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Permeability Classification of Representative Fluoroquinolones by a Cell Culture Method

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ABSTRACT

This study was undertaken to categorize representative fluoroquinolone drug substance permeability based on the methods outlined in the Food and Drug Administration's biopharmaceutic classification system (BCS) Guidance for Industry. The permeability of ciprofloxacin, levofloxacin, lomefloxacin, and ofloxacin was measured in an in vitro Caco-2 assay with previously demonstrated method suitability. The permeability class and efflux potential were ascertained by comparing test drug results with standard compounds (metoprolol, atenolol, labetalol, and rhodamine-123). All 4 quinolones drugs demonstrated concentration-dependent permeability, indicating active drug transport. In comparing absorptive versus secretive in vitro transport, the tested fluoroquinolones were found to be subject to efflux in varying degrees (ciprofloxacin > lomefloxacin > rhodamine 123 > levofloxacin > ofloxacin). Based on comparison to labetalol, the high permeability internal standard, ciprofloxacin was classified as a low permeability drug, whereas lomefloxacin, levofloxacin, and ofloxacin were classified as high permeability drugs. The in vitro permeability results matched human in vivo data based on absolute bioavailabilities. This laboratory exercise demonstrated the applicability of an in vitro permeability method for classifying drugs as outlined in the BCS Guidance.

KEYWORDS: permeability, Caco-2, biopharmaceutics classification system, fluoroquinolones

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INTRODUCTION

The biopharmaceutics classification system (BCS) is a scientific outline for classifying drug substances based on their aqueous solubility and intestinal permeability.[1] The Food and Drug Administration applied the principles of BCS to a regulatory bioavailability and bioequivalence guidance that recommends methods for classifying drug substances and products.[2] The Guidance explains when a waiver (biowaiver) for in vivo bioavailability and bioequivalence studies may be requested based on the approach of BCS. In the current BCS Guidance, high solubility, high permeability, rapid and similar dissolution, wide therapeutic window, and previously used excipients are required for justifying a biowaiver request. The BCS Guidance provides methods and acceptance criteria for classifying a drug substance and product based on its solubility, permeability, and dissolution.

The permeability class boundary is based indirectly on the extent of absorption ($\%f_a$) of a drug substance in humans and directly on measurements of the rate of mass transfer across human intestinal membrane. A drug substance is considered to be highly permeable when the extent of absorption in humans is determined to be ≥90% of an administered dose based on a mass balance determination or in comparison with an intravenous reference dose.[2] Acceptable methods for permeability classification are categorized as human (mass balance or absolute bioavailability studies), animal (in vivo or in situ intestinal perfusion), and in vitro (excised human or animal tissue, or epithelial cell monolayers).

To establish the method suitability of a permeability assay intended for application of the BCS, a rank-order relationship between drug substance permeability values and human extent of absorption must be ascertained using a sufficient number of model drugs.[2] This relationship is required to differentiate between high (HP) and low permeability (LP) drug substances utilizing a sufficient number of animals, excised tissue samples, or cell monolayers for each drug evaluated, due to variability.

Table 1. Bioavailability of Fluoroquinolones

Drug	Highest Dose Strength	Bioavailability	References
Ciprofloxacin	750 mg	70%-85%	6, 8, 12
Levofloxacin	750 mg	99%-100%	6, 9, 12
Lomefloxacin	400 mg	95%-98%	10
Ofloxacin	400 mg	96%-100%	6, 11, 12

After demonstrating method suitability and maintaining the same experimental protocol, the method can be used to classify a drug substance's permeability. Test drugs are classified by selecting an HP internal standard (HP-IS) with permeability in close proximity to the LP/HP class boundary. A test drug substance is classified as highly permeable when its permeability is equal to or greater than that of the HP-IS.

Using an in vitro Caco-2 assay with demonstrated method suitability according to the BCS Guidance,[3] 4 model fluoroquinolone drug substances were classified for permeability. The model used was previously shown to correctly categorize over 20 model drugs in the 4 BSC classes.[3] Fluoroquinolones are synthetic antibacterial agents with rapid bactericidal effect against most susceptible organisms. Structural modifications to the core quinolone have led to expanded microbiological activity, optimal pharmacokinetics, and increased safety profile.[4] Their mode of action involves interactions with DNA gyrase and topoisomerase IV.[5] The fluoroquinolones typically have moderate to excellent bioavailability (Table 1), large volumes of distribution, extensive tissue penetration, and low plasma protein binding.[6,7] Ciprofloxacin oral tablets are rapidly and well absorbed from the gastrointestinal tract with an absolute bioavailability of ~70%.[8] Levofloxacin is rapidly and essentially completely absorbed after oral administration, with an absolute bioavailability of ~99% at 500 and 750 mg.[9] Both lomefloxacin and ofloxacin have bioavailabilities approaching 98%.[10,11]

MATERIALS AND METHODS

Drug Stock Solutions

Stock solutions were based on the highest dose strength of the drug product (Table 1) dissolved in 250 mL to give final solutions of 1X, 0.1X, and 0.01X. Lomefloxacin (16 mg/mL), levofloxacin (20 mg/mL), and ciprofloxacin (30 mg/mL) were dissolved in water, vortexed, and sonicated to mix thoroughly. The drug solution was then centrifuged at 2500 rpm for 5 minutes. The supernatant was collected and used as the 10X drug stock for the permeability and stability experiments. Ofloxacin was dissolved in water at 16 mg/mL with a few drops of hydrochloric acid to reduce the pH and improve solubility.

Permeability Experiments

The protocol used had demonstrated method suitability according to BCS Guidance.[3] Cell culture media, buffers, and reagents were from Invitrogen (Grand Island, NY). The cells and monolayers were cultivated at 37°C in a humidified atmosphere with 5% CO_2. Media and buffer were warmed to 37°C in a water bath before use on the cells. Caco-2 cells were purchased from American Type Culture Collection (HTB-37, Manassas, VA) and used in the transport experiments at passages 55 through 66. Culture media was composed of Dulbecco's modified Eagle's medium containing 4.5 g/L glucose, 5.84 g/L glutamine, 0.1 mM nonessential amino acids, 1% penicillin-streptomycin solution, 10 mM sodium bicarbonate, and 10% fetal bovine serum. The culture media on the Caco-2 cells was replaced 3 times a week, and the cells were passaged at or before 90% confluency with a trypsin-EDTA solution (0.05% trypsin and 0.53 mM EDTA).

To prepare the monolayers, Caco-2 cells were harvested, centrifuged, and resuspended in fresh media. Costar Transwell systems (Corning Inc, Corning, NY) with polycarbonate filters were used to cultivate the cell monolayers in a 12-well plate format (12-mm filter diameter, 0.4-μm pore size). Media were added to the basolateral (BL, 1.5 mL) and apical chambers (AP, 0.5 mL) of each well. Caco-2 cells were initially seeded at a density of ~75 000 cells/cm^2 (~84 750 cells/insert) and the monolayers fed 3 times per week.

The buffer solutions were composed of Hank's balanced salt solution (HBSS) containing calcium and magnesium supplemented with 10 mM 2-(N-morpholino)ethanesulfonic acid (MES) or 25 mM N-(2-hydroxyethyl)piperazine-N'-2-ethane-sulfonic acid (HEPES). The buffer pH was adjusted with sodium hydroxide or hydrochloric acid. The BL and AP chambers contained HBSS/HEPES pH 7.4 and HBSS/MES pH 6.8 buffers, respectively.

Caco-2 cell monolayers were used 20 to 22 days after initial seeding in the transport studies. The media were

removed from both chambers and replaced with HBSS/HEPES buffer solution (pH 7.4). The monolayers were then incubated with the buffer for 60 minutes at 37°C. Following incubation, transepithelial electrical resistance (TEER) was measured for each monolayer using a Millicell-ERS apparatus (Millipore Corp, Bedford, MA). TEER ($\Omega \times cm^2$) was calculated from the following equation:

$$TEER = (R_{mono} - R_{blank}) \times A \qquad (1)$$

where R_{mono} is the cell monolayer and filter resistance, R_{blank} is the filter resistance, and A the filter surface area (1.13 cm^2). Monolayers having TEER values less than 250 $\Omega \times cm^2$ were not included in the study.

For absorptive (AP→BL) permeability experiments, the buffer was aspirated from the AP chamber of each well. Drug solution (0.5 mL, pH 6.8 HBSS/MES) was added to the AP chamber, and the plates were returned to the 37°C incubator on a plate shaker for agitation during the transport experiment. The filter insert was transferred to a well in a new plate containing fresh buffer in the BL chamber (pH 7.4) at 15, 30, 45, 60, 90, and 120 minutes. At the end of the study, a sample was also taken from the AP chamber. For secretive (BL→AP) permeability experiments, the buffer was aspirated from the BL chamber of each well. Drug solution (1.5 mL, pH 7.4 HBSS/HEPES) was added to the BL chamber, and the plates were returned to the 37°C incubator on a plate shaker for agitation during the transport experiment. At 15, 30, 45, 60, 90, and 120 minutes, 0.5 mL was removed from the AP chamber and replaced with 0.5 mL fresh buffer (pH 6.8). At the end of the study, a sample was also taken from the BL chamber. All donor and receiver samples were stored at -20°C until analysis.

The concentrations of test compound added to the donor well at the beginning of experiment (initial donor concentration) and transport samples was measured by high-performance liquid chromatography (HPLC) methods. Cumulated transported drug concentration was plotted against sampling time to yield a value for the initial slope of the resulting curve. Apparent permeability (P_{app}, $\times 10^{-6}$ cm/sec) was calculated from the following equation:

$$P_{app} = \frac{V_R}{A \times C_0} \times \frac{dC}{dt} \qquad (2)$$

where V_R is the volume in the receiver chamber (mL), A is the filter surface area (1.13 cm^2), C_0 is the initial concentration in the donor chamber, and $^{dC}/_{dt}$ is the initial slope of the concentration vs time (seconds) curve. The slope was calculated from the interval yielding the initial linear rate of transfer spanning 2 sampling intervals (ie, 3 data points).

Stability Experiments

Drug solutions (1X) in HBSS/MES and HBSS/HEPES buffers were incubated at 37°C ± 1°C for 3 hours and then stored at -20°C until analysis (n = 3). The concentration of the drug substance in the buffers was measured using a validated HPLC assay that could distinguish the drug substance from its degradation products. The 4 fluoroquinolone drugs remained stable for 3 hours at 37°C in the 2 HBSS buffer solutions used in the permeability experiments.

HPLC Analysis

All 4 drugs and the reference standards were analyzed in reverse-phase HPLC assays on a 250-mm Luna $C_{18(2)}$ column (Phenomenex, Torrance, CA). The mobile phase consisted of phosphate buffer with methanol or acetonitrile as the organic solvent. Ciprofloxacin (278 nm), ofloxacin (294 nm), levofloxacin (290 nm), lomefloxacin (286 nm), metoprolol (222 nm), atenolol (224 nm), and labetalol (224 nm) were measured by UV detection. Fluorescence light detection (excitation/emission) was utilized for fluorescein isothiocyanate (FITC)-dextran (490/510 nm) and rhodamine 123 (501/524 nm). The accuracy was >97% and linearity >0.95 for the fluoroquinolone and standards assays. For the fluoroquinolones and standards, precision was ≤0.02% and <5%, respectively.

RESULTS

Using a Caco-2 assay[3] shown to have method suitability according to the criteria in the BCS Guidance, the in vitro permeability of several standard compounds and model fluoroquinolone drugs was evaluated. The permeability of the standard compounds (Table 2) was similar to our previous results, which had established method suitability of the Caco-2 assay.[3] Metoprolol and atenolol served as the HP and LP reference standards, respectively, with labetalol as the HP-IS that was used to classify a test drug as HP or LP. FITC-dextran was utilized as a marker of cell monolayer integrity and rhodamine 123 served as a marker of efflux potential in the Caco-2 cells.

All 4 fluoroquinolone drugs demonstrated some concentration-dependent permeability (Table 3) indicative of active drug transport, especially levofloxacin and lome-

Table 2. Permeability of Standard Compounds*

Drug	Direction	Concentration (mg/mL)	P_{app}	n
Metoprolol	AP→BL	0.4	29.88 ± 3.17	18
Atenolol	AP→BL	0.4	1.86 ± 0.47	4
Labetalol	AP→BL	1.2	18.05 ± 1.90	12
FITC-dextran	AP→BL	0.8	0.83 ± 0.30	20
Rhodamine 123	AP→BL	0.1	10.61 ± 2.10	18
Rhodamine 123	BL→AP	0.1	43.80 ± 19.80	18

*P_{app} = mean ± SD (× 10^6 cm/s); n = number of wells.

Table 3. Permeability of Fluoroquinolones*

Drug	Direction	Concentration (mg/mL)	P_{app}
Ciprofloxacin	AP→BL	3.0	2.49 ± 0.43
	AP→BL	0.3	0.42 ± 0.06
	AP→BL	0.03	1.82 ± 0.41
	BL→AP	3.0	11.41 ± 2.19
Levofloxacin	AP→BL	3.0	28.36 ± 1.93
	AP→BL	0.3	10.60 ± 0.02
	AP→BL	0.03	1.08 ± 0.01
	BL→AP	3.0	93.66 ± 1.89
Lomefloxacin	AP→BL	1.6	15.88 ± 0.84
	AP→BL	0.16	5.39 ± 0.26
	AP→BL	0.016	3.61 ± 0.40
	BL→AP	1.6	71.26 ± 4.14
Ofloxacin	AP→BL	1.6	21.44 ± 2.94
	AP→BL	0.16	5.29 ± 0.69
	AP→BL	0.016	8.78 ± 0.97
	BL→AP	1.6	36.16 ± 1.47

*P_{app} = mean ± SD (× 10^6 cm/s); n = 6 wells.

floxacin. The rank order of permeability values of the drugs at their highest (1X) concentration was levofloxacin > ofloxacin > lomefloxacin > ciprofloxacin.

All 4 quinolone drugs were subject to efflux in the transport experiments (Table 4) as evidenced by comparing the absorptive (AP→BL) versus secretive (BL→AP) transport (ie, efflux ratio). The rank order of efflux for the drugs was ciprofloxacin ≈ lomefloxacin > levofloxacin > ofloxacin. Both ciprofloxacin and lomefloxacin had an efflux ratio greater than rhodamine 123.

Table 4. Efflux Ratios*

Drug	Efflux Ratio
Ciprofloxacin	4.6
Levofloxacin	3.3
Lomefloxacin	4.5
Ofloxacin	1.7
Rhodamine 123	4.1

*Efflux ratio = $P_{app}^{BL\rightarrow AP}/_{AP\rightarrow BL}$.

In this particular Caco-2 assay, a test drug is considered to be highly permeable when its in vitro P_{app} value is equal to or greater than that of the HP-IS. Based upon comparison to labetalol (Table 2), ciprofloxacin is classified as an LP drug, whereas levofloxacin, lomefloxacin, and ofloxacin are classified as HP drugs (Table 2).

DISCUSSION

This project was undertaken to demonstrate the feasibility of the methods and specification limits for permeability determination as outlined in BCS guidance.[2] The 4 fluoroquinolone drug substances served as a model set to show the utility of a Caco-2 assay. This cell culture model was previously evaluated and determined to be a suitable method according to the BCS Guidance as it demonstrated a rank-order correlation between in vitro permeability and human extent of absorption for the model drugs, with clear segregation between high and low permeability drug substances.[3]

Based on the previous reports of human absolute bioavailability, it was expected that levofloxacin, lomefloxacin, and ofloxacin would be classified as HP drugs and ciprofloxacin as an LP drug.[6,8-12] In the Caco-2 per-

meability assays, ciprofloxacin was classified as an LP drug, whereas levofloxacin, lomefloxacin, and ofloxacin were classified as HP drugs. Thus, the in vitro results matched human in vivo data based on absolute bioavailabilities. Ciprofloxacin, levofloxacin, and lomefloxacin, and to a lesser extent ofloxacin, were subject to secretive transport as seen by their efflux ratios. In addition, there was evidence that the drugs underwent some active transport as their P_{app} values decreased with concentration, especially lomefloxacin, ofloxacin, and levofloxacin.

Given the data showing active transport and/or efflux of the 4 quinolones in the Caco-2 cells, the use of a cellular model would not be acceptable for a biowaiver application with these drugs according to the BCS Guidance. In vivo or in situ animal models and in vitro methods, such as those using cultured monolayers of animal or human epithelial cells, are considered appropriate for passively transported drugs.[2] At this time, there is no acceptance criterion for the degree of intestinal efflux or active transport that should be present in a test system. Instead, the Guidance recommends limiting the use of nonhuman permeability test methods for drug substances that are shown to be transported by passive mechanisms.[2]

Other investigators have noted the role of efflux and active transport of the fluoroquinolones in cellular permeability models. Cavet et al[13] stated that P-glycoprotein functions as a common secretory transporter for some quinolones in Caco-2 cells. The net secretion of ciprofloxacin was partially inhibited by verapamil and its substrate specificity in Caco-2 cells was distinct from renal organic anion and cation transport.[13] Griffiths et al[14,15] found that norfloxacin and ciprofloxacin were subject to active transepithelial transport in Caco-2 cells. Yamaguchi et al, ascertained that levofloxacin uptake in Caco-2 cells was mediated by a specific transport system distinct from organic cations and anions, amino acids, dipeptides, and monocarboxylic acids.[16,17] In TC7 cells, ofloxacin displayed concentration-dependent permeability and was actively absorbed.[18] Other researchers found the efflux ratio for ciprofloxacin in Caco-2 to be ~2[19] and 6,[14] bracketing the value (4.6) we found in our own study.

CONCLUSION

The FDA Center for Drug Evaluation and Research BCS Guidance provides methods and acceptance criteria for classifying a drug substance based on its solubility and permeability as well as the drug product's dissolution profile. This laboratory exercise demonstrated the applicability of using an in vitro laboratory method and relevant acceptance criteria outlined in the BCS guidance to classify a series of drug substances based on their permeability for biowaiver applications. The results we obtained in our Caco-2 assay with the fluoroquinolone drugs appropriately classified the drugs in comparison with their human oral bioavailabilities.

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