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Determination of ofloxacin in human aqueous humour by high-performance liquid chromatography with fluorescence detection

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Abstract

A reversed-phase high-performance liquid chromatographic method is described for the determination of ofloxacin in human aqueous humour; the method involves fluroescence detection (excitation at 290 nm; emission at 500 nm) after direct injection of samples. The method utilized a 100 mm × 8 mm i.d. cartridge column packed with 4 μm Novapak C_{18} with a mobile phase methanol–acetonitrile–0.4 M citric acid (3:1:10, v/v/v) and a flow rate of 1 ml min^{-1} at ambient temperature. The retention times for the internal standard pipemidic acid and for ofloxacin were 4.82 and 7.32 min respectively. The mean recovery ($\pm$ ISD) from human aqueous humour was 103.24 $\pm$ 4.45% for ofloxacin at 1 μg ml^{-1} ($n = 6$). The within-day and day-to-day RSDs at 0.1 μg ml^{-1} and 1 μg ml^{-1} were less than 6.71% ($n = 6$) and the lower limit of reliable determination corresponding to a signal-to-noise ratio of 2.5:1 was 20 ng ml^{-1}. The assay was shown to be suitable for measuring ofloxacin levels in human aqueous humour samples after topical, oral and intravenous administration.

Keywords: Aqueous humour; High-performance liquid chromatography; Ofloxacin

1. Introduction

Ofloxacin is a fluorinated 4-quinolone and has a wide spectrum of antibacterial activity [1]. Ofloxacin is among the fluoroquinolones considered promising for the treatment of ocular infections [2]. Oral administration of ofloxacin produces concentrations in the aqueous humour that may have therapeutic value [3]. An ophthalmic solution of ofloxacin was introduced for the topical treatment of ocular infections caused by susceptible Gram-negative and Gram-positive bacteria [4].

Analysis of fluoroquinolones in pharmacokinetic studies has relied mainly on a variety of microbiological methods [5] which are non-selective and imprecise compared with more recent approaches using high-performance liquid chromatography (HPLC) [6]. Ofloxacin in biological specimens such as plasma and urine has also been

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determined by microbiological methods [7] or by HPLC with ultraviolet [5,8] or fluorescence detection [9–12]. To investigators' knowledge, no technique has been published for the measurement of ofloxacin in aqueous humour. In the present paper, sensitive, precise and simple HPLC method is described for the analysis of ofloxacin in human aqueous humour after direct injection of samples.

2. Materials and methods

2.1. Chemicals and reagents

Analytical-grade citric acid (Sigma, St. Louis, MO) and HPLC-grade methanol and acetonitrile (Baker, Phillipsburg, NJ) were used. Pipemidic acid was purchased from Sigma (St. Louis, MO) and ofloxacin was kindly donated by Hoechst (Istanbul, Turkey). Stock solutions of ofloxacin (1 mg ml^{-1}) and pipemidic acid (1 mg ml^{-1}) were prepared in HCl (0.01 M) and NaOH (0.2 M) respectively. Standard solutions were prepared daily by diluting stock solutions in water to concentrations of 0.05, 0.1, 1, 5, 10 and 20 $\mu g\ ml^{-1}$ ofloxacin containing pipemidic acid (20 μl, 100 $\mu g\ ml^{-1}$) as the internal standard. Aqueous humour standard containing added ofloxacin (1 $\mu g\ ml^{-1}$) and pipemidic acid (20 μl, 100 $\mu g\ ml^{-1}$) was prepared in the same way as the samples. Validation studies were conducted with aqueous humour pooled from different patients to whom no ofloxacin had been administered. Stock solutions and samples were stored at −25°C until analysis and all solutions were protected from light because of the light sensitivity of pipemidic acid. Construction of a calibration curve in drug-free aqueous humour was not possible because insufficient drug-free aqueous humour was available. A calibration curve of the peak-area ratio of ofloxacin to pipemidic acid versus concentration of ofloxacin in water was used to determine ofloxacin levels in samples of human aqueous humour.

2.2. Chromatography

The HPLC equipment comprised a solvent delivery system (Jasco, Model PU-980, Tokyo, Japan), a Rheodyne injection block (Model 7125, Cotati, CA), a fluorescence detector (Waters, Model 470, MA) and a data module (Waters, Model 746, MA). The analytical column was a 100 mm × 8 mm i.d. cartridge packed with 4 μm Novapak C_{18} (Waters) compressed in a Radial-Pak cartridge holder (RCM 8 × 10, Waters) in conjunction with a pre-column module (Guard-Pak, Waters) containing a NovaPak C_{18} insert. The mobile phase was methanol–acetonitrile–0.4 M citric acid (3:1:10, v/v/v) and the flow rate was 1 ml min^{-1} at ambient temperature. The excitation and emission wavelengths were set to 290 nm and 500 nm respectively.

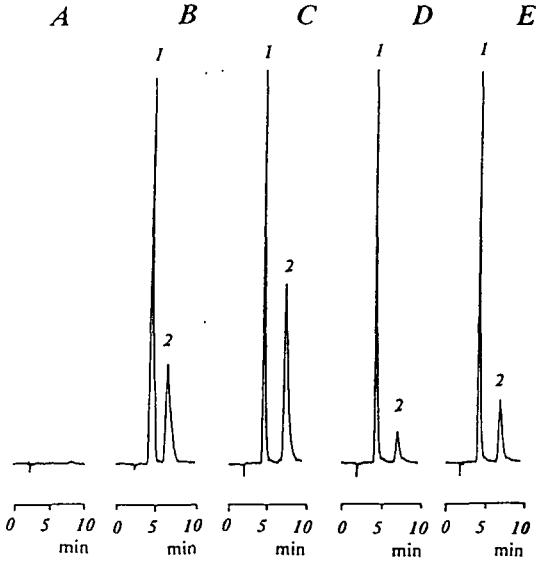

Fig. 1. Representative chromatograms of (A) drug-free aqueous humour, (B) spiked aqueous humour containing 1 $\mu g\ ml^{-1}$ of ofloxacin, (C) a patient's aqueous humour containing 2.16 $\mu g\ ml^{-1}$ of ofloxacin after topical administration, (D) a patient's aqueous humour containing 0.44 $\mu g\ ml^{-1}$ of ofloxacin after oral administration and (E) a patient's aqueous humour containing 0.70 $\mu g\ ml^{-1}$ of ofloxacin after intravenous administration. Chromatograms were monitored with a fluorescence detector at 290 nm (excitation) and 500 nm (emission). The signal of the detector was constant at 10 min. Peaks: 1, pipemidic acid (internal standard); 2, ofloxacin.

Table 1
Aqueous humour levels (μg ml^{-1}) of ofloxacin in subjects after topical, oral and intravenous administration of the drug

Subject code	Topical administration	Subject code	Oral administration	Subject code	Intravenous administration
F.A.	2.16	A.S.	0.26	M.T.	0.47
B.A.B.	0.65	N.G.	0.31	R.Y.	0.70
A.U.	0.81	G.K.	0.44	H.T.	0.48
H.K.	0.45	A.T.	0.41	B.Ç.	0.57
Mean	1.02	Mean	0.36	Mean	0.56
SD	0.67	SD	0.07	SD	0.09

2.3. Sample preparation

Aqueous humour samples were diluted with distilled water to ratio of 1:8 (v/v) and pipemidic acid (20 μl, 100 μg ml^{-1}) was added as the internal standard. After mixing on a vortex-mixer for 30 s, 20 μl of the solution was injected on to the column. All analyses were performed in duplicate.

2.4. Recovery

The recovery of ofloxacin was determined by comparison of an aqueous humour standard with a standard solution in water prepared at the same concentration. The recovery was the mean of six replicates (mean $\pm$ standard deviation).

2.5. Collection of aqueous humour samples from subjects

Patients aged 55–80 years were administered topical, oral and intravenous ofloxacin. In the topical administration procedure, one drop of ofloxacin ophthalmic drops (0.3%, Ocuflox, Allergan, Irvine CA) was instilled every hour for five doses. Aqueous humour samples were drawn by paracentesis 30 min after the last dose. In the oral administration procedure, patients were given a single dose of ofloxacin (200 mg Tarivid; Hoechst, Istanbul, Turkey) 12 h before cataract extraction. In the intravenous treatment, patients were injected a single dose of ofloxacin (200 mg Tarivid) 2.5 h before surgery. All administrations were done by a nurse to ensure compliance. None of the patients had any ocular pathology other than cataracts. Written informed consent was given by all patients.

3. Results and discussion

Fig. 1A shows a fluorescence chromatogram obtained after direct injection of a drug-free aqueous humour sample; no peak occurred. Similarly, it has been reported that although a high level of background absorption and many interfering peaks were observed with aqueous humour samples after direct injection when using a UV detector, but not with a fluorescence detector [6]. Pipemidic acid and ofloxacin appeared as well-resolved peaks in both the standard aqueous humour sample (Fig. 1B) and in the aqueous samples from patients after topical, oral and intravenous administration of ofloxacin (Figs. 1C–1E). The retention times of pipemidic acid and ofloxacin were 4.82 and 7.32 min respectively.

The mean recovery ($\pm$ SD) from aqueous humour was 103.24 $\pm$ 4.45% for ofloxacin at 1 μg ml^{-1} ($n = 6$). The calibration curve for ofloxacin was linear over the concentration range 0.05–20 μg ml^{-1} ($y = -0.1152 + 0.5622x$; $r = 0.9995$); standard errors for the slope and intercept of the calibration curve were 0.0079

and 0.0682 respectively. The within-day reproducibility was determined for samples containing 0.1 and 1 μg ml^{-1} of ofloxacin ($n = 6$); the relative standard deviations (RSDs) were 1.59% and 5.26% respectively. For the day-to-day reproducibility the RSDs were 4.53% and 6.71% at 0.1 μg ml^{-1} of ofloxacin ($n = 6$) respectively. The lower detection limit was 20 ng ml^{-1} for ofloxacin (signal-to-noise ratio = 2.5).

Ofloxacin levels in aqueous humour samples after topical, oral and intravenous administrations of ofloxacin are given in Table 1. The mean ($\pm$S.D.) ofloxacin levels in aqueous humour after topical, oral and intravenous administration were 1.02 ± 0.67, 0.36 ± 0.07 and 0.56 ± 0.09 μg ml^{-1} respectively. The concentration range was 0.26–2.16 μg ml^{-1}, indicating that an approximately eightfold difference between the highest and lowest concentrations was easily detectable. The results demonstrate the usefulness of the present method for the determination of ofloxacin in aqueous humour samples after administration of ofloxacin by different routes.

In conclusion, a fast, sensitive and reliable method has been developed for the determination of ofloxacin in human aqueous humour samples after topical, oral and intravenous administration; this simple approach permits a reduction in sample processing.

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