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Short Communication

Determination of ciprofloxacin levels in chinchilla middle ear effusion and plasma by high-performance liquid chromatography with fluorescence detection

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ABSTRACT

An isocratic high-performance liquid chromatographic method has been developed to determine ciprofloxacin levels in chinchilla plasma and middle ear fluid. Ciprofloxacin and the internal standard, difloxacin, were separated on a Keystone ODS column (100 × 2.1 mm I.D., 5 μm Hypersil) using a mobile phase of 30 m*M* phosphate buffer (pH 3), 20 m*M* triethylamine, 20 m*M* sodium dodecyl sulphate–acetonitrile (60:40, v/v). The retention times were 3.0 min for ciprofloxacin and 5.2 min for difloxacin. This fast, efficient protein precipitation procedure together with fluorescence detection allows a quantification limit of 25 ng/ml with a 50 μl sample size. The detection limit is 5 ng/ml with a signal-to-noise ratio of 5:1. Recoveries (mean ± S.D., $n = 5$) at 100 ng/ml in plasma and middle ear fluid were 89.4 ± 1.2% and 91.4 ± 1.6%, respectively. The method was evaluated with biological samples taken from chinchillas with middle ear infections after administering ciprofloxacin.

INTRODUCTION

Antibiotic treatment of acute otitis media (AOM) has a failure rate of 5–10%. About 30% of patients develop recurrent infection [1]. In an effort to explain these treatment failures and possibly prevent recurrence, we have conducted experimental studies on the effect of antibiotic penetration and antimicrobial treatment response in models of AOM [2,3]. To study antimicrobial penetration into and out of the middle ear, it is essential to develop analytical methods that allow us to precisely measure various antimicrobials at low concentrations in very small volumes of middle ear fluid (MEF) [4,5].

Ciprofloxacin, a new fluoroquinolone, exhibits activity against a broad range of bacteria. Highly effective in treating a wide variety of infectious diseases, it appears to function by inhibiting the bacterial DNA gyrase (topoisomerase II) [6]. Its relatively high apparent volume of distribution (> 2 l/kg) implies good penetration into extravascular space such as the middle ear, hence its relevance to AOM. In addition, ciprofloxacin could help treat chronic otitis media and mastoiditis caused by a *Pseudomonas* infection (a serious and difficult clinical problem) [7].

Prevalent methods to determine ciprofloxacin levels use reversed-phase high-performance

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liquid chromatography (HPLC), fluorescence detection and a variety of internal standards. HPLC methods were developed for use in determining ciprofloxacin levels in biological fluids such as plasma and urine. However, a method to determine ciprofloxacin concentrations in a matrix such as MEF has not been reported. Weber *et al.* [8] used a combination of fluorescence and UV detection to determine ciprofloxacin in human plasma without the aid of an internal standard.

A comparison of HPLC and bioassays for determining ciprofloxacin levels in plasma and urine [9] suggests that bioassays yield equivalent values for plasma samples, but overestimate ciprofloxacin concentrations in urine.

Difloxacin has been used as an internal standard for determining ciprofloxacin concentrations in human plasma and urine [10]. The method requires 0.5 ml of plasma for analysis. El-Yazigi and Al-Rawithy [11] also determined ciprofloxacin in human plasma with quinine bisulphate as the internal standard and a limit of detection (LOD) of 50 ng/ml. Mack [12] developed an assay for ciprofloxacin and its three metabolites in human serum, urine, saliva and sputum. This method used UV detection with a detection limit of 50 ng/ml for ciprofloxacin and 250 ng/ml for the metabolites, for a 1-ml sample volume. Fast LC has also been done using a 30 × 4.6 mm ODS column and relying on UV detection [13]; it had a detection limit of 100 ng/ml for a 1-ml sample volume. Ciprofloxacin concentration has also been determined in the presence of non-steroidal anti-inflammatory drugs, including fenbufen and felbinac, in rat plasma [14]. This method uses 50 μl of sample with a ciprofloxacin detection limit of 200 ng/ml.

EXPERIMENTAL

Chemicals

All chemicals were of analytical grade and included phosphoric acid, sodium phosphate, triethylamine and sodium dodecyl sulphate (Sigma, St. Louis, MO, USA). Acetonitrile (Fisher, Fairlawn, NJ, USA) was HPLC grade. Ciprofloxacin (Sigma) was United States Pharmacopoeia grade; difloxacin was a gift from Abbott Laboratories (North Chicago, IL, USA). Chinchilla plasma and MEF samples were obtained from the University of Minnesota Otitis Media Research Center.

Instrumentation and chromatography

Chromatography was done with a Hewlett Packard 1090 L liquid chromatograph (Hewlett Packard, Palo Alto, CA, USA) and an HP 1046 A programmable fluorescence detector set at 278 nm excitation and 456 nm emission with a 399 nm cut-off filter. The mobile phase was pumped at 0.35 ml/min through a Keystone Scientific (Bellefonte, PA, USA) 100 × 2.1 mm I.D. column and matching 10 × 2.1 mm I.D. precolumn, packed with 5-μm C_{18} Hypersil packing material and maintained at 45°C. The mobile phase consisted of 30 m*M* NaH_2PO_4, 20 m*M* triethylamine (TEA) and 20 m*M* sodium dodecyl sulphate (SDS) buffer solution, adjusted to pH 3.0 with phosphoric acid and acetonitrile (60:40, v/v). Data were collected with an HP 3396A integrator and analysed using Chrom-Perfect software (Justice Innovations, Palo Alto, CA, USA).

Stock solutions and standards

Ciprofloxacin and difloxacin (internal standard) were made up as 0.1 mg/ml stock solutions in methanol and distilled water (1:10, v/v). Ciprofloxacin was diluted with distilled water to make additional working stocks of 1 and 10 μg/ml. Difloxacin was diluted with distilled water to make a single working internal standard stock solution of 10 μg/ml. Standards for the calibration curve were prepared from 25 ng/ml to 1500 ng/ml by spiking pooled MEF or plasma with an appropriate amount of ciprofloxacin.

Sample preparation

A 50-μl aliquot of plasma, MEF or standard matrix was pipetted into a 12 × 75 mm disposable culture tube to which 20 μl of the 10 μg/ml internal standard solution were added. Protein precipitation was carried out by adding 2 ml of acetonitrile, vortex-mixing briefly and centrifug-

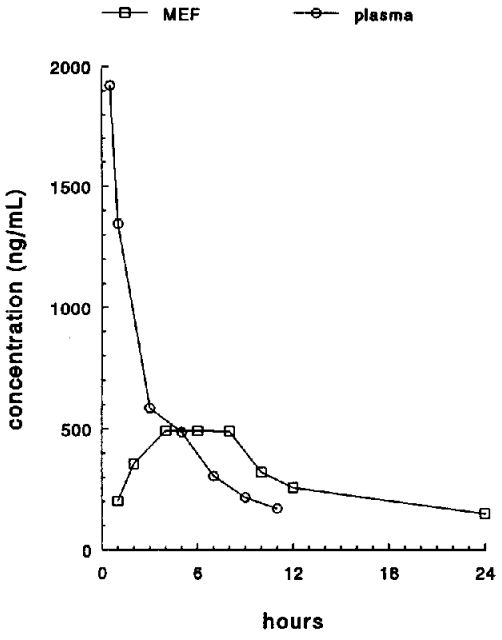

Fig. 1. Concentration–time profile of ciprofloxacin in chinchilla plasma and middle ear fluid after an intramuscular injection of 6 mg/kg ciprofloxacin.

ing at 1500 *g* for 10 min. The acetonitrile was then transferred to a clean 10 × 75 mm disposable culture tube and evaporated to dryness under nitrogen in a 50°C water bath. The residue was then reconstituted in 75 μl of mobile phase and transferred to autoinjector vials; 5 μl were injected on the column.

Application of the method

To test the assay in experimental conditions, twenty chinchillas were given 6 mg/kg ciprofloxacin by intramuscular injection. Samples of plasma and MEF were collected from 0.5 to 24 h after the dose. The log concentration *versus* time data were plotted and the half-life of elimination was calculated from the linear portion of each animal's curve.

RESULTS

Fig. 1 shows the mean concentration at each sample collection time point. In plasma, ciprofloxacin had a mean ± S.D. disappearance half-life of 3.2 ± 1.1 h. The concentration of the drug in MEF was significantly lower than in plasma. The time required to achieve maximum concentration was also prolonged: a maximum MEF concentration of 500 ng/ml occurred at about 6 h post dose (Fig. 1). Ciprofloxacin disappearance half-life from the MEF was 15.0 ± 11.8 h.

Recovery, precision and accuracy

Recovery was determined by comparing the peak heights of treated plasma and lavage samples with the peak heights of standard injections of the same concentration. Recoveries (mean ± S.D., $n = 5$) at 100 ng/ml in plasma and middle ear effusion were 89.4 ± 1.2% and 91.4 ± 1.6%, respectively.

Peak heights were used for quantitation. Linear regression of the peak-height ratios *versus* the drug concentration was performed on the standard curve to determine the slope, intercept, variability and strength of correlation. The detection limit was 5 ng/ml at a signal-to-noise ratio of 5:1. The standard curve spanned a range from 25 to 1500 ng/ml. A typical standard curve had a slope of $2.01 \cdot 10^{-4}$, an intercept of $1.3 \cdot 10^{-5}$ and a correlation coefficient of 0.995.

Owing, in large part, to the selectivity afforded by the fluorescence detection, there was no interference from endogenous sample components with this method (see Fig. 2a, a chromatogram of blank chinchilla plasma, and Fig. 2b, a chromatogram of blank chinchilla MEF). Fig. 3a is a chinchilla plasma sample and Fig. 3b is a chinchilla MEF sample, both collected by the University of Minnesota Otitis Media Research Center.

Intra-day calibration curves consisted of three replicates at each calibration level. Inter-day calibration standards and quality control standards were assayed over five days. Analysis was done five times intra-assay and five times inter-assay in plasma and three times intra-assay for ear effusion (Table I). Generation of the blank matrix requires sacrifice of the animals for a very small amount and the matrix is therefore in limited supply.

No degradation of quality control samples was noticed over a two-week period. All real samples

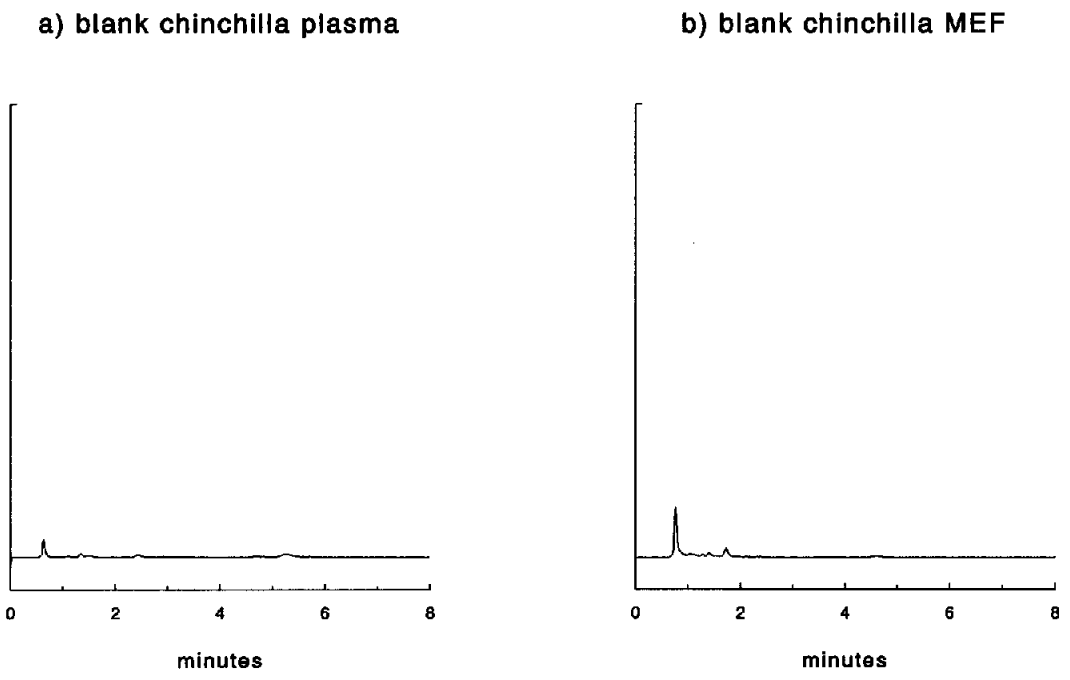

Fig. 2. Chromatograms of (a) blank chinchilla plasma chromatogram and (b) blank chinchilla MEF. Separation of ciprofloxacin and difloxacin, the internal standard, occurred on a Keystone ODS column (100 × 2.1 mm I.D., 5 μm Hypersil), using a mobile phase of 30 m*M* phosphate buffer (pH 3), 20 m*M* TEA, 20 m*M* SDS–acetonitrile (60:40, v/v). The retention times were 3.0 min for ciprofloxacin and 5.2 min for difloxacin. This fast, efficient protein precipitation procedure together with fluorescence detection allows a quantitation limit of 25 ng/ml with a 50 μl sample size; 5 μl injected.

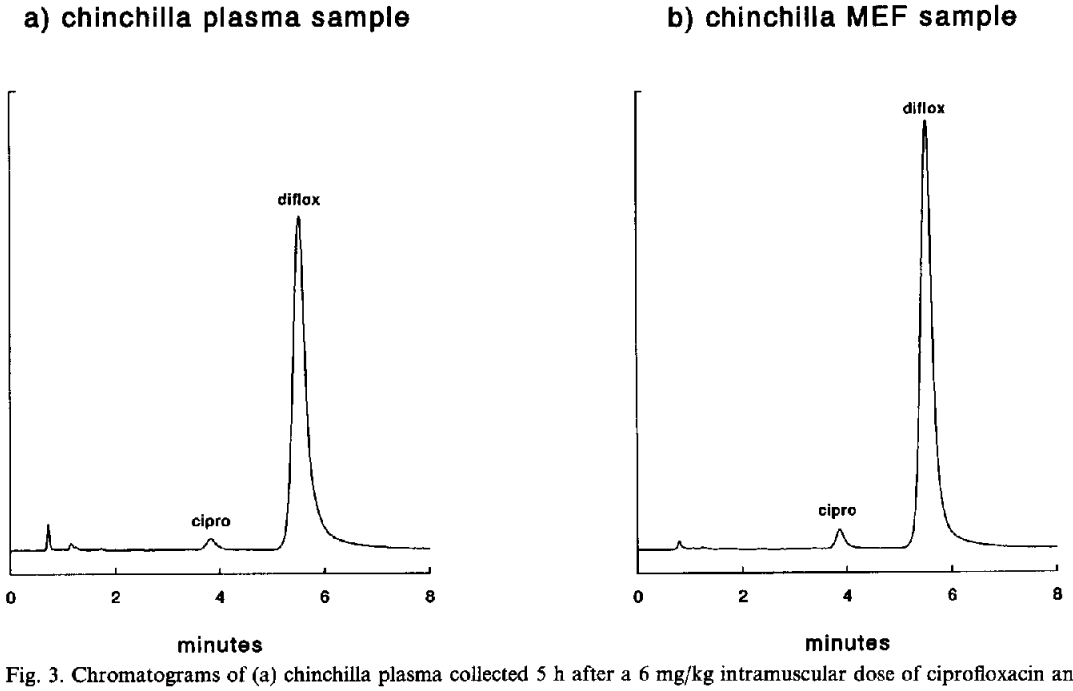

Fig. 3. Chromatograms of (a) chinchilla plasma collected 5 h after a 6 mg/kg intramuscular dose of ciprofloxacin and (b) chinchilla MEF collected 6 h after a 6 mg/kg intramuscular dose of ciprofloxacin. Conditions as in Fig. 2.

TABLE I

INTRA-DAY AND INTER-DAY PRECISION OF CIPROFLOXACIN DETERMINATION IN CHINCHILLA PLASMA AND MIDDLE EAR FLUID

Analysis conditions as in Fig. 2. Quantitation was by a weighted linear calibration curve of peak-height ratios of ciprofloxacin/difloxacin *versus* concentration over the range of 25–1500 ng/ml

Theoretical concentration (ng/ml)	Plasma (n = 5)			Middle ear fluid (n = 3)		
	Concentration found (mean ± S.D.) (ng/ml)	R.S.D. (%)	Accuracy (%)	Concentration found (mean ± S.D.) (ng/ml)	R.S.D. (%)	Accuracy (%)
Intra-day (n = 3)						
50	51.61 ± 1.44	2.79	3.97	49.68 ± 3.79	7.62	−0.63
250	245.00 ± 13.69	5.59	−1.97	248.88 ± 5.34	2.15	−0.45
1500	1487.52 ± 51.67	3.47	−0.83	1465.82 ± 22.38	1.53	−2.28
Inter-day (n = 5)						
50	50.98 ± 4.39	8.61	1.97	50.48 ± 6.65	11.19	0.96
250	244.69 ± 11.77	4.81	−2.13	252.54 ± 8.92	3.53	1.02
1500	1485.46 ± 55.62	3.74	−0.97	1487.94 ± 25.57	1.72	−0.80

were run within two days of collection. Working stocks were made fresh each day.

CONCLUSION

We have described an HPLC method for analysing ciprofloxacin in microlitre volumes of chinchilla plasma and MEF. This method is sensitive and rapid, yet requires as little as a 50-μl sample volume. It involves protein precipitation with acetonitrile and fluorescence detection, with an excitation wavelength of 278 nm and an emission wavelength of 456 nm, which provides excellent analyte selectivity. We use this method to quantify ciprofloxacin in chinchilla plasma and MEF, and are currently using it to study the efficacy and penetration of ciprofloxacin in the middle ear of chinchillas with AOM.

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