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High-Performance Liquid Chromatography Measurement of Antimicrobial Concentrations in Polymorphonuclear Leukocytes

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High-performance liquid chromatography was used to determine the penetration of 19 antimicrobial agents into human polymorphonuclear leukocytes. The ratios of the intracellular concentration to the extracellular concentration of ampicillin, piperacillin, cefazolin, ceftizoxime, cefpimizole, and ceftazidime were all less than 0.6. Lincomycin showed a high intracellular-to-extracellular ratio (3.0), while clindamycin achieved a ratio of 15.5, which was the highest ratio of all of the 19 tested antibiotics. Ratios for rifampin, isoniazid, chloramphenicol, and trimethoprim were 8.2, 1.1, 9.6, and 6.1, respectively. Six quinolone-class antimicrobial agents had ratios from 2.2 to 8.2. Flucytosine showed a penetration ratio of 4.6. Clindamycin uptake was temperature dependent and occurred best with live polymorphonuclear leukocytes; sodium fluoride, adenosine, and puromycin were inhibitory. The results obtained in this study correlate well with the results of other studies involving radioisotopic methods. This indicates that high-performance liquid chromatography is a useful method for determining the intracellular penetration of antimicrobial agents.

Some bacteria are able to remain viable even after they have been phagocytized by phagocytes by protecting themselves from the bactericidal activities of the phagocytes and by being shielded from extracellular antibiotics (3, 10, 11, 15, 18). Accordingly, knowledge of the ability of various antimicrobial agents to be transferred into phagocytes is essential when attempting to treat infections caused by bacteria capable of remaining alive inside cells. Several methods exist to measure antibiotic concentrations in body fluids and tissues. However, measurement of intracellular antibiotic concentration is difficult, since it is often very low. Because of this, previous investigations have involved the use of radioisotopic methods (5, 7, 8, 12).

In this study I used high-performance liquid chromatography (HPLC) to measure intracellular concentrations of various antibiotics in human polymorphonuclear leukocytes (PMNs).

MATERIALS AND METHODS

Collection of human PMNs. Approximately 30 ml of venous blood was collected from healthy adult volunteers by using a heparinized syringe (final heparin concentration, 10 to 20 U/ml). Mono-Poly Resolving Medium (Flow Laboratories, Inc., McLean, Va.) was used to purify the PMNs. Fresh, heparinized blood (3.5 ml) was layered on top of the Mono-Poly Resolving Medium (3 ml) in a sterile plastic tube (17 by 100 mm; Becton Dickinson Labware, Oxnard, Calif.). The mixture was centrifuged at 300 × g for 30 min at room temperature (15 to 30°C). The PMN fraction was removed and washed with Hanks balanced salt solution (pH 7.4; GIBCO Laboratories, Grand Island, N.Y.). When erythrocytes contaminated the PMNs, they were lysed by the addition of 0.2% NaCl. Finally, the PMNs were suspended in 10 ml of Hanks balanced salt solution so that the cell concentration was about 2×10^6/ml to 5×10^6/ml. The PMN fraction contained 95% or more PMNs, and their viability was 99% or greater as demonstrated by trypan blue exclusion.

Incubation of PMNs with antibiotics. The antibiotics investigated in this study were ampicillin sodium (ABPC; Meiji Seika Kaisha, Ltd.), piperacillin sodium (PIPC; Toyama Chemical Co., Ltd.), cefazolin sodium (CEZ) and ceftizoxime sodium (CZX) (Fujisawa Pharmaceutical Co., Ltd.), cefpimizole sodium (CPIZ; Ajinomoto Co., Inc.), ceftazidime (CAZ; Glaxo Inc.), lincomycin hydrochloride (LCM) and clindamycin hydrochloride (CLDM) (Japan Upjohn Ltd.), rifampin (RFP), isoniazid (INH), and ofloxacin (OFLX) (Daiichi Seiyaku Co. Ltd.), chloramphenicol (CP; Sankyo Co., Ltd.), trimethoprim, flucytosine (5FC; Roche Products Inc.), pipemidic acid (PPA; Dainippon Pharmaceutical Inc.), norfloxacin (NFLX; Kyorin Pharmaceutical Co., Ltd.), pefloxacin mesylate (PFLX; Yoshitomi Pharmaceutical Industries Ltd.), ciprofloxacin hydrochloride (CPFX; Bayer Yakuhin, Ltd.), and NY-198 (Hokuriku Seiyaku Co., Ltd.). Each of these antibiotics was dissolved in Hanks balanced salt solution to give concentrations of 100 μg/ml.

Antibiotic solutions (10 ml) were added to purified PMN suspensions (10 ml) (final antibiotic concentration, 50 μg/ml), mixed, incubated with agitation at 37°C for 30 min, and then centrifuged at 250 × g for 10 min at 4°C. After all of the supernatant except for 1 ml had been removed, the cells were resuspended therein and the cell count was determined in a hemacytometer once again (approximately 2×10^7 to 5×10^7 PMNs were contained in the suspension).

Separation of PMNs from extracellular solution. To separate the extracellular antibiotic from the PMNs, into which some of the antibiotic had entered, the velocity gradient technique was used (4, 8). Silicone oil (0.5 ml) (William F. Nye, Inc., New Bedford, Mass.; a 6:5 mixture of no. DC 550 and no. DC 556) was placed in a microcentrifuge tube, and 0.5 ml of the PMN suspension was layered on top. This was then centrifuged at 12,000 × g for 3 min at 4°C. After a pellet had been formed at the bottom of the tube, the preparation was stored frozen at −80°C. The extracellular solution layer and the PMN layer were obtained by cutting the frozen tube above and below the oil layers, respectively. The extracel-

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lular solution was thawed at room temperature and assayed for antibiotic solution. The PMN layer was suspended in 1 ml of distilled water and then disrupted by two cycles of freezing and thawing. The lysate was passed through a membrane filter (pore size, 0.22 μm; Millex-GS [Millex-GV for RFP], Millipore Corp., Bedford, Mass.) to remove the cell debris, and the antibiotic concentration in this solution was determined.

HPLC. HPLC was performed by using an ALC/GPC model 206 (model 6000A pump, U6K injector, and model 441 detector) (Waters Associates, Inc., Milford, Mass.). Table 1 shows the conditions and column used for each drug or drug group. Quantitative standards were run for each drug, and a standard curve was determined by using the total area under the peak of interest, as determined by electronic integration.

Determination of intracellular volume. The intracellular water space was measured by using tritiated water and the extracellular marker [^{14}C]polyethylene glycol (^3H_2O, 1.0 mCi/g; [^{14}C]polyethylene glycol, 1.4 mCi/g; New England Nuclear Corp., Boston, Mass.). The PMNs were incubated with these radiolabeled compounds for 2 min at 37°C, and then cells were separated from extracellular fluid by velocity gradient centrifugation as described above and counted in a liquid scintillation counter. The total water content of the PMN pellet was corrected for trapped extracellular water, i.e., polyethylene glycol space, to obtain the intracellular water space (8, 9, 12).

Characterization of antibiotic uptake. Further studies were performed to elucidate the mechanism of antibiotic uptake by PMNs, as described previously (6, 8, 9, 12), to determine whether HPLC measurement of intracellular concentrations gave results comparable to those of other studies. We examined the effect of cell viability by using PMNs killed by exposure to 10% Formalin for 30 min. These cells were then washed and suspended in fresh medium. In addition, the influence of temperature, pH, a metabolic inhibitor, and potential competitive inhibitors was examined. The influence of temperature was examined by comparing antibiotic uptake at 4 and 37°C. The pH profile of CLDM uptake was measured in media that had been preadjusted to different external pH values (from 5.0 to 8.5) by the addition of 10 N HCl or 10 N NaOH. Sodium fluoride (Sigma Chemical Co., St. Louis, Mo.) was used as a metabolic inhibitor at 5×10^{-3} and 1×10^{-3} M. The PMNs in Hanks balanced salt solution with or without sodium fluoride were incubated for 30 min at 37°C. CLDM was then added, and uptake was measured as described above. Nucleosides (adenosine, puromycin dihydrochloride; Sigma) were evaluated for competitive inhibition of CLDM uptake by PMNs. These materials were preincubated for 30 min with PMNs. CLDM was then added, and uptake was measured as described above.

The efflux of CLDM was also studied. The PMNs were incubated for 30 min at 37°C with CLDM, collected by centrifugation, and rapidly suspended in antibiotic-free medium. The intracellular concentration of CLDM was quantitated at various intervals (5, 10, 20, and 30 min) after removal of extracellular antibiotic.

RESULTS

Intracellular volume of PMNs. The intracellular volume of 10^7 PMNs was 2.15 ± 0.05 μl, calculated by subtracting the extracellular volume (25% of the total) from the total volume. This result was in fairly good agreement with the result of another study (9).

Uptake of antibiotics by PMNs. Table 2 shows the ratios of the intracellular concentration to the extracellular concentration (I/E ratio) of antibiotics incubated with human PMNs. Temperature and viability dependence of accumulation in PMNs of CLDM, LCM, RFP, CP, and six quinolone-class antimicrobial agents are shown in Table 3. The uptake of LCM, RFP, CP, PPA, NFLX, and NY-198 was independent of the viability of PMNs at 37°C. In contrast, the uptake of CLDM, OFLX, CPFX, and PFLX by Formalin-killed cells was markedly decreased. The uptake of CLDM at 4°C was also markedly decreased (Fig. 1).

The uptake of CLDM by PMNs under various pH conditions is shown in Fig. 2. At pH 5.0, CLDM uptake was markedly inhibited and the I/E ratio was 1.0. There were significant differences in uptake between pH 5.0 and pH 8.0. CLDM uptake increased gradually with increasing alkalinity of the medium, reaching a maximum I/E ratio of 14 at pH 8.0.

I used NaF as an inhibitor of glycolysis to elucidate the active CLDM transport system (Fig. 3). There was no statistically significant effect on CLDM uptake after a 5-min exposure of PMNs to the antibiotic. However, at 15 or 30 min, CLDM uptake was significantly reduced.

TABLE 1. HPLC apparatus and conditions used for each drug or drug group

Antibiotic[a]	UV wavelength (nm)	Mobile phase mixed with phosphate buffer[b]	Retention time (min)	Flow rate (ml/min)
CLDM	214	CH_3CN (35%)	3.8	1.0
LCM	214	CH_3CN (12%)	3.9	1.5
RFP	254	CH_3CN (40%)	3.3	1.0
Penicillins[c]	214	CH_3CN (6–18%)	7.7–9.0	1.0
Quinolones[d]	313	CH_3CN (80–85%)[e]	2.2–3.0	1.5
Cephems[f]	254	CH_3OH (3–25%)	4.3–5.5	0.8–1.7
CP	313	CH_3OH (50%)	4.2	0.6
Trimethoprim	254	CH_3OH (35%)	5.8	0.6
5FC	280	CH_3OH (3%)	2.5	1.0
INH	254	CH_3OH (3%)	3.5	3.0

[a] A NOVA PAK C_{18} column was used for all antibiotics but INH: particle size, 5 μm; length, 15 cm; inside diameter, 3.9 mm. A μ-Bondapak C_{18} column was used for INH: particle size, 5 μm; length, 30 cm; inside diameter, 3.9 mm. A Guard-PAK precolumn module, C_{18} cartridge, was also used. All were obtained from Waters Associates.

[b] Mixed with 0.05 M KH_2PO_4 (pH 6.0).

[c] ABPC and PIPC.

[d] PPA, NFLX, OFLX, CPFX, NY-198, and PFLX.

[e] Mixed with 0.04 M H_3PO_4 (pH 2.0).

[f] CEZ, CZX, CPIZ, and CAZ.

TABLE 2. I/E ratios in human PMNs for various antimicrobial agents

Antibiotic[a]	Mean I/E ratio (*n*)[b]
APBC	0.26 ± 0.03 (4)
PIPC	<0.01 (4)
CEZ	0.10 ± 0.09 (4)
CZX	0.09 ± 0.05 (4)
CPIZ	<0.01 (4)
CAZ	0.56 ± 0.13 (4)
LCM	3.01 ± 0.34 (3)
CLDM	15.46 ± 1.30 (10)
RFP	8.23 ± 0.78 (4)
INH	1.06 ± 0.24 (4)
CP	9.64 ± 0.59 (4)
Trimethoprim	6.10 ± 0.63 (3)
PPA	5.50 ± 0.52 (4)
NFLX	2.24 ± 0.30 (3)
OFLX	8.15 ± 2.00 (4)
CPFX	3.49 ± 0.60 (3)
NY-198	5.94 ± 0.74 (3)
PFLX	2.90 ± 0.38 (3)
5FC	4.57 ± 0.67 (3)

[a] Incubations were for 30 min, except for trimethoprim, which was incubated with PMN for 15 min.

[b] Values are mean ± standard errors of the I/E ratios; *n* is the number of experiments.

Potential competitive inhibitors of CLDM uptake, adenosine and puromycin, were preincubated with PMNs for 30 min before the determination of CLDM uptake. Both nucleosides had an inhibitory effect on early uptake of CLDM by PMN (Fig. 4).

To determine whether CLDM that had been taken up by PMNs was tightly bound to cellular components, the kinetics of efflux were studied (Fig. 5). The CLDM efflux was rapid, with loss of 60% of the intracellular drug by 5 min, and by 10 to 30 min only 20% of the CLDM remained in cells.

DISCUSSION

This study demonstrates that HPLC can be used instead of radioassay to determine intraleukocytic concentrations of many antimicrobial agents. The I/E ratios that were observed in our study are similar to those found by others using radioassay (5, 7, 8, 12). The HPLC method avoids the hazards associated with the use of radioisotopes and does not require the use of specially labeled antimicrobial agents.

TABLE 3. Influence of cell viability and environmental temperature on antibiotic uptake by human PMNs

Antibiotic	I/E ratios for[a]:		
	Viable cells		Dead cells (37°C)
	37°C	4°C	
CLDM	15.5	2.8	4.5
LCM	3.0	0.6	2.9
RFP	8.2	2.8	6.9
CP	9.6	6.7	8.5
PPA	5.5	1.7	5.3
NFLX	2.2	0.4	1.7
OFLX	8.2	4.8	2.6
CPFX	3.5	1.3	1.1
NY-198	5.9	4.2	3.6
PFLX	2.9	2.9	<0.1

[a] Values are means of three experiments. Incubations were carried out for 30 min.

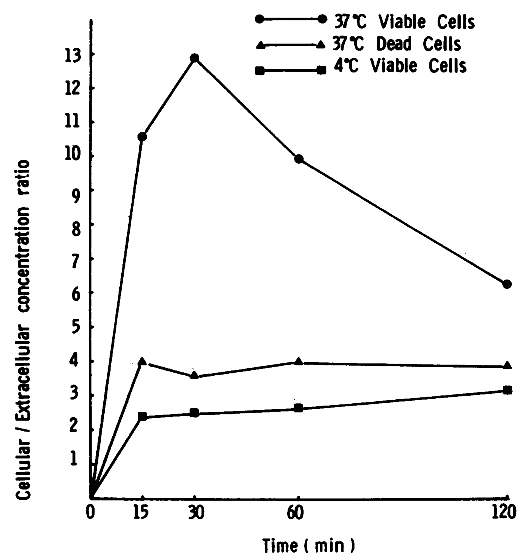

FIG. 1. Influence of cell viability and environmental temperature on CLDM uptake by human PMNs. Data are the means of two experiments at each time point.

Since some drugs, such as erythromycin and the aminoglycosides, cannot be measured directly by HPLC without derivitization (1, 2, 17, 19), radioassay may be easier to perform in these cases.

A velocity gradient centrifugation technique used in this study, which had previously been used by others (8, 12), was simple, rapid, reproducible, and useful for measurement of antibiotic concentrations in PMNs by HPLC.

CLDM uptake by the PMNs showed a high I/E ratio and energy requirement as demonstrated by dependency of cellular viability and environmental temperature. Accumulation of CLDM at 37°C was rapid and saturable, and then a gradual decrease in the I/E ratio was observed after 30 min of incubation because of a slight drop in extracellular pH and PMN clumping, as described by others (8, 9). CLDM uptake at 4°C or by Formalin-killed cells, reflecting entry due to

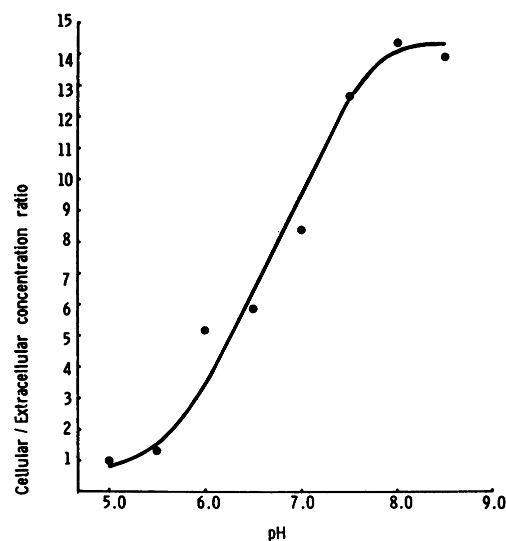

FIG. 2. Effect of pH on CLDM uptake by human PMNs after a 10-min incubation period. Data are the means of two experiments at each pH.

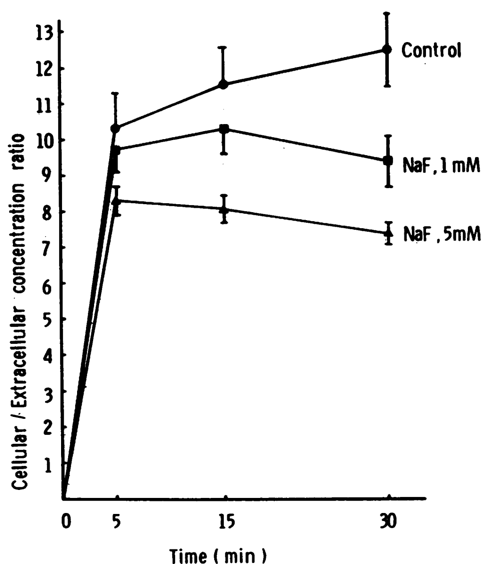

FIG. 3. Influence of inhibitor of cellular metabolism (NaF) on CLDM uptake by human PMNs. Mean I/E values ± standard errors of the means are shown.

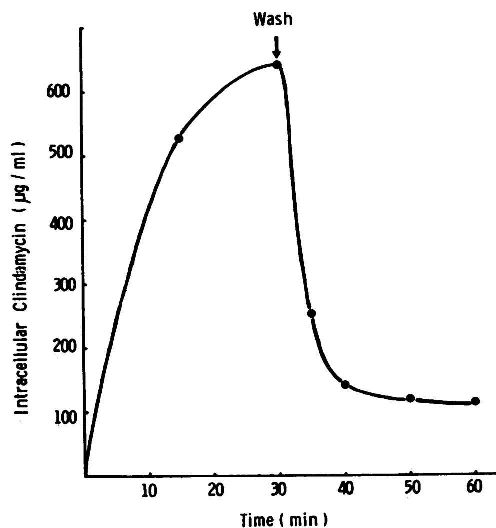

FIG. 5. Efflux of intracellular CLDM from human PMNs after removal of the extracellular drug. Data are the means of two experiments at each time point.

simple solubility partition, was markedly decreased and showed a discrepancy of accumulation depending upon plasma membrane alteration by low temperature or Formalin. In addition, pH dependence indicating a carrier-mediated transport system, and effects of metabolic inhibitor and potential competitive inhibitors were observed. The findings of this study suggest that CLDM is actively transported into the PMNs via the nucleoside transport system and that efflux occurs rapidly after removal of extracellular drug. Consequently, the use of HPLC gave results for CLDM cell transport similar to those found by others (6, 8, 9, 12, 16).

The finding that quinolone-group antimicrobial agents are concentrated in PMNs supports clinical and experimental evidence that these drugs are effective for the treatment of disease caused by intracellular bacteria such as mycobacteria and *Legionella pneumophila* (13, 14). Because this group of drugs was concentrated in PMNs with various degrees of dependence on cell viability and temperature, it is possible that the group is heterogenous in terms of transport mechanisms.

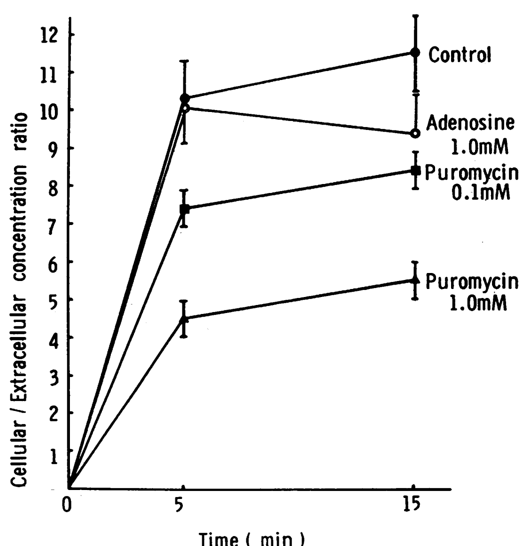

FIG. 4. Uptake of CLDM in the presence of adenosine and puromycin. Mean I/E values ± standard errors of the means are shown.

Although this study involved the use of radioisotopes to measure PMN volume, such information is not essential to determine approximate intracellular concentrations of antimicrobial agents. If the knowledge of intracellular antimicrobial concentrations is used in a semiquantitative fashion for predicting the efficacy of a particular antimicrobial agent in the treatment of infections caused by intracellular pathogens, a margin of error in measurement of intracellular volume is permissible. The acceptable error of estimating the true intracellular antimicrobial concentration is unknown, but certainly must be at least 20 to 30%, which is probably the error of estimating, rather than directly measuring, the cell volume. A reasonable fixed estimate of PMN volume is about 0.27 pl per cell, assuming a cell diameter of 8 μm and a true spherical shape. This value for cell volume is close to that found by Klempner and Styrt (9), 0.20 pl per cell, and to that found in this study, 0.22 pl per cell.

In summary, HPLC can be substituted for radioassay for determination of I/E ratios for most antimicrobial agents. This is of particular benefit when radiolabeled compounds are not available.

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