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Fluorometric Assay for Fleroxacin Uptake by Bacterial Cells

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A sensitive and convenient method for quinolone determination has been developed, based on the natural fluorescence of the quinolone nucleus. Fleroxacin (Ro 23-6240; AM 833), used as a prototype quinolone in these studies, had an excitation maximum at 282 nm and an admission maximum at 442 nm (pH 3.0). Fluorescence intensity was pH dependent, being maximal at pH 3.0 and linear at quinolone concentrations between 1 and 200 ng/ml. A protocol for the fluorometric monitoring of fleroxacin uptake in *Escherichia coli* was developed. Intracellular quinolone concentrations measured by the fluorometric assay correlated well with values obtained by the bioassay. The results indicate that the fluorometric assay is an attractive alternative to the more laborious bioassay.

Quinolones are antibacterial agents whose primary mechanism of action is the inhibition of DNA gyrase, an essential enzyme involved in the supercoiling of DNA (3). The newer compounds have as common structural features a carboxyl group at C2 and a fluorine at C6; in addition they usually have a piperazinyl moiety at C7 (Fig. 1) (9). The older quinolones such as a nalidixic and oxolinic acids were moderately active, and development of resistance was a problem. However, the newer compounds are highly active and have a broad spectrum of activity and lower rates of resistance development (8).

The increased use of quinolones as experimental and therapeutic antibacterial agents has created a need for a rapid, easy, and sensitive method of assaying quinolones and their uptake by bacterial and eucaryotic systems. Quinolone determination in biological samples has been performed by UV absorbance and fluorescence emission on samples separated by high-pressure liquid chromatography (6, 7), radiometry (1), and bioassay (4, 5). However, preparation of samples for high-pressure liquid chromatography requires several manipulations such as deproteinization and ion-pair extraction; sensitivity of UV detection is limited to concentrations in the microgram-per-milliliter range. Radiometric assays are sensitive and accurate but require radiolabeled quinolones. Bioassay techniques are relatively insensitive, laborious, and generally inappropriate for kinetic studies.

In the present study, a fluorometric method for measuring quinolone concentrations based on the natural fluorescence of the quinolone nucleus was developed. Parameters affecting fluorescence and optimal conditions for a fluorometric assay are described by using fleroxacin as a model quinolone. The method, which is comparable in ease and rapidity to the radiometric assay, was used to monitor quinolone uptake by bacterial cells.

MATERIALS AND METHODS

Materials. Fleroxacin (Ro 23-6240; AM 833) was obtained from Hoffmann-La Roche, Inc. (Nutley, N.J.). Norfloxacin was from Merck & Co., Inc. (Rahway, N.J.); ofloxacin was from Ortho Diagnostics, Inc. (Raritan, N.J.); pefloxacin was from Rhone-Poulenc Pharmaceuticals (Monmouth Junction, N.J.); difloxacin (A-56619) and A-56620 were from Abbott Laboratories (North Chicago, Ill.); flumequine was from Riker Laboratories; nalidixic and oxolinic acids, MES (morpholineethanesulfonic acid), MOPS (morpholinepropanesulfonic acid), HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), and Tris hydrochloride were from Sigma Chemical Co. (St. Louis, Mo.). Buffers were adjusted to the desired pH with either HCl or NaOH.

Fluorescence was measured with a Perkin-Elmer LS-5 fluorescence spectrophotometer; the excitation slit width was 5 mm, and the emission slit width was 10 nm. The emission spectra were recorded with excitation at 282 nm.

Escherichia coli JF568 was a K-12 strain obtained from John Foulds (2). Organisms were routinely grown at 37°C in Luria broth until the midlog phase (A_{660}, ~0.4). Bacterial growth was measured by A_{660} with an LKB Ultraspec 4050 spectrophotometer.

RESULTS AND DISCUSSION

Quinolone fluorescence. The excitation and emission spectra of fleroxacin are shown in Fig. 1. In 0.1 M glycine-HCl, pH 3.0, the excitation maximum was 282 nm and the single emission peak occurred at 442 nm. Other fluoroquinolones had similar excitation and emission maxima, whereas the excitation and emission maxima of nalidixic acid, flumequine, and oxolinic acid were at lower wavelengths (Table 1).

The fluorescence intensity of fleroxacin varied with pH. The fluorescence intensity was maximal at pH 3.0; it was decreased nearly ninefold at pH 9.0 and twofold at pH 7.0. Lowering the pH from 9.0 to 2.0 shifted the emission peak to a longer wavelength, from 413 nm to 459 nm. The nature of the buffer had minor effects on fluorescence intensity and emission maxima. At pH 6.0, 7.0, and 8.0, phosphate-buffered solutions of fleroxacin had lower fluorescence intensities than those obtained in MES, MOPS, HEPES, or Tris hydrochloride.

Reagents commonly used to lyse bacterial cells or deproteinize samples were examined for their effect on the fluorescence of fleroxacin. In pH 3.0 and 7.0 buffered solutions, methanol or acetonitrile at 50% final concentration had minor effects (5 to 13% difference) on the intensity of fleroxacin fluorescence. Sodium dodecyl sulfate at 1% increased fluorescence by 16% at pH 3.0 and 287% at pH 7.0 due to the fluorescence of sodium dodecyl sulfate itself.

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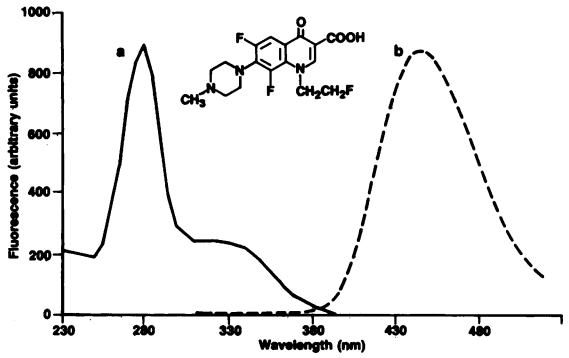

FIG. 1. Structure of fleroxacin and its excitation and emission spectra (200 ng/ml in 0.1 M glycine hydrochloride [pH 3.0]). The excitation spectrum (a) was recorded with emission at 442 nm; the emission spectrum (b) was recorded with excitation at 282 nm.

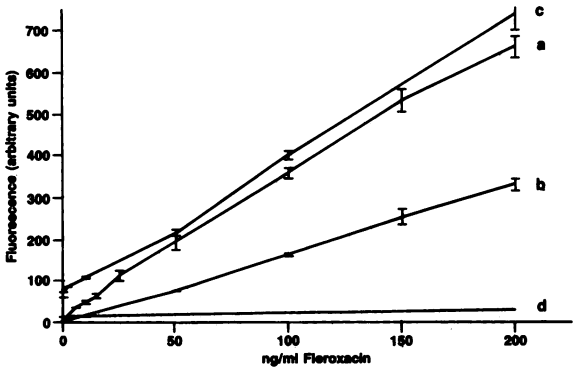

FIG. 2. Plots of fluorescence versus fleroxacin concentration at pH 3.0 (a) and pH 7.0 (b) and at pH 3.0 with acid-treated cell extracts (c) or boiled cell extracts (d). Values are shown with standard errors.

Trichloroacetic acid at 1% decreased fluorescence by 17% at pH 3.0, whereas in pH 7.0 buffer (final pH 5.5) the emission peak was shifted to 438 nm from 417 nm. Acetone at 10% totally quenched the fluorescence of fleroxacin at both pH 3.0 and 7.0.

Sensitivity and precision. A plot of fluorescence intensity versus fleroxacin concentration at pH 3.0 and pH 7.0 was linear up to 200 ng/ml, with a detection limit for fleroxacin of 1 ng/ml (Fig. 2). The slope of the plot (fluorescence response) at pH 3.0 was 3.4 ± 0.04 fluorescence units per ng of fleroxacin, and that at pH 7.0 was 1.9 ± 0.2 fluorescence units per ng. A similar standard plot constructed in pH 3.0 buffer containing 1% sodium dodecyl sulfate revealed that although fluorescence intensity increased in the presence of sodium dodecyl sulfate the fluorescence response remains the same, whereas at pH 7.0 the response increased to 3.1 ± 0.03 fluorescence units per ng of fleroxacin.

Extraction of quinolones. Previous protocols for monitoring quinolone uptake by bacterial cells have used boiling to lyse cells and recover intracellular quinolones (4). A standard plot constructed by adding fleroxacin to boiled cell extracts in pH 3.0 buffer revealed that the fluorescence intensity was greatly reduced and the fluorescence response was described to 0.08 fluorescence units per ng of fleroxacin (Fig. 2). On the other hand, when *E. coli* cells were extracted by treatment with 0.1 M glycine hydrochloride (pH 3.0), there was no quenching of fleroxacin fluorescence. The fluorescence of acid-treated cell extracts and fleroxacin were additive, and the fluorescence response was nearly identical to that in the absence of acid-treated cells (3.52 ± 0.26 versus 3.41 ± 0.04 fluorescence units per ng of fleroxacin). Fleroxacin release from preloaded cells was complete after 60 min of treatment with 0.1 glycine hydrochloride (pH 3.0) at room temperature (Fig. 3).

Other assay conditions. With 2 mg (wet weight) of cells per ml, uptake of fleroxacin at an external concentration of 10 μg/ml (27 μM) could be measured. Uptake at an external concentration of 1 μg/ml could be measured with 10 to 40 mg (wet weight) of cells per ml. Quinolone uptake was maximal at pH 7.0 (Fig. 4), and at that pH there was no difference between phosphate, HEPES, or Tris hydrochloride buffer.

The accuracy of the assay was assessed by parallel determinations of fleroxacin in bacterial cell extracts with both spectrofluorimetry and bioassay. *E. coli* cells were exposed to 100 μg of fleroxacin per ml for 30 min, washed, and extracted by incubation in 0.1 M glycine hydrochloride (pH 3.0) for spectrofluorometry or by boiling in incubation buffer for 8 min for the bioassay (4). The amount of fleroxacin in the extract determined by bioassay was 1.2 ± 0.08 μg/ml (n = 4); by spectrofluorometry the amount was 1.13 ± 0.05 μg/ml.

Uptake assay. Based on these results, the following protocol for monitoring quinolone uptake was derived. Midlog-phase bacterial cells were harvested by centrifugation (5,600 × g for 5 min), washed once with 50 mM sodium phosphate buffer (pH 7.0), and suspended to 40 mg (wet weight) per ml. Samples of 10 ml were dispensed into 50-ml Ehrlenmeyer flasks and incubated in a shaking water bath at 37°C for 10

TABLE 1. Fluorescence excitation and emission maxima of quinolones[a]

Quinolone	$\lambda_{excitation}$ (nm)	$\lambda_{emission}$ (nm)
Fleroxacin	282	442
Norfloxacin	281	440
Pefloxacin	277	442
Difloxacin	280	452
A-56620	281	459
Ofloxacin	292	496
Nalidixic acid	246	354
Flumequine	245	365
Oxolinic acid	230	305

[a] Determined in 0.1 M glycine hydrochloride (pH 3.0).

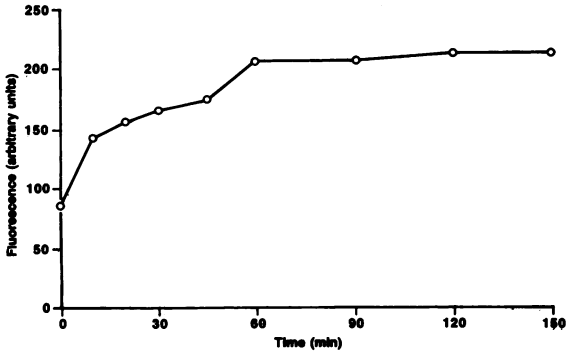

FIG. 3. Release of fleroxacin from *E. coli*, JF568 suspended in 0.1 M glycine hydrochloride (pH 3.0). Cells were incubated with 10 μg of fleroxacin per ml for 30 min, washed once, and suspended in buffer.

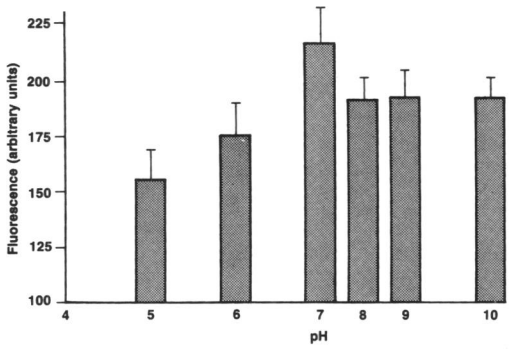

FIG. 4. Effect of pH on fleroxacin uptake by *E. coli* JF568. Cells were incubated with 10 μg of fleroxacin per ml for 30 min at the indicated pHs. Values are shown with the standard error.

min. Quinolone was added; at timed intervals 0.5-ml samples were removed, diluted into 2.0 ml of phosphate buffer, and immediately centrifuged at 7,000 × *g* for 1 min. Samples were washed once with 2.0 ml of buffer and pelleted again. To each cell pellet 2 ml of 0.1 M glycine hydrochloride (pH 3.0) was then added, and the tubes were vortexed vigorously to completely suspend the pellet. Incubation at room temperature for 60 to 90 min was sufficient to fully release intracellular quinolones from *E. coli*. Samples were centrifuged for 5 min at 7,000 × *g*, and the amount of fleroxacin was determined by spectrofluorometry.

This assay presents several advantages over previous methods for determining quinolones and their uptake. Since all quinolones fluoresce, radiolabeled compounds are not required. Although not potentially as sensitive as the radiometric assay, the fluorometric method is suitable for measuring uptake at therapeutic concentrations of quinolones. The fluorometric assay is less laborious than the bioassay, permitting the processing of more samples, and therefore it is more suitable for kinetic studies. Interference from other bioactive substances in cell extracts, common with bioassays, is avoided in the fluorometric assay. Finally, the fluorometric assay is 1,00-fold more sensitive than the UV assay, and unlike high-pressure liquid chromatography methods, requires minimal manipulation of samples.

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