml. They were stable for at least 3 months if stored at 4°C. Standard solutions were prepared from the stock solutions by sequential dilution with Nanopure water.

Drug-free vitreous gel and liquid samples spiked

with known amounts of 5-Fu and 5-Fc were analysed concurrently with each set of unknown samples. At least seven different concentrations of 5-Fu across the working range were measured in quintuplicate. Calibration graphs were calculated by the least-squares method. Peak-area ratios between 5-Fu and 5-Fc were used to generate the least-squares linear regression lines. Concentrations of 5-Fu in the vitreous samples were obtained by interpolation from these calibration graphs using peak-area ratios obtained from unknown samples. Vitreous blanks were used to monitor for interferences.

The recovery of 5-Fu was determined at concentrations between 1 and 100 μg/ml. Vitreous samples (0.2 ml) were spiked with appropriate volumes of 5-Fu solution and extracted. A constant concentration (25 μg/ml) of internal standard was added to each sample prior to extraction and analysis, completing to the final volume, always 1 ml, with Nanopure water.

Sample preparation

Homogenized vitreous gel and liquid (0.2 ml) was placed in a glass tube, 25 μg/ml of internal standard (5-Fc) were added and Nanopure water was added to a final volume of 1 ml. Then 50 μl of 0.1 *M* acetic acid–acetate buffer (pH 4.6) and 0.5 ml of a saturated solution of sodium sulphate were added. The mixture was placed in a 50-ml separating funnel and 15 ml of *n*-propanol–diethyl ether (16:84, v/v) were added and mechanically shaken for 15 min. The two layers were separated and the organic phase was concentrated to dryness in a rotary evaporator. The residue was dissolved in 1 ml of the mobile phase, the solution was filtered through a 0.2-μm filter and 20 μl were injected into the HPLC system.

RESULTS AND DISCUSSION

There are two bands between 190 and 300 nm in the molecular absorption spectra of 5-Fu and 5-Fc, which make the detection of these compounds possible over a wide range of wavelengths. In order to obtain an economical and simple procedure 254 nm was chosen although when working at 266 nm the sensitivity is multiplied by 1.4 and at 200 nm by 1.8, but at 200 and 266 nm there are more instrumental limitations and only a few mobile phases are useful.

Different types of columns were used in order to isolate the peaks of 5-Fu and 5-Fc: reversed-phase (Ultramex C_{18}, Vydac C_{18}, Spherisorb 5 ODS 2), exclusion reversed-phase (Pinkerton GFF II) and ion exchange (Spherisorb SAX), using mobile phases of acetic acid–acetate or phosphate solutions in different concentrations and pH values.

The best separation was obtained with the Sphe-

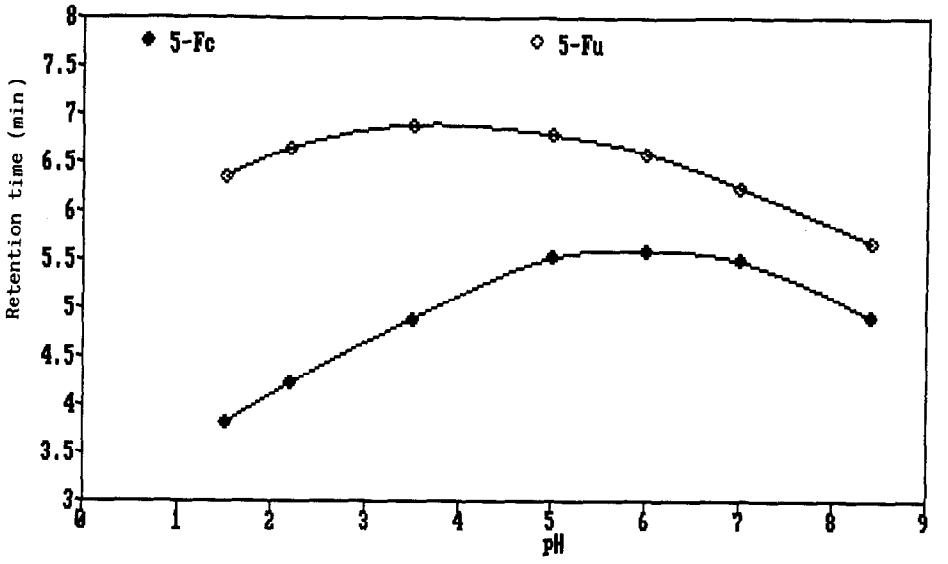

Fig. 1. Variations in the retention times of 5-Fu and 5-Fc *vs.* the pH of the mobile phase.

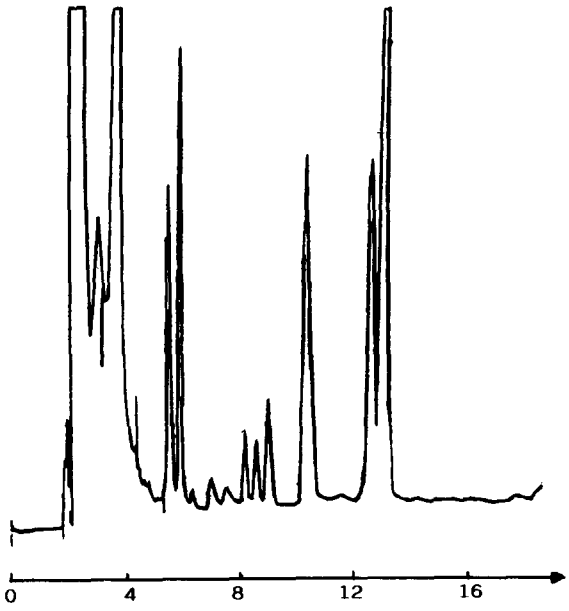

Fig. 2. Chromatogram of a drug-free vitreous gel and liquid sample after sonication, centrifugation and filtration.

risorb 5 ODS 2 column and 0.05 *M* phosphate solution. From these experiments it was found that the pH of the mobile phase affected the retention of different solutes considerably (Fig. 1). The most suitable pH seems to be 3.5, where the retention times are 4.89 min for 5-Fc and 6.89 min for 5-Fu.

Extraction clean-up

When a filtered vitreous sample without any other treatment is injected, various peaks that will coelute with 5-Fu (6.89 min) appear in the chromatogram (Fig. 2), so an extraction clean-up procedure is necessary. In order to obtain an appropriate method to determine 5-Fu without interferences, solid–liquid and liquid–liquid extractions were used.

In the solid–liquid extraction different minicolumns were used (C_{18}, C_8, NH_2, CN and phenyl). Using C_{18} cartridges most interfering compounds were retained and so was the 5-Fu (Fig. 3). However, it was not possible to carry out the selective elution of the compounds. With other cartridges it was also not possible to achieve the separation.

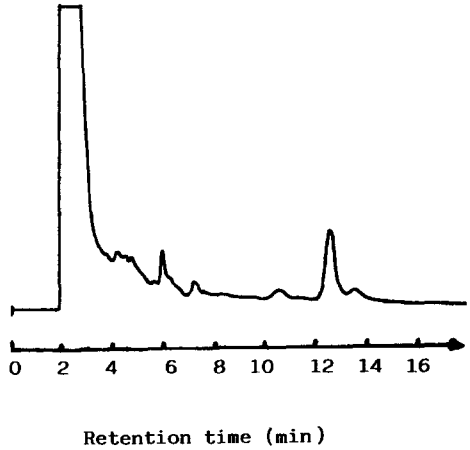

Fig. 3. Chromatogram of a vitreous gel and liquid sample spiked with 5-Fu and 5-Fc after clean-up through a C_{18} cartridge.

In the liquid–liquid extractions two solvents were used, ethyl acetate and *n*-propanol–diethyl ether.

In the former instance, the peaks that interfered with 5-Fu were eliminated (Fig. 4a) but the 5-Fu recovery was only about 30%. In order to increase the extraction yield, the influence of different parameters was studied: pH of the medium (2–9), volume of extractant (5–20 ml) and shaking time (10–30 min), but the recovery of the method was not improved.

With the latter solvent, in a first test 5 ml of *n*-propanol–diethyl ether (16:84, v/v) were added to a vitreous sample spiked with 5-Fu, shaking and rejecting the aqueous phase. Subsequently a new extraction of the organic phase was performed by adding 1 ml of phosphate buffer (pH 11), then the aqueous phase was separated, filtered and injected into the HPLC system. Many interfering peaks disappeared (Fig. 4b) but the efficiency of this procedure was only about 38%. To improve this, different volumes of acetic acid–acetate buffers at several pH values and different amounts of a sodium sulphate solution were also added to a vitreous sample prior to the extraction. When 0.1 *M* acetic acid–acetate buffer (pH 4.6) and 0.5 ml of the saturated solution of sodium sulphate were used, the recovery was about 55%. The effects of the volume of *n*-propanol–diethyl ether used and the shaking time were also studied, in addition to the influence of drying the organic phase from the first extraction

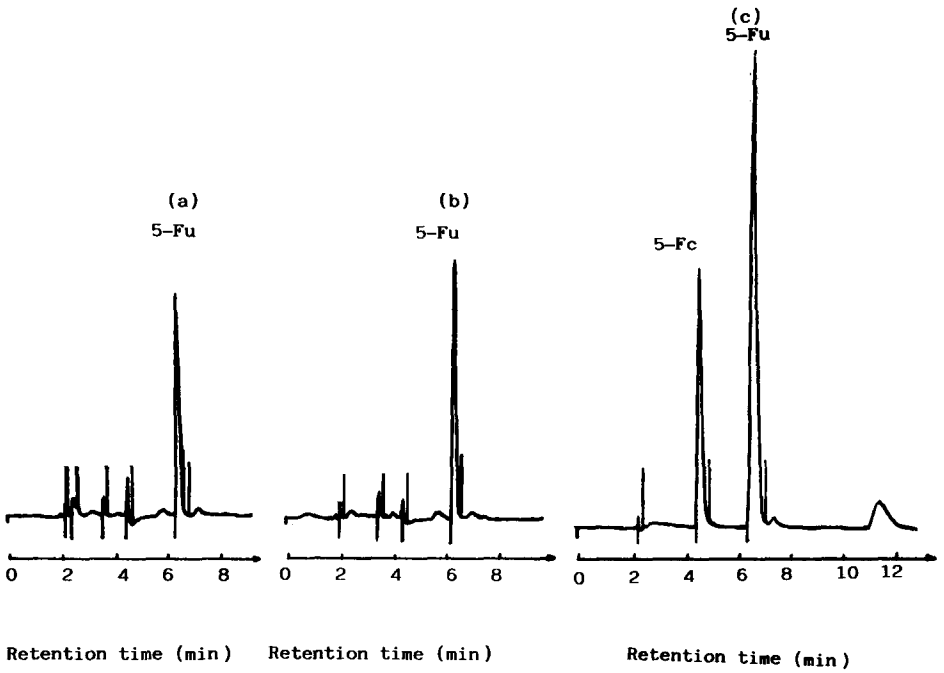

Fig. 4. Chromatogram of vitreous gel and liquid sample spiked with 5-Fu and 5-Fc (a) after extraction with ethyl acetate, (b) after extraction with *n*-propanol–diethyl ether followed by a second extraction with phosphate buffer and (c) after extraction according to sample preparation.

step and dissolving the residue in phosphate afterwards. The optimum conditions obtained, which were introduced in the sample preparation stage, were applied to different vitreous samples fortified with 5-Fu, giving recoveries around 95% (Table I), and the interfering peaks in the chromatogram disappeared, as can be seen in Fig. 4c.

Calibration graphs

The calibration graphs for 5-Fu obtained from the extraction of spiked vitreous samples was linear over the concentration range 0.5–100 μg/ml. The regression equation for this line was concentration = 0.28(peak area) − 2.68 with a correlation coefficient of 0.998. By using these conditions the detec-

TABLE I

EXTRACTION RECOVERY OF 5-Fu IN VITREOUS GEL AND LIQUID

Concentration (μg/ml)	Recovery (%) (mean ± S.D., n = 5)
1	94.61 ± 0.88
5	95.33 ± 0.91
10	94.96 ± 0.85
25	95.24 ± 0.95
50	94.91 ± 0.93
100	94.67 ± 0.89
Mean ± S.D.	94.95 ± 0.29

TABLE II

5-Fu CONCENTRATIONS IN VITREOUS GEL AND LIQUID AFTER A SINGLE 1-mg INTRAVITREAL INJECTION OF 5-Fu

Time after administration (h)	Concentration (μg/ml) (mean ± S.D., n = 3)
0.1	983.97 ± 41.87
2	609.69 ± 31.53
6	188.36 ± 3.63
12	9.21 ± 1.25
24	1.19 ± 0.18
48	0.39 ± 0.11
72	0.35 ± 0.10

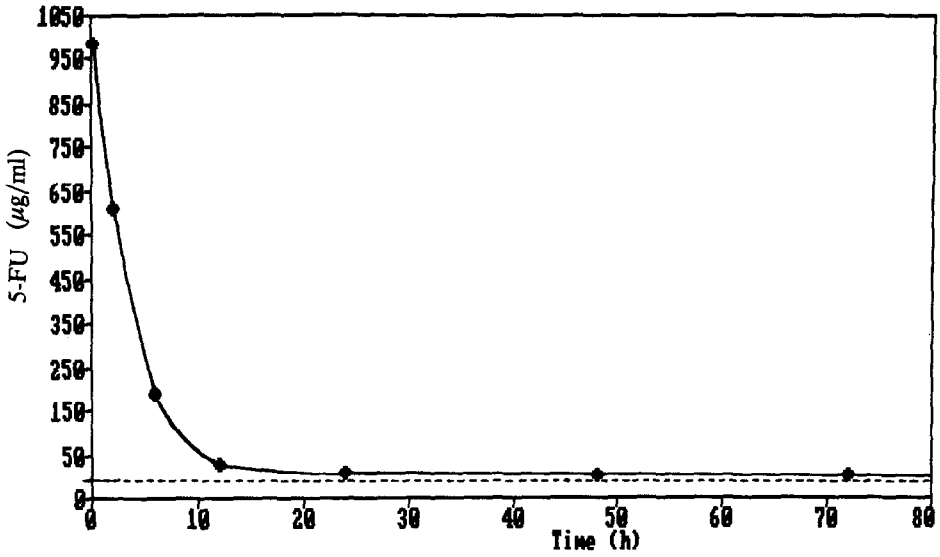

Fig. 5. Vitreous gel and liquid concentration–time profile of 5-Fu after a single 1-mg intravitreal injection of 5-Fu.

tion limit was 0.25 μg/ml and the limit of determination was 0.75 μg/ml.

Application to vitreous gel and liquid samples

The results obtained are given in Table II. The highest concentration of 5-Fu was obtained immediately after the injection of the drug. Data for vitreous sample clearance of 5-Fu *versus* time are plotted in Fig. 5 and suggest a biphasic character in which clearance is accelerated during the first 12 h and then is more moderate. Both phases closely approximate first-order kinetics, $\ln C = A - K_e t$, where C is the 5-Fu concentration in μg/ml, t is the time in which the samples were obtained and K_e is the elimination constant.

The pharmacokinetic parameters are given in Table III.

TABLE III

PHARMACOKINETIC PARAMETERS OF 5-Fu

Phase	Elimination constant, K_e (h^{-1})	Half-life time, $t_{1/2}$ (h)
First	0.282	2.45
Second	0.025	27.72

CONCLUSION

Reversed-phase HPLC is suitable for the determination of the remaining 5-Fu in vitreous gel and liquid samples after intravitreal therapy. The method requires a previous clean-up of the sample in which the addition of sodium sulphate solution and the drying step are extremely important.

This method is very useful for following the pharmacokinetic behaviour of intravitreal therapy with 5-Fu. There is almost no drug left in the vitreous cavity of rabbit eyes 12 h after injection of 5-Fu through this route.

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