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A ^{19}F NMR study of lomefloxacin in human erythrocytes and its interaction with hemoglobin☆

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Abstract

^{19}F NMR spectroscopy of a model fluoroquinolone, lomefloxacin, in an erythrocyte suspension showed separate resonances for the intra- and extra-cellular compartments. The intra-cellular peak revealed significant line broadening of the fluorine signals of lomefloxacin. Line broadening also occurred in the presence of oxyhemoglobin (HbO_2), hematin, globin and iron. This evidence indicated that lomefloxacin interacted with these compounds; however, ultrafiltration experiments indicated that there was only weak binding (5%) of lomefloxacin to HbO_2. ^{19}F and ^{31}P NMR spectroscopy revealed that lomefloxacin may compete with 2,3-diphosphoglycerate for its binding site on HbO_2. An apparent partition coefficient of 1.90 ± 0.15 was observed for lomefloxacin in human erythrocytes, utilizing LC analysis.

Keywords: 2,3-Diphosphoglycerate; Erythrocyte; Fluoroquinolone; ^{19}F NMR; Hemoglobin binding; Lomefloxacin; ^{31}P NMR

1. Introduction

The fluorinated 4-quinolones (fluoroquinolones) are a group of orally active antimicrobials, which are active against a wide range of gram-negative organisms and gram-positive cocci [1]. They contain two relevant ionizable functional groups, one acidic and one basic, and exist mainly as zwitterions at physiological pH. At pH 7, the fluoroquinolones are in their lowest state of aqueous solubility and in their highest state of lipophilicity. For the model fluoroquinolone, lomefloxacin (Fig. 1), the 1-octanol–water apparent partition coefficient was determined to be 0.14 at pH 7 [2]. The fluoroquinolones complex with metal ions and are therefore contraindicated with antacid therapy, iron supplements, and dairy products [3–

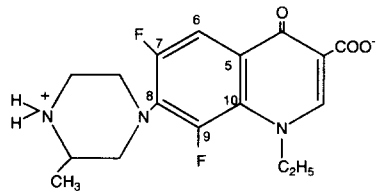

Fig. 1. Structure of the zwitterionic form of lomefloxacin.

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6]. They are not readily metabolized in the body and are excreted virtually unchanged in the urine. They are approximately 20% bound to plasma proteins [7].

In 1992, one fluoroquinolone, temafloxacin (Omniflox), was withdrawn from the market owing to serious adverse reactions. Approximately 50 reports of serious reactions and three deaths were reportedly caused by the drug. Reports included hemolytic anemia and other blood cell abnormalities [8].

The purpose of the present studies was to investigate the partitioning of lomefloxacin across a biological membrane using human erythrocytes as a model, and utilizing ^{19}F NMR and liquid chromatography (LC) as the methods of analysis. Simanjuntak et al. [9] reported an active transport system for lomefloxacin in rat erythrocytes in which lomefloxacin utilizes the nicotinic acid transport system in order to cross erythrocyte membranes. Erythrocyte partitioning of one fluoroquinolone, fleroxacin, has previously been investigated by liquid chromatography [10].

^{19}F NMR is a useful analytical method in studying the interaction of fluorinated compounds with macromolecules in erythrocytes because of the lack of interference due to background signals often observed in ^{1}H or ^{13}C studies [11–14]. ^{19}F NMR has also been utilized with this class of drugs to study the tissue pharmacokinetics of fleroxacin in vivo [15].

When a molecule is incorporated into an erythrocyte suspension, the different intra- and extra-cellular surroundings give rise to separate resonances, enabling the estimation of transmembrane distributions of the drug without the need to separate the extra-cellular fluid from the cells [11,12]. Also, when a species interacts with a macromolecule in solution, this interaction can be measured by the extent of line broadening of the fluorine signals [16–19].

2,3-Diphosphoglycerate (2,3-DPG) resides in erythrocytes and binds to hemoglobin, decreasing its affinity for oxygen and facilitating the release of oxygen from hemoglobin to the tissues [20,21]. Previous ^{31}P NMR studies with 2,3-DPG revealed a significant signal shift when hemoglobin is present [20,22,23]. This effect allows the 2,3-DPG binding site to be explored by NMR spectroscopy.

2. Materials and methods

2.1. Materials

Lomefloxacin mesylate was supplied by G.D. Searle and Co. (Skokie, IL). Bovine hematin, globin from bovine hemoglobin, human serum albumin, myoglobin from horse skeletal muscle, hematoporphyrin · 2HCl, and 2,3-DPG pentasodium salt were purchased from Sigma (St. Louis, MO). All other chemicals were reagent grade or better and were obtained from commercial sources. Water was purified in a Milli-Q Water System (Millipore Corp., Bedford, MA) and stored in glass containers until use. All glassware was washed with nitric acid and rinsed with metal-free water before use to eliminate any trace metal contamination [3]. All samples containing lomefloxacin were wrapped in aluminum foil to protect them from light. pH measurements were made using an Orion SA 520 pH meter (Orion Research, Inc., Boston, MA) and a calomel pH combination glass microelectrode (Markson, Phoenix, AZ).

2.2. Erythrocyte suspensions

Fresh human blood was collected from a consenting healthy adult in heparinized tubes and centrifuged at 450*g* for 15 min. The supernatant was discarded. The erthrocytes were washed three times with phosphate buffered saline (pH 7.4; 0.01 M) containing 10 mM glucose (PBS–glucose) and centrifuged, discarding the supernatant. The erythrocyte pellet was resuspended in PBS–glucose to achieve a hematocrit of 0.45.

2.3. NMR partitioning studies

The erthrocyte pellet (above) was resuspended in 2.3 mM lomefloxacin in PBS–glucose at pH 7.4 and 25 °C, and bubbled with CO to minimized the paramagnetic effect of deoxyhemoglobin [11]. Ten per cent D_2O was added to the lomefloxacin–erythrocyte suspension and the sample was analyzed by ^{19}F NMR. The external and internal surroundings of the erythrocyte suspension were observed by NMR.

2.4. LC partitioning studies

The method of Brunt et al. [10] was employed to study the partitioning of lomefloxacin across an erythrocyte membrane with liquid chromatographic analysis. Lome-floxacin was added to the erythrocyte suspension to yield a total drug concentration of 0.5–10 μg ml^{-1} while maintaining a hematocrit of 0.45 and a pH of 7.4. The suspension was incubated at 25 °C for 0–90 min, including a centrifugation for 3 min at 15 000*g*. The supernatant was analyzed for lomefloxacin concentration by HPLC. For each concentration, a reference or control solution without erythrocytes was also analyzed for drug concentration. The apparent erythrocyte partition coefficient (D) was calculated using the following equation:

$$D = \frac{C_{ref} - C_{pw}(1 - H)}{C_{pw} \cdot H} \quad (1)$$

where C_{ref} is the concentration in the reference solution, C_{pw} is the concentration in the plasma water (supernatant) and H is the hematocrit [10].

2.5. Ghost cell preparation

Erthrocyte ghosts cells were prepared by the method of Steck and Kant [24]. The erythrocytes were washed as described for the erythrocyte suspensions. Hemolysis was initiated by mixing 1 ml of packed red blood cells with approximately 40 ml of sodium phosphate buffer (pH 8.0; 5 mM). The ghost cell suspension was centrifuged at 22 000*g* for 10 min. The supernatant was discarded. The tube containing ghost cells was tipped on its axis so that the loosely packed ghosts slid away from a pellet of proteases, which were aspirated from the tube. The ghost cells were resuspended in 40 ml of PBS and incubated 40 min at 37 °C to induce resealing. The ghosts were then pelleted and washed twice more. Finally, the ghosts were resuspended in 1 ml of a solution containing 2.3 mM lomefloxacin in PBS–glucose. Ten per cent D_2O was added to the sample. The ghosts were allowed to equilibrate with the drug for 1 h. The suspension was analyzed by ^{19}F NMR.

2.6. Hemoglobin purification

Human blood was centrifuged for 15 min at 450*g* and the supernatant was discarded. The erythocytes were washed three to five times with four volumes of ice-cold PBS containing 10 mM glucose and centrifuged for 10 min at 450*g*. The supernatant was aspirated each time. The procedure was considered complete when the supernatant was clear and colorless. The erythrocytes were then diluted in two volumes of ice-cold PBS containing 10 mM glucose, and centrifuged for a final time at 800*g* for 15 min. Again, the clear supernatant was removed.

The erthrocytes were lysed with two volumes of ice-cold, deionized, distilled water. The solution was agitated and allowed to stand at 4 °C for 2 h with occasional agitation. The hemoglobin solution was centrifuged at 4 °C, 1300*g* for 30 min to remove the cell debris. The supernatant containing hemoglobin was prepared for lyophilization by the addition of 250 mM glucose [25]. Lyophilization was performed on a Vertis Sentry Freezemobile 5SL (Gardiner, NY) and was complete in 24 h. A sample of the lyophilized cake was dissolved in water and the concentration was measured by a Beckman DU 650 Spectrophotometer (Beckman Instruments) at 540 nm using the formula

$$C = 1.4 f A_{540} \quad (2)$$

where C is the concentration (mg ml^{-1}) of hemoglobin, f is the dilution factor, and A_{540} is the absorbance at 540 nm [26].

2.7. ^{19}F NMR binding studies

The NMR binding studies were performed at pH 7.4 and 25 °C, unless otherwise indicated. Binding studies were performed on solutions of oxyhemoglobin (HbO_2), hematin, globin, myoglobin, human serum albumin, hematoporphyrin, and $FeCl_2$. The lomefloxacin concentrations were maintained at 2.3 mM. All solutions contained lomefloxacin and were prepared in PBS with the exception of globin and hematoporphyrin. Globin was prepared in water owing to its poor solubility in PBS. Hematoporphyrin was dissolved in PBS–MeOH (75:25, v/v), owing to its low solubility in water. Controls containing 2.3 mM lomefloxacin were prepared in the appropriate buffer for all of the above conditions. When EDTA was added, its concentration was approximately ten times that of lomefloxacin. Ten per cent D_2O was added to each sample before NMR analysis. Each experiment was performed in triplicate.

2.8. 2,3-DPG binding studies

All solutions were prepared in 0.01 M TRIS buffer (pH 7.4; 0.01 M; 25 °C). The samples

contained 2,3-DPG at 5 mM, HbO_2 at 2.6 mM, and lomefloxacin at 2.3 mM. Ten per cent D_2O was added to each sample prior to NMR analysis. All samples were analyzed by ^{31}P and ^{19}F NMR.

2.9. ^{19}F NMR

Spectral analysis was performed on a Bruker AM 500 spectrometer operating at 470.5 MHz for ^{19}F. The sweep width was 10 000 Hz and 4K data points were acquired. The spectra were acquired without proton decoupling. The acquisition time was 205 ms with 256–3000 scans, depending on the line width or concentration of the macromolecule. The pulse width was 6 μs (30° flip angle) and 10 Hz of line broadening was applied prior to the Fourier transform. The samples were referenced to external $CFCl_3$.

2.10. ^{31}P NMR

Spectral analysis was performed on a Bruker AM 500 spectrometer operating at 202.457 MHz for ^{31}P. The sweep width was 10 000 Hz and 32K data points were acquired. Proton decoupling was observed to have no effect on line widths or positions. The acquisition time was 2.3 s with 200–600 scans. The pulse width was 6 μs (90° flip angle) and 1 Hz of line broadening was used. The samples were referenced to external phosphoric acid.

2.11. Ultrafiltration

While maintaining the HbO_2 concentration at zero or 1.3 mM, the lomefloxacin concentration was varied from 0.022 to 0.14 mM. The volume of each sample remained constant at 0.9 ml in each Centrifree ultrafiltration device with 10 000 MW cutoff membrane (Amicon, Beverly, MA). The samples were centrifuged at 1250*g* for 20 min [7] in a Dynac II Centrifuge (Clay Adams, B-D). The experiment was performed in triplicate. Each sample was diluted in mobile phase prior to LC analysis.

2.12. Chromatographic conditions

Erythrocyte suspensions and ultrafiltration samples were analyzed by a stability-indicating assay for lomefloxacin content by LC using a method described previously by Ross et al. [4]. A Beckman Model 110A Pump (Beckman Instruments, Inc.), an Altex Injector fitted with a 20 μl loop, and a Spectroflow 980 Fluorescence Detector (Kratos Analytical, Ramasey, NJ) were employed. The chromatographic conditions for the ultrafiltration and erythrocyte partitioning analysis consisted of an MOS Hypersil (C8) reversed phase column (5 μm, 15 cm × 4.6 mm i.d.), a Perisorb RP-18 Guard Column (P.J. Cobert, St. Louis, MO), and fluorescence detection (Ex 286 nm, Em 418 nm cutoff filter). The mobile phase was tetrahydrofuran–acetonitrile–H_3PO_4 (100 mM)–triethylamine–water (10:30:10:0.03:qs 100, v/v/v/v/v) with a flow rate of 1.5 ml min^{-1}. All injections were made in triplicate. The peak height of each sample was determined.

3. Results and discussion

When analyzing the erythrocyte suspension by ^{19}F NMR it was observed that the extra-cellular fluorine resonances were represented by comparatively sharp signals of approximately 20 Hz width without ^1H decoupling, while the intra-cellular resonances were broad, of about 400 Hz width (Fig. 2). With only the erythrocyte lysate present, line broadening of the fluorine signals of lomefloxacin was again observed.

An accurate partition coefficient could not be determined by NMR owing to the broad lines and overlap of the intra- and extra-cellular signals. However, it was determined that the apparent partition coefficient (D) for lomefloxacin into human erythrocytes was approximately 1.90 ± 0.15 ($n = 60$) for concentrations of 1–10 μg ml^{-1} at pH 7.4 and 25 °C by LC analysis. This was higher than the 1-octanol partition coefficient of 0.14 which was previously determined [2]. This was also the case for fleroxacin, whose erythrocyte partition coefficient was determined to be 1.45 by LC analysis [10], which was higher than its corresponding 1-octanol–water apparent partition coefficient of 0.27 at pH 7 [2].

The apparent partition coefficient (D) for lomefloxacin was found to be independent of concentration in the range 1–10 μg ml^{-1} (Fig. 3(a)), although there was a statistical difference between the partition coefficient value observed at 0.5 μg ml^{-1} (1.6 ± 0.16, $n = 15$) and those values for D obtained at 1, 3, 5, and 10 μg ml^{-1} (T-method or Tukey's honestly significant difference method to compare the means, $P \leqslant 0.05$). There also appeared to be a

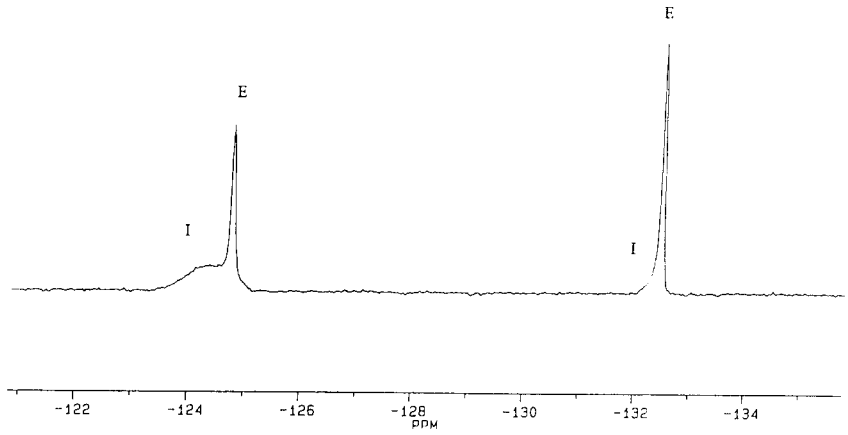


Fig. 2. ^{19}F NMR spectrum of the intra- and extra-cellular regions of lomefloxacin in an erythrocyte suspension at pH 7.4. The extra-cellular signals (E) are sharp and narrow while the intra-cellular signals (I) are flat and broad.

weak dependence of the apparent partition coefficient on time, such that the value of D increased slightly with time (Fig. 3(b)). Statistically, the difference was only between the 5 and 90 min time points (T-method or Tukey's honestly significant difference method to compare the means, $P \leqslant 0.05$). It was also found that the value of D decreased in glucose-starved, aged erythrocytes (more than three days old). The apparent partition coefficient for lomefloxacin in these cells was found to be 1.7 ± 0.12 ($n = 6$) with a drug concentration of 3 μg ml^{-1} and an incubation time of 30 min.

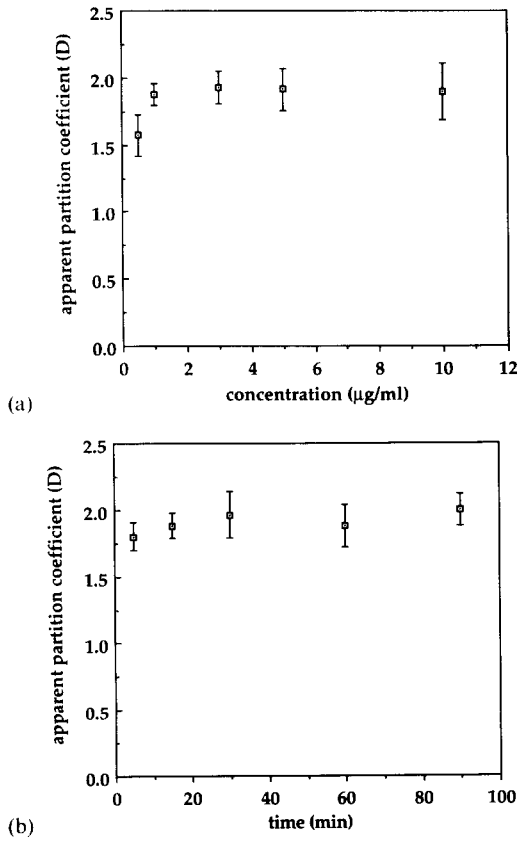
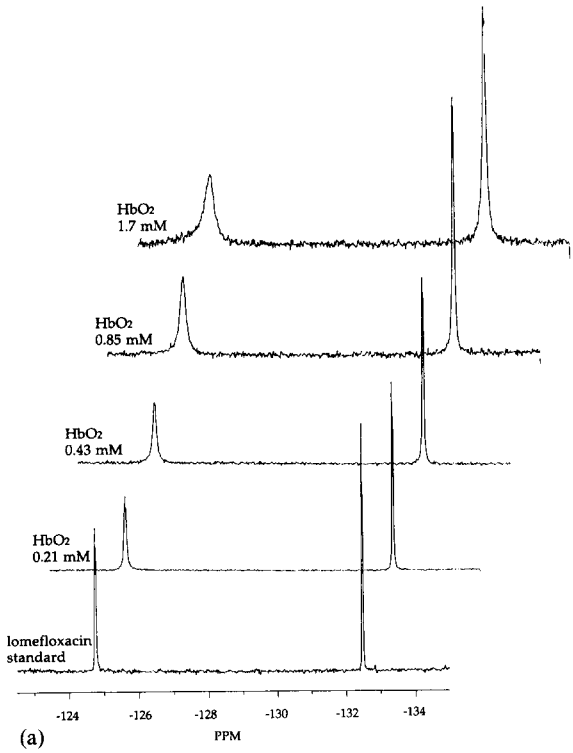


Fig. 3. Plot of the effect of the apparent partition coefficient (D) at pH 7.4 due to concentration (a) and time (b). For (a) $n = 15$, and for (b) $n = 12$.

In the presence of increasing concentrations of HbO_2, the fluorine signals of lomefloxacin by ^{19}F NMR broadened in a linear fashion over the concentration range studied (Fig. 4). There was a modest shift of approximately 0.5 ppm for the broadened signal. The same line broadening effect was observed with both carbonmonoxyhemoglobin (HbCO) and oxyhemoglobin (HbO_2); therefore, subsequent studies were performed with HbO_2. Line broadening of the fluorine signals also occurred when lomefloxacin was added to solutions of globin, hematin, and Fe^{2+} (Fig. 5).

There are three possible explanations for the observed line broadening of the NMR signals: (1) paramagnetic broadening, due to an interaction of the ligand with metal ions; (2) the intermediate rate of exchange between the bound and unbound states of the fluorinated species; and (3) restriction of rotational freedom of the fluorinated species when bound to a larger molecule, or macromolecule, causing an increase in the spin–spin relaxation rate of the species [14,18,19].

No line broadening of the fluorine signals of lomefloxacin was observed while in the presence of ghost cell membranes. This indicated that lomefloxacin was not bound to the ghost cell membrane, and therefore not to the erythrocyte membrane. Had there been any cell membrane association by the drug, the signals would have broadened owing to the slowing of the spin of the lomefloxacin molecule when in



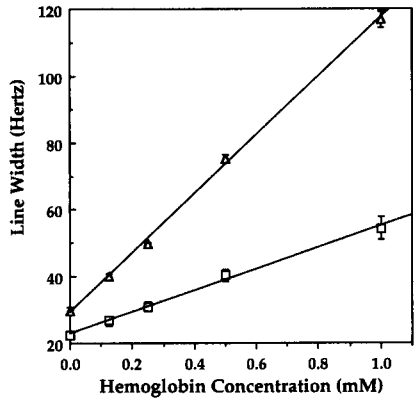


Fig. 4. (a) ^{19}F NMR stacked spectra of lomefloxacin in the presence of increasing concentrations of HbO_2 at pH 7.4. The spectra are offset slightly. The lomefloxacin concentration remained constant at 2.3 mM. The left signal represents the fluorine at the C7 position of lomefloxacin while the right signal represents the fluorine at C9. (b) Plot of the observed ^{19}F NMR line widths of the fluorine signals of lomefloxacin at varying concentrations of HbO_2 at the C7 (△) and C9 (□) positions of lomefloxacin ($n = 3$).

association with a larger species. Simanjuntak et al. [9] also reported that lomefloxacin passes through the membrane and enters the interior of the rat erythrocyte.

When free iron was in solution with lomefloxacin, paramagnetic broadening effects were observed owing to chelation of lomefloxacin to the metal ion [3,6]. This was probably also the case when hematin was present. Chelation and paramagnetic broadening could not have been a factor when lomefloxacin was in the presence of HbO_2, since it was found that the effect of line broadening of the fluorine signals was not influenced by the presence or absence of CO, which was added to make the heme iron diamagnetic [11]. Paramagnetic broadening could not have been the explanation for the observed fluorine signal line broadening of lomefloxacin in the presence of globin either, since globin does not contain a porphyrin moiety, and therefore does not contain iron. Owing to the dependence of line broadening of the fluorine signals of lomefloxacin on the concentration of the macromolecule, it was determined that the observed line broadening in the presence of HbO_2 and globin was due to the restriction of rotational freedom of lomefloxacin when bound to the proteins [14,18,19].

It was observed in the cases of HbO_2 and globin that the signal at −124 ppm, which represents the fluorine at the C7 position of lomefloxacin, broadened to a much greater extent than did the signal at −132 ppm. However, when examining the spectra of lomefloxacin with hematin and with Fe^{2+}, the fluorine signal at −132 ppm, representing the

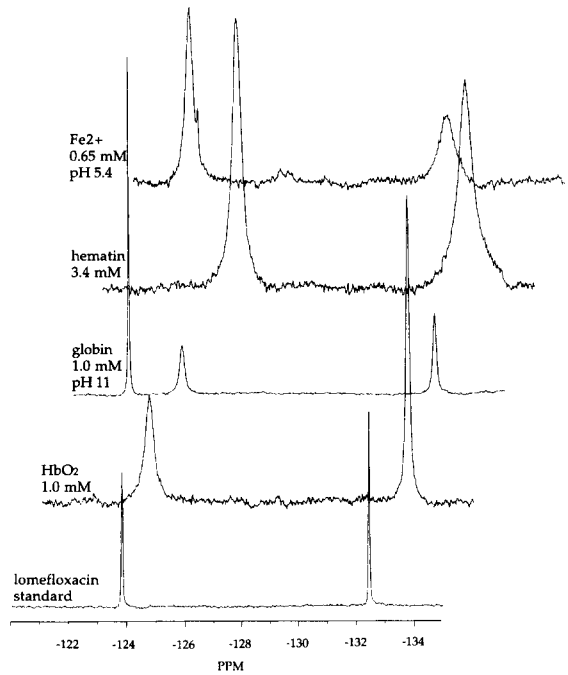


Fig. 5. ^{19}F NMR stacked spectra of lomefloxacin in the presence of HbO_2, globin, hematin, and Fe^{2+} at pH 7.4 (except where stated). The spectra are offset slightly. The lomefloxacin concentration remained constant at 2.3 mM. The additional peak in the globin spectrum was attributed to residual trifluoroacetate in the sample.

fluorine at the C9 position of lomefloxacin, broadened to a much greater extent (see Fig. 5). This differential line broadening of the fluorine signals of lomefloxacin in the ^{19}F NMR spectra indicated that one fluorine may have been more perturbed than the other, depending on the molecular orientation of lomefloxacin at the binding site of the macromolecule, porphyrin ring, or iron molecule. Further, it was concluded that there was a difference in the mode of interaction of the drug molecule with HbO_2 and globin vs. hematin and iron. The explanation of the differential broadening of the fluorine signals of lomefloxacin is unknown.

Broadening of the fluorine signals of lomeflox-acin occurred to a very small extent in the presence of albumin and no line broadening occurred in the presence of myoglobin or hematoporphyrin.

In summary, it was observed that, owing to line broadening, lomefloxacin interacted with iron, which is consistent with previous reports [3,4,6]. It was also observed that lomefloxacin interacted with hematin, the oxidized form of heme, and therefore it is reasonable to assume that there was an interaction between the fluoroquinolone and heme. However, no binding occurred with hematoporphyrin, which does not contain iron, suggesting that the iron moiety is necessary in order for lomefloxacin to bind to the porphyrin ring. Interestingly, lomefloxacin did not bind to myoglobin, despite the presence of an iron-containing porphyrin moiety. However, lomefloxacin did interact with globin, which does not contain iron or a porphyrin ring. Finally, lomefloxacin did bind to HbO_2, as indicated by the amount of line broadening that occurred during ^{19}F NMR analysis.

It was concluded that whereas lomefloxacin interacts with heme, it cannot interact with the heme portion of the intact HbO_2 molecule owing to steric effects. It would appear, therefore, that lomefloxacin binds to the protein moiety of HbO_2.

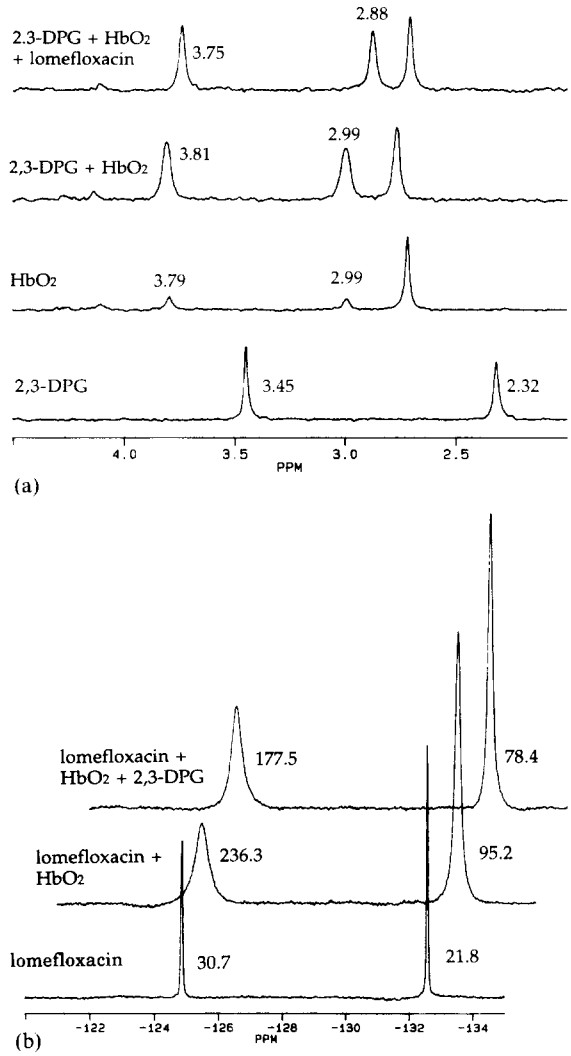


Fig. 6. (a) ^{31}P NMR stacked spectra of 2,3-DPG (bottom) with HbO_2 (second from top) and with HbO_2 and lomefloxacin (top) at pH 7.4. The corresponding concentrations are 5 mM DPG, 2.6 mM HbO_2, and 2.3 mM lomefloxacin. The right signal in the top three spectra is due to residual phosphate in the samples. The HbO_2 purified in this laboratory contained some residual 2,3-DPG (second from bottom). The signal shifts (ppm) are given. (b) ^{19}F NMR stacked spectra of lomefloxacin (bottom) with HbO_2 (middle) and with HbO_2 and 2,3-DPG (top) at pH 7.4. The corresponding concentrations are 2.3 mM lomefloxacin, 2.6 mM HbO_2 and 5.1 mM DPG. The spectra are offset slightly. The line widths (Hz) are given.

There is a distinct binding site for small anionic molecules on hemoglobin. 2,3-Diphosphoglycerate (2,3-DPG) is known to bind to the central cavity between the β-chains of hemoglobin [21,27]. Other anions are known to bind at this site, namely inositol hexaphosphate [28], NADPH [29], and chloride [30]. Lomefloxacin has an anionic carboxyl moiety at pH 7.4 and is therefore, structurally, a candidate for binding at the 2,3-DPG site of hemoglobin. To probe whether lomefloxacin binds to this cavity on HbO_2, competition studies with 2,3-DPG and lomefloxacin were performed by ^{31}P and ^{19}F NMR spectroscopy.

The addition of HbO_2 to a solution of 2,3-DPG caused a shift in the phosphorus signals of 2,3-DPG by ^{31}P NMR (Fig. 6(a), bottom three spectra). When lomefloxacin was added

to the solution of 2,3-DPG and HbO_2, the effective shift of the phosphorous signals decreased only slightly (Fig. 6(a), top). When analyzing the same solutions by ^{19}F NMR, it was again noted that the addition of HbO_2 to a lomefloxacin solution induced substantial broadening of the fluorine signals of the drug (Fig. 6(b), bottom and middle). It was then observed that the addition of 2,3-DPG to the HbO_2–lomefloxacin solution decreased the observed line broadening of the fluorine signals of lomefloxacin significantly (Fig. 6(b)). These two studies suggest that lomefloxacin and 2,3-DPG are competing for the same binding site of HbO_2; however, 2,3-DPG binds much more strongly to HbO_2 than does lomefloxacin, even though 2,3-DPG is known to bind only weakly to HbO_2 [31]. Further, since the line broadening of lomefloxacin was not completely eliminated by an excess of 2,3-DPG, there may also be non-specific binding of the drug to the protein, which could account for part of the observed line broadening.

NADPH also binds to the 2,3-DPG binding site of hemoglobin and is structurally similar to nicotinic acid. It has been reported that lomefloxacin utilizes the nicotinic acid transport system in rat erythrocytes [9], suggesting a similarity in the three-dimensional structures of lomefloxacin and nicotinic acid.

Addition of EDTA abolished the effect of line broadening in the cases of iron, HbO_2, and globin; however, this was not the case with hematin. The explanation of this phenomenon is not clear, especially since the effect of EDTA cannot be attributed only to chelation of the molecule with metal ions because, again, there were no metal ions present in the case of globin. EDTA is a polyanion and it is possible that it could have some affinity for the anionic binding site on hemoglobin. In this case, EDTA would be competing with lomefloxacin for the 2,3-DPG binding site on HbO_2. It was hypothesized that EDTA did not disrupt the interaction between lomefloxacin and hematin owing to the steric effects of the porphyrin ring of hematin prohibiting EDTA from binding.

The results of the ultrafiltration studies showed that there was very little physical binding between lomefloxacin and HbO_2, or that the binding is very weak. The apparent association constant for the binding of lomefloxacin to HbO_2 was less than unity; however, the true association constant for lomefloxacin to HbO_2 was impossible to determine since 2,3-DPG was always associated with the HbO_2 molecule. In conclusion, the physical association between lomefloxacin and HbO_2 detectable by ^{19}F NMR is weak and does not explain why the partition coefficient for lomefloxacin into human erythrocytes is greater than unity.

Acknowledgments

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