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Determination of quinolone antibiotics in growth media by reversed-phase high-performance liquid chromatography

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Abstract

A simple, accurate, precise, and versatile high-performance liquid chromatographic (HPLC) method was developed and validated for the determination of three quinolone antibiotics in Mueller–Hinton broth. The fluoroquinolone agents studied were ciprofloxacin, ofloxacin, and sparfloxacin; other quinolone agents have been identified using this method but not validated in this matrix (levofloxacin, clinafloxacin, temafloxacin, and trovafloxacin). In addition, several other biological growth mediums have been investigated (human serum, human urine, Todd–Hewitt growth media, Ensure enteral feeding solution, and *Haemophilus* growth media). This method uses UV detection (280 nm), a simple, one-step protein precipitation extraction, and separation using a C_{18} column with an isocratic, ion-pairing mobile phase. An appropriate internal standard was obtained by using another quinolone antibiotic of differing retention time. The calibration curves were linear ($r^2 \geq 0.999$) over a concentration range of 0.0625–20.0 μg/ml with a lower limit of quantification of 0.1 μg/ml. The intra-day and inter-day coefficients of variation were less than 15%.

Keywords: Ciprofloxacin; Ofloxacin; Sparfloxacin

1. Introduction

The fluoroquinolone antibiotics are synthetic antimicrobial agents with a broad spectrum of activity against Gram-positive and Gram-negative bacteria used in the treatment of a wide range of infections [1]. The beneficial characteristics of the fluoroquinolones include a unique spectra of antimicrobial activity, favorable side effect profiles, infrequent dosing (long half-lives), and the advantages of both oral and parenteral routes of administration. A number of fluoroquinolone antimicrobial agents have recently been introduced for clinical use, and other members of this class of drugs will soon be made available. There is a need, therefore, for clinical assays that can be applied to a range of these compounds. Specifically, an assay for the determination of quinolone concentrations in complex growth media is necessary for in vitro work in such areas as pharmacokinetic and pharmacodynamic research.

High-performance liquid chromatography (HPLC) assays have been developed to quantify some of the quinolones with ultraviolet [2–6] or fluorescence

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[2,5–9,13–17] detection. Microbiological methods [3,6] have also been used to determine quinolone levels. Of these, the highly specific and sensitive HPLC methods are preferable to the more time-consuming and less specific microbiological methods. In the latter, active metabolites or co-administered drugs can interfere to give high results for the drug [10,11]. Microbiological methods also suffer from poor reproducibility and accuracy [5,12].

Many HPLC assays have been designed to measure a specific fluoroquinolone and often require complicated sample preparation [3,5–17]. The lack of internal standard is reflected in some of the published methods [6,13]. Other limitations noted in some of the present methods include the use of two different mobile phases to separate the metabolites [6,13] and tedious sample preparation [13,14]. Additionally, a majority of these methods utilize fluorescence detection [2,5–9,13–17], which is not commonly available in every laboratory.

Characterization of antimicrobials with in vitro modeling continues to advance and expand the fields of pharmacodynamics and pharmacokinetics. These in vitro models can simulate disease states and physiological conditions via a continuous infusion of complex growth media. An assay is therefore required for the determination of fluoroquinolone antibiotics in commonly used growth media, such as Todd–Hewitt broth. The vast majority of quinolone assays currently available, however, are designed for the evaluation of samples in biological fluids, such as serum and urine.

We have developed a rapid, specific reversed-phase HPLC method for the clinical assay of a variety of quinolones in complex growth media. This isocratic HPLC method uses a simple mobile phase, UV/Vis detection, and a single sample preparation step of deproteination.

2. Experimental

2.1. Chemicals and reagents

Three fluoroquinolones were analyzed: ciprofloxacin from Bayer Pharmaceuticals (West Haven, CT, USA), ofloxacin from R.W. Johnson (Spring House, PA, USA), and sparfloxacin from Rhone Poulenc Rorer, Inc. (Collegeville, PA, USA). Sodium phosphate monobasic was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI, USA). Triethylamine (TEA) and HPLC-grade acetonitrile were purchased from Fisher Scientific, Inc. (Fair Lawn, NJ, USA). Phosphoric acid (85%) and sodium dodecyl sulfate were purchased from Mallinckrodt Chemicals, Inc. (Paris, KY, USA). Mueller–Hinton broth was purchased from Difco, Inc. (Detroit, MI, USA) and steam sterilized in an American CyclomatiControl, Model 57CR, steam sterilization system (American Sterilizer, Erie, PA, USA) before use. All chemicals were reagent grade and used as received without further purification. The water used in the preparations of reagent solutions and mobile phase was purified with a Milli-Q SP TOP system obtained commercially from Millipore (Bedford, MA, USA).

2.2. Standard solutions

Stock solutions of each quinolone were prepared individually by dissolving 25 mg of each quinolone in 25 ml of purified water with a few drops of 1 *M* sodium hydroxide to enhance solubility. The solutions were shaken completely by hand and sonicated for 10 min each in a BranSonic ultrasonic cleaner, Model 1210 (Branson, Danbury, CT, USA). These stock solutions were equivalent to 1000 μg/ml of drug. A stock solution of internal standard was prepared by selecting an appropriate quinolone to be used as an internal standard based on relative retention time. Generally, sparfloxacin served as the internal standard for ciprofloxacin and ofloxacin assays, and ciprofloxacin served as the internal standard for sparfloxacin assays. Exactly 100 ml of the selected internal standard drug stock was added to 900 ml of purified water resulting in a final concentration of 100 μg/ml of drug. All drug stock solutions were stored at −20°C for no longer than 1 month and protected from light in amber flasks covered with aluminum foil.

2.3. Chromatographic conditions

The HPLC system consisted of a Hewlett-Packard series 1050 (HP 1050) (Hewlett-Packard Company,

Wilmington, DE, USA) quaternary solvent delivery system with an HP 1050 autoinjector, an HP 1050 variable wavelength UV/Vis detector, and an HP 1050 column heater and solvent sparging system using compressed helium at 75 p.s.i. from Genex (Des Moines, IA, USA). Data were captured with Hewlett-Packard ChemStation software, version A.00.33, on a generic DOS computer (486/33). The detector was operated at a wavelength of 280 nm and a peak width of 0.27 min. Chromatographic separation was accomplished using a Alltech Adsorbosphere HS C_{18} 7U (150×4.6 mm I.D., 7 μm particle size) HPLC column (stock # 288114, Alltech Associates, Inc., Deerfield, IL, USA) with an Alltech direct connect universal C_{18} 5U cartridge pre-column (stock # 28013 & 28015, Alltech Associates, Inc., Deerfield, IL, USA). In addition, the guard column contained a 2-μm, 4-mm filter element on the inlet side (stock # 28640, Alltech Associates, Inc., Deerfield, IL, USA). The mobile phase consisted of 0.02 *M* sodium phosphate buffer–acetonitrile (65:35, v/v), pH 3, containing 0.2% triethylamine and 0.2% sodium dodecyl sulfate as an ion-pairing agent. The mobile phase was delivered at a constant flow-rate of 1.75 ml/min at 25°C with an Upchurch A-315, 0.45-μm in-line precolumn filter (Upchurch Scientific, Oak Harbor, WA, USA) prior to the autoinjector. Average column backpressure was approximately 1200 p.s.i. (83 bar).

2.4. Mobile phase

A 0.02 *M* solution of sodium phosphate monohydrate (2.76 g in 1 l of purified water) was prepared, and 2 g of sodium dodecyl sulfate was added along with 2 ml of TEA for peak tailing. This solution was adjusted to pH 3.0 with 85% phosphoric acid. Pump A delivered 65% sodium phosphate buffer, while pump B delivered 35% acetonitrile at 1.75 ml/min isocratic flow-rate. The mobile phase mixture can easily be pre-mixed in the appropriate proportions and delivered with a single pump if a binary or quaternary pumping system is unavailable. All solvents were filtered (0.45 μm) and degassed before use. In addition, the mobile phase was sparged with compressed helium at 75 p.s.i. throughout the analysis.

2.5. Calibration standards

Calibration standards for the assay were prepared by adding different volumes of quinolone standard solutions to sterile, drug-free Mueller–Hinton broth. Calibration standards were prepared at concentrations of 0.0625, 0.1, 0.2, 0.5, 1, 2, 5, 10, and 20 μg/ml, in replicates of six. Quality control standards were prepared at 0.2, 1, and 10 μg/ml in replicates of six, for determination of assay precision and accuracy. All calibration standards were prepared daily.

2.6. Sample preparation

A 500-μl aliquot of the blank, calibration standard, quality control sample or unknown sample was transferred to a 13×100 ml glass disposable test tube (Baxter Scientific Products, Deerfield, IL, USA) with an Eppendorf reference-series 2000 adjustable volume pipette (Brinkman Instruments, Inc., Westbury, NY, USA). A 500-μl aliquot of cold HPLC-grade acetonitrile (4°C) with internal standard (100 μg/ml) was added to the test tube and mixed completely via a vortex mixer (Baxter) to achieve deproteinization. The mixed solution was centrifuged at 3000 *g* in a Clinifuge centrifuge (Baxter) for 5 min. A 500-μl aliquot of the supernatant was filtered through an Acrodisc syringe filter (LC 13PVDF, 13 mm, 0.45 μm, Gelman Sciences, Inc., Ann Arbor, MI, USA) on a B-D 3cc Luer-lok syringe (Baxter). A 30-μl aliquot of the filtered solution was injected onto the HPLC column via the HP 1050 autoinjector. All specimens were stored at −20°C and protected from light in crimp-cap amber glass autoinjector vials (Chromacol, Inc., Trumbull, CT, USA) until assayed.

2.7. Calibration and calculation procedure

Calibration curves were determined via direct linear regression. A standard curve was constructed to relate the untransformed peak–height ratios of sample quinolone to internal standard to the untransformed standard concentrations of the calibration quinolone. The unknown concentrations of the quinolone-of-interest in Mueller–Hinton broth were calculated by interpolation using these daily cali-

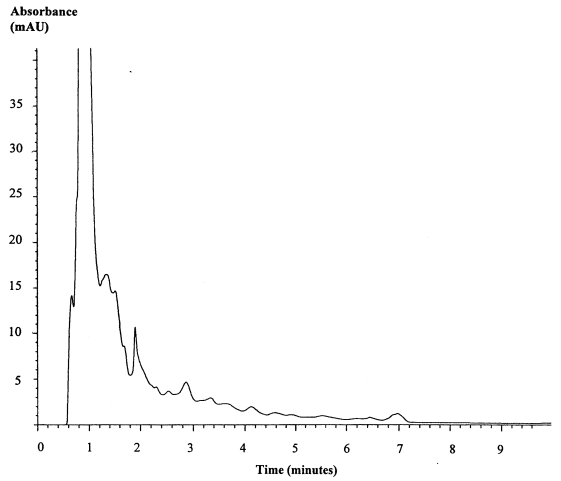

Fig. 1. Chromatogram of blank Mueller–Hinton broth.

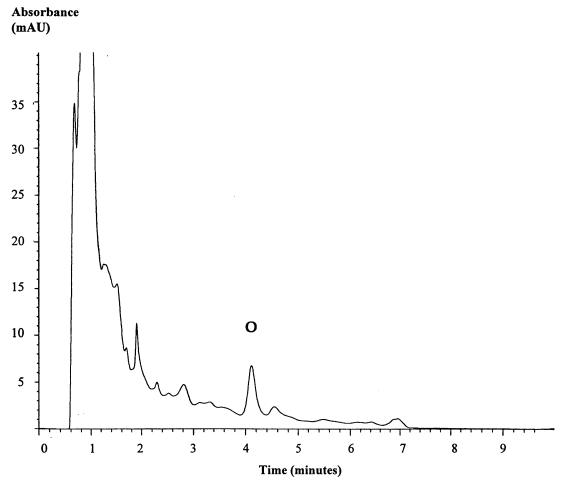

Fig. 3. Chromatogram of Mueller–Hinton broth spiked with 0.1 μg/ml of ofloxacin (O).

bration curves. All calculations were preformed using a Microsoft Excel, version 5.0, spreadsheet.

3. Results and discussion

3.1. HPLC profiles

Adequate chromatographic separation was obtained using the above method. Figs. 1–4 show the chromatograms from the analysis of blank Mueller–Hinton broth and Mueller–Hinton broth blanks spiked with 0.1 μg/ml of each quinolone (ciprofloxacin, ofloxacin, and sparfloxacin). In a 1-day validation, the retention times (mean±S.D., $n=54$) were: for ciprofloxacin, 4.67±0.12 min; ofloxacin, 4.15±0.11 min; and sparfloxacin, 7.09±0.16 min. Using the above sample preparation procedure, the blank chromatogram contained no peaks at the retention times corresponding to any of the quinolones tested. Peaks of interest were resolved

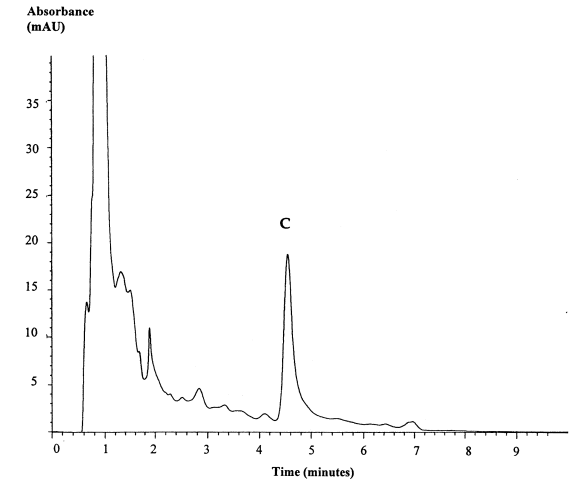

Fig. 2. Chromatogram of Mueller–Hinton broth spiked with 0.1 μg/ml of ciprofloxacin (C).

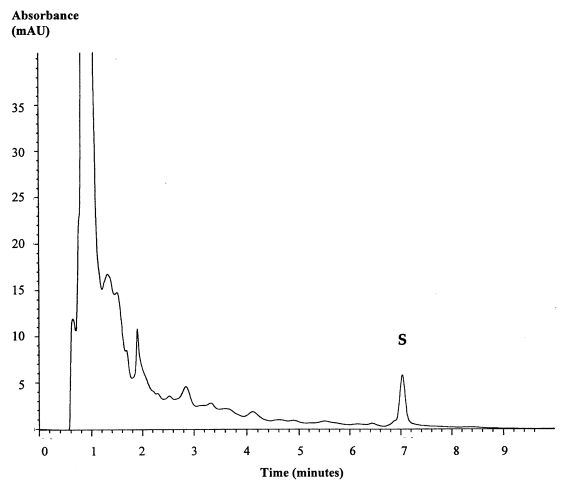

Fig. 4. Chromatogram of Mueller–Hinton broth spiked with 0.1 μg/ml of sparfloxacin (S).

from other components in the extracts suggesting a lack of interference from endogenous compounds in Mueller–Hinton broth.

3.2. Calibration curve and limit of quantification

The assays exhibited linearity ($r^2 \geq 0.9994$) over the 0.1–20.0 μg/ml range for all three quinolone agents. Table 1 indicates the mean r^2, intercept, correlation, and slope values for each of the quinolone agents studied. In practice, a series of standards ($n=18$) are analyzed together among the samples. These calibration curves are used for calibration and calculation purposes of the assay. The standards are randomly dispersed among the samples over the length of the entire run to ensure accuracy and precision of daily analyses. The injection order of each analysis is completely randomized between calibration standards and samples.

The lower limit of quantification for each fluoroquinolone was assessed with analyses of calibration standards at concentrations of 0.0625 and 0.1 μg/ml. Both precision and accuracy data were taken into account when determining the lower limit of quantitation for each quinolone studied. The mean precision of the 0.0625 μg/ml standard was 5.24% (4.76%–5.79% range, $n=3$) for ciprofloxacin, 7.12% (4.72%–9.58% range, $n=3$) for ofloxacin, and 5.37% (1.09%–8.93% range, $n=3$) for sparfloxacin. The mean precision of the 0.1 μg/ml calibration standard was 2.47% (1.74%–3.61% range, $n=3$) for ciprofloxacin, 2.92% (2.42%–3.63% range, $n=3$) for ofloxacin, and 2.18% (1.49%–3.32% range, $n=3$) for sparfloxacin. Mean accuracy for the 0.0625 μg/ml calibration standard was −14.60% (−12.97%–−15.65% range, $n=3$) for ciprofloxacin, 1.78% (−22.35%–48.18% range, $n=3$) for ofloxacin, and −18.24% (−47.02%–1.93% range, $n=3$) for sparfloxacin. Mean accuracy for the 0.1 μg/ml calibration standard was −5.96% (−14.96%–−1.42% range, $n=3$) for ciprofloxacin, 4.90% (−13.90%–27.37% range, $n=3$) for ofloxacin, and 1.29% (−9.10%–11.41% range, $n=3$) for sparfloxacin. Even though these results indicate satisfactory precision for the 0.0625 μg/ml calibration standard by conventional criteria, 0.1 μg/ml was considered to be the lower limit of quantification for all quinolones tested.

3.3. Recovery

Calculation of extraction recovery for each fluoroquinolone was calculated with comparison of peak heights of unextracted injected standards and those from Mueller–Hinton broth-spiked standards subjected to the sample preparation procedures ($n=6$). The extraction recoveries of fluoroquinolone agents in Mueller–Hinton broth at a concentration of 5 μg/ml was 97.31% for ciprofloxacin, 96.81% for ofloxacin, and 99.55% for sparfloxacin.

3.4. Accuracy and precision

Accuracy and precision of the method were estimated by comparison of calculated concentrations versus actual concentrations. The results of back-calculated standard concentrations are shown in Table 2. Overall mean precision, as defined by the coefficient of variation, ranged from 0.36 to 7.06% for the range of 0.1–20 μg/ml concentrations in all the quinolone agents studied. The accuracy of these calculations ranged from 0.13 to 22.25%. Analysis of independent calibration standards were used to determine intra-day and inter-day precision (0.2, 1 and 10 μg/ml). Analysis of intra-day and inter-day precision and accuracy are shown in Table 3. The coefficient of variation values for all the quinolone agents studied were less than 3% and the accuracy

Table 1
Mean r^2, y-intercept, slope and correlation values of fluoroquinolones in Mueller–Hinton broth

Fluoroquinolone	Mean r^2 value ($n=3$)	Mean y-intercept value ($n=3$)	Mean slope value ($n=3$)	Mean correlation value ($n=3$)
Ciprofloxacin	0.9994	0.0622	4.2593	0.9997
Ofloxacin	0.9996	−0.0147	2.4337	0.9997
Sparfloxacin	0.9998	−0.0044	2.4426	0.9999

Table 2
Accuracy and precision of the HPLC determination of fluoroquinolones in Mueller–Hinton broth

Concentration		C.V. (%)	Accuracy (%)
Actual (μg/ml)	Calculated (mean±S.D., $n=6$) (μg/ml)		
Ciprofloxacin			
0.0625	0.0544±0.0061	4.76	−12.97
0.1	0.0985±0.0086	3.61	−1.50
0.2	0.1972±0.0093	1.81	−1.40
0.5	0.5122±0.0265	1.77	2.44
1	0.1005±+0.0297	1.26	0.48
2	2.0060±0.1025	1.66	0.30
5	5.0200±0.1590	1.19	0.40
10	9.9507±0.4258	1.96	−0.49
20	20.0185±1.2093	2.34	0.09
Ofloxacin			
0.0625	0.0485±0.0095	7.06	−22.25
0.1	0.0861±0.0050	2.42	−13.90
0.2	0.1863±0.0059	1.08	−6.83
0.5	0.4857±0.0050	1.07	−2.87
1	1.0083±0.0376	1.31	0.83
2	1.9712±0.0655	1.19	−1.44
5	5.0630±0.0923	0.70	1.26
10	10.0521±0.1942	0.74	0.52
20	19.9613±0.6195	1.24	−0.19
Sparfloxacin			
0.0625	0.0637±0.0103	6.10	1.93
0.1	0.1016±0.0035	1.49	1.55
0.2	0.2112±0.0032	0.53	5.62
0.5	0.5055±0.0041	0.40	1.11
1	1.0108±0.0482	1.79	1.08
2	1.9949±0.0473	0.91	−0.25
5	5.0019±0.0389	0.36	0.04
10	9.9473±0.2307	0.86	−0.53
20	20.0256±0.4780	1.08	0.13

C.V., coefficient of variance.

less than 5%. These results indicate that the assay is reliable and reproducible.

3.5. Storage stability

We did not attempt to directly determine the storage stability of the fluoroquinolones studied. According to results of a recent study, freeze–thaw stability of trovafloxacin samples in urine and serum stored at −20°C was considered to be stable for up to 12 months [18]. We extrapolate that similar findings would be seen with other fluoroquinolones in growth media. All samples were stored for less than 2 months at −20°C before analysis, new stock standards were created monthly, and no degradation was observed at any point during this validation.

4. Conclusion

In summary, the analytical method described is rapid, sensitive, selective, reliable, and reproducible. Other fluoroquinolone antibiotics have been studied with this methodology but not validated to date.

Table 3
Intra-day and inter-day precision of fluoroquinolones in Mueller–Hinton broth

Concentration (μg/ml)	Intra-day precision		Inter-day precision	
	C.V. (n=6) (%)	Accuracy (%)	C.V. (n=18) (%)	Accuracy (%)
Ciprofloxacin				
0.2	1.11	−2.21	1.45	−2.04
1	1.25	−1.06	2.34	1.58
10	2.18	−0.17	2.04	1.75
Ofloxacin				
0.2	1.09	−2.99	2.81	0.36
1	1.00	3.61	2.21	3.27
10	1.40	0.16	1.36	1.08
Sparfloxacin				
0.2	0.55	4.93	2.60	2.05
1	0.80	1.06	1.04	1.08
10	1.53	0.57	1.29	0.47

C.V., coefficient of variation.

Temafloxacin, levofloxacin, clinafloxacin, and trovafloxacin have been successfully identified without modification of this methodology. Other matrices have also been studied with this assay. Human serum, human urine, Todd–Hewitt growth media, Ensure enteral feeding solution, and *Haemophilus* growth media have all been used with slight modifications of the extraction methodology in this assay. This method has been used in several published clinical bioequivalence and pharmacokinetics studies [19–23], with minimal modifications.

The versatility of UV/Vis detection, an isocratic mobile phase, minimal sample preparation, and short run times (<10 min) allow this methodology to serve as a valuable adjunct with in vitro pharmacodynamic and pharmacokinetic analysis.

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