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High-performance liquid chromatographic determination of vancomycin in rabbit serum, vitreous and aqueous humour after intravitreal injection of the drug[1]

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Abstract

A high-performance liquid chromatographic method for the determination of vancomycin in rabbit serum, vitreous and aqueous humour has been developed. No clean-up step was necessary for vitreous and aqueous humour samples. For serum samples liquid–liquid and solid-phase extraction were tested and the best results were achieved using C_{18} cartridges. The extracts were analyzed on a C_{18} reversed-phase column, using a mixture of 0.05 *M* phosphate buffer (pH 4) with 10% of acetonitrile as mobile phase. The detection was carried out at 198 nm, which allows higher sensitivity. The average quantitation limit obtained was 0.03 μg/ml. The method has been applied to the study of the residual quantities of vancomycin in serum and rabbit eyes after intravitreal administration of the drug in endophthalmitis treatment.

Keywords: Sample preparation; Optimization; Vancomycin; Antibiotics

1. Introduction

Bacterial endophthalmitis is a serious complication of intraocular surgery and eye trauma. Many bacteria have been documented as etiologic agent in this process. *Staphylococcus epidermidis* and *S. aureus* account for more than 50% of the pathogens in this disease [1]. Vancomycin, an antibiotic effective against all Gram-positive bacteria, has been increasingly recommended for endophthalmitis treatment [2]. Despite its increasing therapeutic role, there are few published studies on the concentrations of intravitreal vancomycin in healthy and in infected eyes.

Therefore, it is necessary to have an exact method to adequately measure the concentrations of vancomycin in serum, vitreous and aqueous humour of rabbits.

Different methods for determining vancomycin in biological fluids have been developed. These include enzyme-multiplied immunoassay techniques (EMIT) [3], radioimmunoassay (RIA) [4] and fluorescence polarization immunoassay (FPIA) [5–8]; however, these methods had insufficient specificity and sensitivity and were expensive [4,9].

Several HPLC methods have also been described; some of those use liquid–liquid [10–13] or solid-

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phase [14,15] extraction followed by reversed-phase chromatography on a C_8 [14] or C_{18} [7,10,16] column, or by normal-phase on an aminopropyl column [17]; detection is carried out on a UV–Vis detector at different wavelengths as 235 nm [10], 240 nm [17], 254 nm [14], or 280 nm [11]. From the absorption spectrum of vancomycin it can be seen that absorption at these wavelenghts is less than that at 198 nm, and thus the sensitivity of these methods can be improved.

In the present work an HPLC procedure to evaluate residual amounts of drug in serum, vitreous and aqueous humour from rabbit has been developed, in an attempt to get a shorter procedure with improved sensitivity.

2. Experimental

2.1. Reagents

Vancomycin hydrochloride and caffeine were supplied by Sigma (St. Louis, MO, USA). Monobasic ammonium phosphate and all other chemicals used for the preparation of buffers were of analytical grade (Merck, Darmstad, Germany). HPLC-grade acetonitrile was obtained from Scharlau (Barcelona, Spain). The water used was purified by passage through a Compact Milli-RO and Milli-Q water system from Millipore (Milford, MA, USA). Ethyl ether, *n*-propanol, ethyl acetate, chloroform, dichloromethane and methanol were obtained from S.D.S. (Peypin, France).

2.2. Apparatus 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 column used was 15×0.46 cm I.D. packed with 5-μm particles of Spherisorb 5 ODS 1 from Phenomenex (Torrance, CA, USA). It was termostated as required by using a CAL 9900 advanced air oven from CAL Controls (Libertyville, IL, USA). As the vancomycin could be interfered by peaks from the matrix, the different experimental parameters influencing retention (pH of the phosphate buffer 0.05 *M*, percentage of acetonitrile and column temperature) were optimized, and the most suitable wavelength to detect the compound studied was selected. The mobile phase was pumped at a flow-rate of 1.0 ml/min. Samples were injected by means of a Rheodyne 7125 injector (Berkeley, CA, USA) with a fixed-volume loop of 20 μl.

2.3. Animal study

The animals were maintained and used in accordance with the Association for Research in Vision and Ophthalmology resolution on the use of animals in research.

A total of 54 pigmented rabbits (54 eyes), each weighing 1.5 to 2 kg, were divided into two groups. Group I (n=27) included normal eyes and group II infected eyes. All operations and intraocular injections were performed with the animal under general anaesthesia by using a mix of intramuscular ketamine hydrochloride (35 mg/kg of body mass) and xylazine hydrochloride (3 mg/kg of body mass). In addition, one drop of proparacaine hydrochloride (0.5%) was applied topically. To induce an experimental endophtalmitis (group II) the vitreous of the right eye of these animals was inoculated with 0.1 ml of solution with 2000 cfu/ml of *S. aureus*. Four days after inoculation endophtalmitis was developed and the eyes were prepared to the study. A 0.1-ml aliquot of a solution containing 1 mg of vancomycin was injected into the vitreous of the right eyes of all the rabbits using a 27 gauge needle. Following drug administration samples were obtained at 0, 2, 4, 6, 12, 24, 48, 72 and 84 h. At each time point three animals were killed by using intracardiac injection and samples of serum, aqueous and vitreous humor were taken.

2.4. Internal standard calibration

Stock solutions of vancomycin and caffeine were prepared in deionized water at a concentration of 100 μg/ml. Standard solutions were prepared from the stock solutions by sequential dilution with water. Drug-free samples spiked with known amounts of vancomycin and caffeine were analysed concurrently with each set of unknown samples. At least seven

different concentrations of vancomycin across the working range were measured in quintuplicate. Calibration graphs were obtained by using the least-squares method. Peak-area ratios between vancomycin and caffeine were used to make the least-squares regression line. The concentrations of vancomycin in the samples were determined by interpolation from the graphs using the peak-area ratios obtained from unknown samples. Blank samples were used to monitor potential interfering substances.

2.5. Sample extraction

Extraction procedures for serum 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 of 1 ml (C_{18} Sep-Pak cartridges, Millipore, Milford, MA, USA). The cartridges were preconditioned by flushing with 3 ml of methanol and 3 ml of water. The samples were loaded on the cartridges and the unwanted components were eluted with 0.5 ml of water. The vancomycin was eluted with 1 ml of methanol and injected in the chromatographic system.

In the liquid–liquid extraction different mixtures of organic solvents were added to serum samples (1 ml), raw samples and samples spiked with vancomycin, were placed into separatory funnels and shaken for 10 min. Then the organic phase was separated and evaporated to dryness in a rotatory evaporator from Büchi (Flawil, Switzerland). The residues obtained were dissolved in 1 ml of mobile phase, filtered on 0.2-μm filters and injected onto the chromatographic system.

2.6. Sample preparation

Aliquots of vitreous or aqueous humour (200 μl) were taken, and 25 μl of caffeine (100 μg/ml) as internal standard were added. The mixture was filtered through a 0.2-μm filter and then injected onto the chromatographic system.

To analyze serum samples, 1 ml of serum was loaded on a C_{18} cartridge previously conditioned. The unwanted compounds were eluted with 0.5 ml of water and after that, vancomycin was eluted with 1 ml of methanol. This solution, with the internal standard added, was injected.

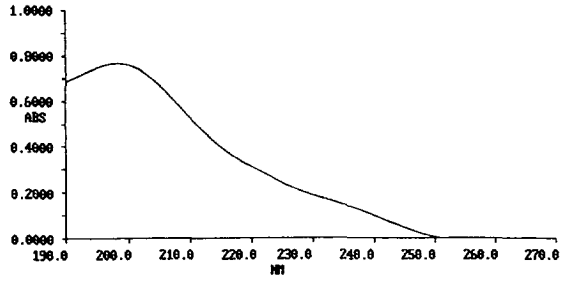

Fig. 1. Absorption spectrum of vancomycin.

3. Results and discussion

The absorption spectrum of vancomycin (Fig. 1) shows the highest absorbance at 198 nm followed by a sharp absorbance decrease; thus, the wavelength of 198 nm was chosen in order to obtain the highest sensitivity.

3.1. Influence of separation parameters

As mentioned before, we studied the effect of the mobile-phase pH, the organic modifier content in the phase and the column temperature on the determination in order to optimize the resolution and avoid overlapping with interferent peaks from the matrix.

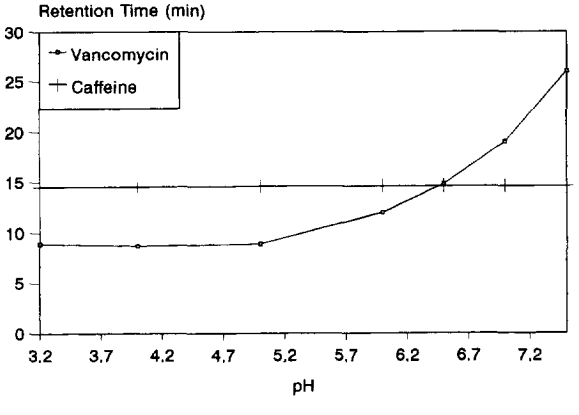

Fig. 2. Variations in the retention time of the compounds as a function of the pH of the mobile phase.

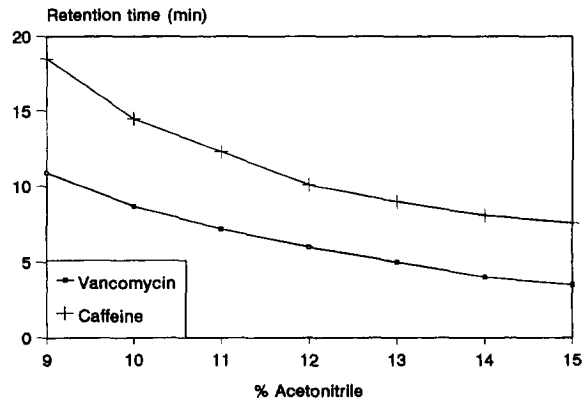

Fig. 3. Variations in retention time of the compounds as a function of the percentage of acetonitrile in the mobile phase.

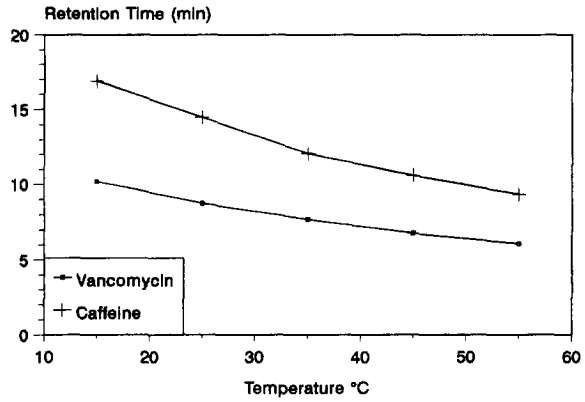

Fig. 4. Influence of column temperature on retention time of the compounds.

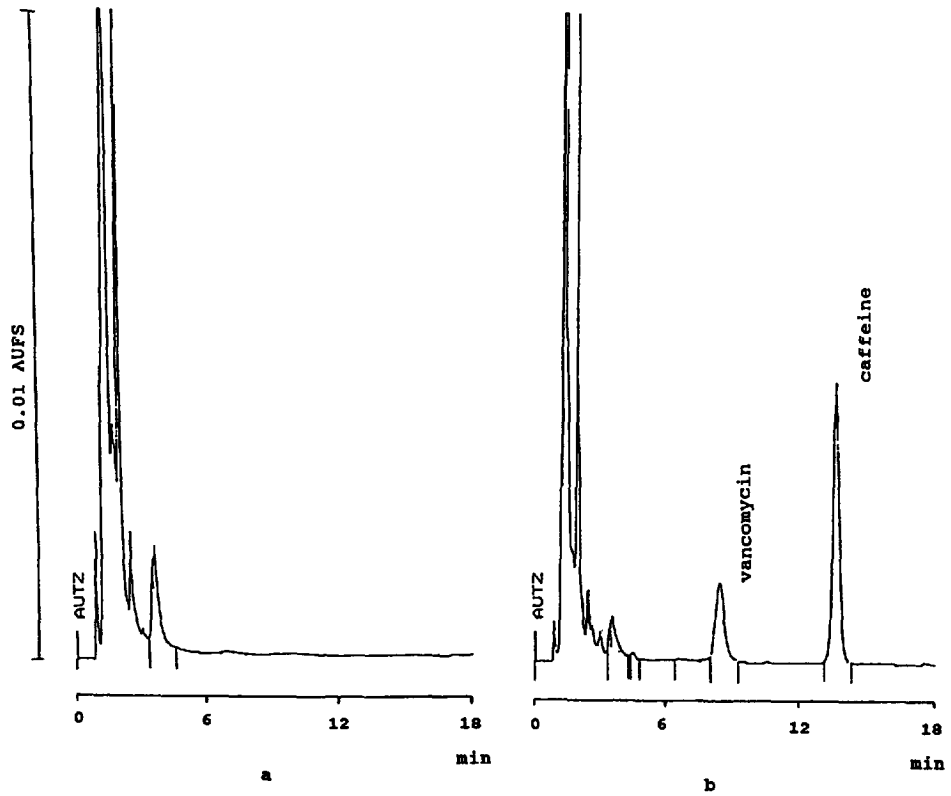

Fig. 5. Chromatogram showing (a) a blank of a vitreous humour sample and (b) a vitreous humour sample spiked with vancomycin and caffeine.

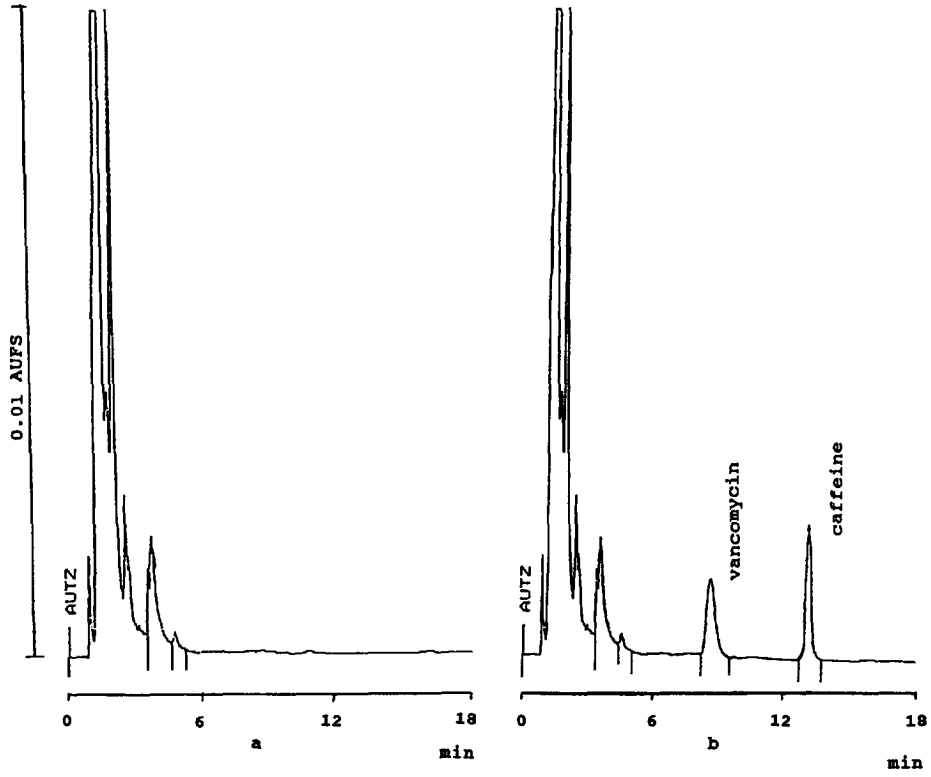

Fig. 6. Chromatogram showing (a) a blank of an aqueous humour sample and (b) an aqueous humour sample spiked with vancomycin and caffeine.

3.1.1. The influence of pH

The influence of the pH of the mobile phase was studied by using 0.05 *M* solutions of ammonium dihydrogenphosphate (10% acetonitrile) at different pHs adjusted to 3–7.5 with 0.1 *M* HCl or 0.1 *M* NaOH. These solutions were used as the mobile phase and a mixture of vancomycin and the internal standard (caffeine) was injected with each mobile phase. Fig. 2 shows the retention time for the compounds at different pH. As can be seen, vancomycin retention time is much more affected by the pH than the internal standard which is practically unaffected.

3.1.2. Organic modifier content

We used acetonitrile at concentrations between 9% and 15% as organic modifier. The mobile phase used was 0.05 *M* phosphate buffer (pH 4) containing different proportions of acetonitrile. The mixture of the standards was thus injected with mobile phase of different composition. Fig. 3 shows the retention times obtained for vancomycin and caffeine as a function of the acetonitrile content in the mobile phase. As can be seen, increasing the proportion of acetonitrile causes a decrease in retention time.

3.1.3. Column temperature

In order to determine the effect of temperature, the column was termostated from 15 to 55°C at intervals of 10°C, and a mobile phase consisting of 0.05 *M* phosphate buffer (pH 4) containing 10% of acetonitrile was used. Fig. 4 shows the retention times obtained as a function of the column temperature. As can be seen, with increasing temperature retention times decrease.

Taking into account the results obtained above, a mobile phase of monobasic ammonium phosphate

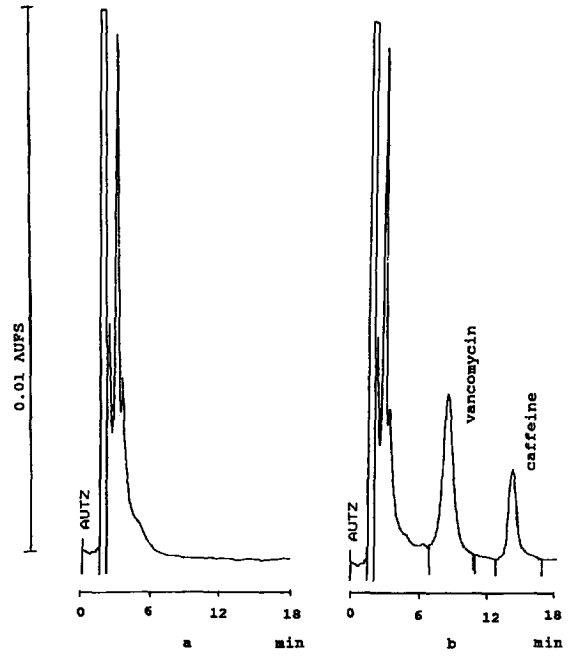

Fig. 7. Chromatogram showing (a) a blank of a serum sample and (b) a serum sample spiked with vancomycin and caffeine after the extraction procedure.

0.05 *M*, pH 4, containing 10% acetonitrile and a working temperature of 25°C were selected.

3.2. Extraction clean-up

To analyze aqueous and vitreous humour samples it was not necessary to perform a clean-up procedure because these samples did not give peaks that could interfere with the vancomycin peak. This can be seen in Fig. 5 and Fig. 6, where chromatograms of blank samples (raw and spiked) are shown.

However, to analyze serum samples a clean-up step must be included to avoid interfering peaks. Liquid–liquid extraction (LLE) and solid-phase extraction (SPE) were studied. Several solvents (dichloromethane, chloroform, ethyl acetate, chloroform–methanol, ethyl acetate–methanol and propanol–diethyl ether) to extract the vancomycin selectively were assayed. The addition of bases, acids and salts to increase the extraction recovery was also studied. The best achieved recovery was not higher than 5% in the most favourable conditions.

In the SPE study C_{18} cartridges were used which were preconditioned by flushing with 3 ml of methanol and 3 ml of water. The experiments were performed in triplicate: 1 ml of a vancomycin solution (10 μg/ml), 1 ml of blank serum and 1 ml of spiked serum were loaded on three different cartridges, the cartridges were dried under a flow of N_2, eluted with 1 ml of methanol and then injected. It could be observed that vancomycin remained in the cartridge while the interfering compounds from the serum were practically not retained. Eluting with 1 ml of methanol the vancomycin recovery was near 97%; adding more volumes of methanol did not improve the recovery. However, if 0.5 ml of water were passed before the elution with methanol the chromatogram was cleaner and at the same time the recovery was the same. This can be seen in Fig. 7 where chromatograms of blank serum samples (raw and spiked) are shown. After making this procedure

Table 1
Non-infected rabbit eyes, group I

Time after administration (h)	Concentration (mean±S.D., n=3) (μg/ml)		
	Vitreous humour	Aqueous humour	Serum
0	1199.50±185.21	–	–
2	1118.12±223.73	16.91±3.30	–
4	1057.45±114.25	42.31±25.25	0.25±0.11
6	619.18±59.71	47.25±18.54	0.47±0.22
12	580.83±39.52	57.38±13.46	0.14±0.10
24	268.57±89.85	29.05±25.91	–
48	295.16±80.67	38.78±14.95	–
72	271.52±96.05	13.83±5.50	–
84	121.82±28.46	12.84±3.17	–

Vancomycin concentrations after a single 1-mg intravitreal injection of vancomycin.

Table 2
Infected rabbit eyes, group II

Time after administration (h)	Concentration (mean±S.D., n=3) (μg/ml)		
	Vitreous humour	Aqueous humour	Serum
0	2084.73±603.85	7.76±0.14	–
2	2053.65±592.04	15.17±1.25	0.13±0.11
4	2024.36±571.87	27.08±0.57	0.39±0.15
6	1587.29±407.65	7.59±0.42	0.53±0.19
12	978.22±69.08	4.20±0.31	0.89±0.15
24	671.50±394.44	3.15±0.59	0.73±0.22
48	254.51±166.73	1.57±0.23	0.55±0.30
72	60.75±40.47	1.58±0.11	0.06±0.05
84	41.03±26.53	1.43±0.16	–

Vancomycin concentrations after a single 1-mg intravitreal injection of vancomycin.

for different concentrations of vancomycin, an average of 97.5±0.3% in recovery was obtained.

3.3. Calibration graphs

The calibration graphs for vancomycin were linear from the limit of quantitation to at least 100 μg/ml. The calibration graph for serum was obtained from the extraction of spiked serum samples. The limits of quantitation obtained ($s_b+10\ \sigma$, s_b being the average signal of the blank and σ the standard deviation) for serum, aqueous and vitreous humour are 0.04, 0.03 and 0.03 μg/ml, respectively.

3.4. Application of the method to experimental samples

In Table 1 and Table 2 the average results obtained to apply the experimental rabbit samples are shown. It can be appreciated that the initial concentration of vancomycin in vitreous humour in infected eyes is nearly twice that of non-infected eyes. Nevertheless vancomycin concentrations in aqueous humour are higher in non-infected eyes than in infected ones. Both concentrations are low in serum samples but they last longer in infected eyes. This could be explained assuming that the inflamation produced in infected eyes made drug distribution worse and so the concentrations in vitreous humour would be higher in infected eyes than in the non-infected ones.

3.5. Conclusion

The proposed procedure is fast and simple for vancomycin analysis in rabbit serum, aqueous and vitreous humour samples. Selecting a 198 nm wavelength for detection allows a higher sensitivity.

To analyze serum samples a clean-up step on C_{18} cartridges is necessary that avoids interference peaks and gives drug recoveries of more than 95%. On the other hand aqueous and vitreous humour samples do not need a special treatment.

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