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Research Article

Cellular To Extra-Cellular Concentrations Of Doxycycline, Enoxacin And Roxithromycin In Human Polymorphonuclear Neutrophils.

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Abstract

Introduction: Since bacteria which can penetrate and reproduce within phagocytes are more likely to cause prolonged and serious infections, this emphasize the use of those antibiotics which concentrate inside cells and kill those bacteria. C/E ratio is a parameter to determine the drug concentration inside and outside the cell. In present study we determined the C/E Ratios of doxycycline, enoxacin and roxithromycin in human polymorphonuclear neutrophils.

Method: Polymorphonuclear neutrophils from the whole blood was separated by the protocol given by Polymorphprep reagent obtained from Progen Company with few modifications. Viability of cells was tested by trypan blue. Later, these neutrophils were allowed to incubate with drugs at concentration prepared by given C max and T max in literature. After that they were micro-centrifuge, freezed at -80℃ and hence been separated into cellular and extracellular concentrations which were quantitatively assayed by RP-High Performance Liquid Chromatography technique.

Results: The result shows that doxycycline, enoxacin and roxithromycin have an outstanding ability to concentrate inside the human polymorphonuclear neutrophils. Hence these drugs are proven to be more effective against intracellular pathogens. However, they are dependent upon the conditions and factors provided to them. These results can prove to be an important milestone for further research and are of great importance. It provides us a fair idea towards treatment approach and allows the comparison with other drugs of same group.

Conclusion: This study demonstrates that enoxacin and doxycycline achieve substantially higher intracellular accumulation in human polymorphonuclear neutrophils than roxithromycin, supporting their greater potential efficacy against intracellular pathogens.

Keywords: Polymorphonuclear neutrophils; Intracellular accumulation; Doxycycline; Enoxacin; Roxithromycin.

INTRODUCTION

Antibiotics, type of antimicrobials is a large and important group of medicine which act by either killing or arresting growth of susceptible microorganisms inside and outside the cells. Some of the antibiotics also have antiprotozoal activity (1). Antibiotics work mainly through four different ways: they can stop cell wall synthesis, block protein synthesis, interfere with folate synthesis, or disrupt DNA gyrase activity. Each of these actions messes with how bacteria maintain their structure, perform essential functions, produce nucleotides, or replicate their DNA (2). Polymorphonuclear neutrophils are type of white blood cells and the first line of defense along with macrophages in any infection, so those microorganisms which multiply and survive in them are more prone to relapse and elongation of disease period. Only some of the antibiotics are capable of internal localization of cells. Those antibiotics which penetrate and accumulate more intracellularly are believed to kill more efficiently the intracellular pathogens (3). Enoxacin, Doxycycline and Roxithromycin are antibiotics which belong to class flouroquinolones, tetracycline and macrolides respectively. These antibiotics are founds to be more effective against intracellular and atypical pathogens (4-6). Doxycycline is a type of tetracycline that stops bacteria from growing by preventing protein synthesis. It does this by binding to the 30S ribosomal subunit, which blocks the attachment of aminoacyl tRNA. Resistance to doxycycline happens when Gram negative bacteria become less permeable, utilize efflux pumps, produce protective ribosomal proteins, modify the drug with enzymes, or develop mutations in the 16S rRNA that reduce how well the drug binds (7-9). Enoxacin gets into bacteria through porins and inhibits DNA replication by attaching to DNA gyrase and topoisomerase IV. This leads to breaks in DNA strands and problems with separating

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newly formed chromosomes. Bacteria can become resistant through mutations in these enzymes or by reducing the levels of the drug inside the cell due to efflux mechanisms (10-12). Roxithromycin works by binding reversibly to the 50S ribosomal subunit. This prevents the ribosome from moving along the mRNA and affects transpeptidation, ultimately suppressing protein synthesis and the growth of bacteria. Resistance can occur through active efflux pumps, the hydrolysis of the drug by enzymes, or through chromosomal mutations that change the 50S ribosomal target (13-17)

This study is finding and comparing ratios of cellular to extracellular concentrations of these three drugs in human PMNs and plasma using HPLC analysis. Cellular to extra cellular Ratio (C/E) is a determining parameter used to compare antibiotics and to find the localization of drug inside the cells and outside in the plasma, Comparison of it give us a fair idea about the drugs cellular uptake and possible advantageous effects against intracellular pathogens.

MATERIAL AND METHODS

Study setting

This study was performed in Pharmacology, Hematology and cell culture laboratories, Institute of Basic Medical Sciences, Khyber Medical University Peshawar. It was approved in the 47th meeting of KMU-AS&RB held on 28 February 2017 under the Reference No. DIR/KMU-AS&RB/EC/000604. Moreover, ethical approval was obtained from the ethical committee of Khyber Medical University under Reference No. DIR/KMU-AS&RB/EC/000604, Held on 06-04-2017.

Blood collection

10ml venous blood was collected for each drug assay from different healthy volunteers, fulfilling the requirements of inclusion/exclusion criteria. Consent form in both Urdu and English languages were signed from volunteers after telling them full details and made sure about complete understanding of it (18).

HPLC analysis

Chemicals, Reagents, and Standards

HPLC-grade methanol, acetonitrile, water, acetic acid, potassium dihydrogen phosphate, and diluted hydrochloric acid were used in the experiments. Doxycycline, enoxacin, and roxithromycin were sourced from local pharmaceutical companies in Pakistan, and diazepam used as an internal standard for the doxycycline analysis.

Preparation of Calibration Standards

Stock solutions (1 mg/mL) of doxycycline, enoxacin, and roxithromycin were prepared using appropriate solvents and serially diluted to obtain calibration ranges of 0.0625–1 mg/mL. was diluted in HPLC-grade water, while enoxacin was prepared using a mix of water, methanol, and acetic acid in a ratio of 2:5:3 (v/v/v) and then further diluted with a mixture of methanol and water at 8:2 (v/v). Roxithromycin dilutions were made in methanol (18).

UV Spectrophotometric Analysis

The maximum absorbance wavelength (λmax) using a UV spectrophotometer was determined and turned out to be 229 nm for doxycycline, 290 nm for enoxacin, and 215 nm for roxithromycin. These wavelengths were used for the chromatographic detection later on (18).

Plasma Sample Preparation

To collect human blood samples, EDTA tubes were used and centrifuged them at 3000 rpm for 10 minutes to separate the plasma. 250 µL of this plasma was taken and spiked it with another 250 µL of the calibration standards. For the doxycycline analysis, 100 µL of the diazepam internal standard was added. Protein precipitation was carried out with acetonitrile (250 µL), mixed it with a vortex, and then centrifuged it at 11,000 rpm for 10 minutes. Finally, the supernatants were filtered through 0.22 µm nylon membrane filters and stored them at −20 °C until ready for the analysis (18).

HPLC Conditions

Reverse-phase high-performance liquid chromatography (RP-HPLC) using Shimadzu HPLC systems fitted with a C18 column (5 µm, 25 × 0.46 cm) was used in this study. The injection volume was set at 20 µL, and kept the flow rate at 1 mL/min. For doxycycline, the mobile phase was a mixture of water and methanol (50:50, v/v; pH 2). In the case of enoxacin, methanol and water (80:20, v/v), while for roxithromycin, a buffer with 0.03 M KH_2PO_4 and methanol (40:60, v/v; pH 4.5) was used. The retention time of the compounds at their respective λmax values, was noted at 7.15 minutes for doxycycline, 1.6 minutes for enoxacin, and 18 minutes for roxithromycin. Calibration curves were constructed using the peak area responses that obtained (18).

Preparation of Drug Suspensions for Assay

Drug suspensions were prepared based on known pharmacokinetic parameters, including maximum plasma concentration (C_max), time to reach maximum concentration (T_max), and elimination half-life. The stock solutions of doxycycline, enoxacin, and roxithromycin at 100 µg/mL in Hanks' Balanced Salt Solution (HBSS) were prepared, then diluted them to reach final assay concentrations that matched their respective C_max values. Specifically, (19) and enoxacin (20) final concentrations were set at 2 µg/mL (C_max ≈ 2 ± 1 mg/L; T_max = 2 h) and 2.741 µg/mL (C_max = 2.741 ± 0.960 mg/L; T_max ≈ 1 h), respectively. For roxithromycin, a stock solution based on a reported C_max of 6.34 mg/L; T_max ≈ 1 h was prepared, and diluted it to get a final assay concentration of 6.34 µg/mL, using 5 mL for the analysis (21).

Isolation, Purification, and Drug Treatment of Human Polymorphonuclear Neutrophils

Isolation of Polymorphonuclear Neutrophils

Polymorphonuclear neutrophils (PMNs) were isolated from peripheral venous blood in two healthy adult volunteers. All reagents and solutions were equilibrated to room temperature prior to use. Blood was collected aseptically using sterile syringes into EDTA-containing blood collection tubes that contained 5 mL of the blood. Whole blood was carefully layered over Polymorphprep (a 13.8 % w/v sodium diatrizoate and 8.0 % w/v polysaccharide solution) using a Pasteur pipette. This procedure used 5 mL of whole blood and was done using a 15 mL Falcon tubes, the two solutions had limited mixing at the interface before centrifugation. Centrifuge was set at 550 g for 30-35 minutes at 19 °C without using the brake to stop the centrifuge. Centrifugation resulted in the sedimentation and separation of whole blood into six distinct layers, including the following: plasma, peripheral blood mononuclear cells (PBMCs), the isolation medium, and polymorphonuclear neutrophils, the isolation medium, and erythrocytes at the bottom of the tube. The plasma and PBMC layers were aspirated using a Pasteur pipette. The neutrophil layer was collected using a serological pipette and included 1 to 2 mL of the isolation medium by orienting the tube upwards and gently rotating it. The erythrocyte layer of the tube was discarded (22, 23).

Washing and Purification of Neutrophils

The neutrophil fractions, from both Falcon tubes, were pooled, using a new 15 mL Falcon tube containing 9 mL of Calcium- and Magnesium-Free, 1x Phosphate buffer Saline (PBS). The total volume was adjusted to 14mL by adding PBS, and centrifuged at 450gav for 10 min at 19°C. After the centrifugation process, red pellet containing neutrophils and residual erythrocytes were formed at the bottom of tube. The supernatant was aspirated very carefully without disrupting the sediment. To lyse the erythrocytes, the sediment was re-suspended in 3 mL of sterile distilled water and agitated for 30 seconds (longer than 30 seconds was avoided to prevent lysis of neutrophils). Immediately after, 700 µL of 5x PBS was added and the mix was agitated gently. The resulting suspension was centrifuged at 250gav for 5 minutes at 19°C, and the supernatant was aspirated to leave a sediment approximately 2mL in diameter. This lysis procedure was repeated three additional times until the white sediment was obtained and identified as purified neutrophils. The remaining neutrophils were washed with 9 mL of Calcium and Magnesium Free PBS and centrifuged at 250g av for 5 minutes. The supernatants were then aspirated to leave approximately 2 mL of neutrophil retentate, which was then re-suspended in 5mL of Hank's Balanced Salt Solution (HBSS) (22, 23).

Assessment of Cell Viability by Trypan Blue Exclusion

The Trypan Blue exclusion test was used to assess neutrophil viability prior to final resuspension in HBSS (i.e. to determine if the cells could be used for functional assays). 10 µl of the neutrophil cell suspension, with equal volume (10 µl) of 0.4% Trypan Blue solution, was added to a cryovial and mixed gently by pipetting. The cell-dye mix was then incubated for 3 min at room temperature. The hemocytometer was prepared by placing a coverslip on it, and the cell-dye mix was carefully added through the notch, being careful not to form air bubbles. The hemocytometer and microscope were cleaned with 70% ethanol and 30% distilled water before use. The cells were viewed on a light microscope at 10X magnification. The viable neutrophils were white and the non-viable cells were blue. Each corner square (total of four) of the hemocytometer (each corner square containing 16 smaller squares) was counted as a total of 64 squares; hence, each corner square contained a viable neutrophil count as well as a total neutrophil count. Cell counts were made using a tallied method (22, 23). Cell viability was calculated using the following formula:

Incubation of Neutrophils with Drugs

Purified and viable neutrophils suspended in 5 mL of HBSS were mixed with an equal volume (5 mL) of previously prepared drug solutions in HBSS in centrifuge tubes. The suspensions were incubated in a shaking incubator (WiseCube WIG-105, Germany) at 37 °C with constant agitation. Incubation durations were based on the maximum time (Tmax) for each drug: doxycycline (2 hours), enoxacin (1 hour), and roxithromycin (1.04 hours) (22, 23).

$$\text{Percentage viability} = \left(\frac{\text{Number of viable cells}}{\text{Total number of cells}}\right) \times 100$$

Separation of Neutrophils and Extracellular Fluid

Following incubation, the tubes were centrifuged at 250 × g for 10 minutes at 4 °C. The supernatant was carefully removed, leaving approximately 1 mL, and the cell pellet was resuspended by gentle tapping. In a microcentrifuge tube, 40 µL of 88% formic acid was placed at the bottom, followed by careful layering of 300 µL of silicone oil. Subsequently, 400 µL of the resuspended drug-treated neutrophil suspension was layered over the silicone oil. The tubes were centrifuged in a refrigerated microcentrifuge (MSE Harrier 18/18, UK) at 12,000 × g for 5 minutes at 4 °C. During centrifugation, neutrophils passed through the silicone oil and were lysed by formic acid, while extracellular fluid remained above the oil layer. The microcentrifuge tubes were then frozen at −80 °C. The following day, frozen tubes were carefully cut at the interface between the two layers using a sharp sterile razor. The upper (extracellular) and lower (cellular) layers were transferred into separate microcentrifuge tubes (22, 23).

RESULTS

The present study was conducted to evaluate the intracellular accumulation of three antibiotics—doxycycline, enoxacin, and roxithromycin—by determining their cellular/extracellular (C/E) concentration ratios at maximum concentration (C_max) and time to maximum concentration (T_max). Quantitative analysis was performed using a validated highperformance liquid chromatography (HPLC) method.

For each antibiotic, a set of standard dilutions was prepared to construct calibration curves. The assay yielded consistent retention times and satisfactory precision and accuracy within all three drugs' concentration ranges. Diazepam (the internal standard) produced consistent retention times of approximately 5.6 minutes. Average accuracy of diazepam was between 74.04% and 95.13% and the coefficients of variation were within acceptable limits confirming the reliability of the analytical method (**Table 1**).

Table 1. Assay of Internal Standard, Diazepam.

Sr #	Actual Concentration on (mg/ml)	Expected Concentration (mg/L)			RT (min)	SD	%Accuracy	Mean ± %Coefficient of variance	%Yield
		C1	C2	C3					
1	10	8.985	8.876	8.924	5.6	0.546	89.28	8.928±0.61	89.28
2	10	7.381	7.452	7.384	5.6	0.04015	74.04	7.406±0.54	74.04
3	10	9.414	9.614	9.512	5.6	0.1000	95.13	9.513±1.05	95.13
4	10	9.137	9.201	9.313	5.6	0.08908	92.17	9.217±0.97	92.17
5	10	8.539	8.541	8.562	5.6	0.01274	85.47	8.547±0.15	85.47

For doxycycline, standard concentrations ranging from 6.25 to 100 mg/L produced mean measured concentrations between 5.92 ± 0.53 mg/L and 95.02 ± 2.25 mg/L. Accuracy ranged from 88.4% to 99.76%, with coefficients of variation below 9%, indicating good linearity and repeatability of the assay (**Table 2**).

Table 2. Assay of Standard Doxycycline Dilutions.

Sr#	Actual Concentration (mg/L)	Expected Concentration (mg/L)			RT (min)	Mean ± SD	% Accuracy	% Coefficient of variance	%Yield
		C1	C2	C3					
1	100	97.507	94.412	93.131	11.6	95.02±2.250	95.02	2.37	95.02
2	50	44.228	49.905	48.403	11.6	47.51±2.942	95.02	6.19	95.02
3	25	25.001	26.459	23.367	11.6	24.94±1.547	99.76	6.20	99.76
4	12.5	11.011	11.518	10.627	11.6	11.052±0.4469	88.4	4.04	88.4
5	6.25	6.451	5.929	5.397	11.6	5.92±0.5270	95.58	8.89	95.58

Similarly, enoxacin calibration standards (6.25–100 mg/L) yielded mean measured concentrations between 6.2 ± 0.10 mg/L and 96.67 ± 1.53 mg/L, with accuracy values ranging from 91.2% to 96.67% (**Table 3**).

Table 3. Assay of Standard Enoxacin dilutions.

Sr #	Actual Concentration (mg/L)	AUC	Expected Concentration (mg/L)			RT (min)	Mean±SD	Accuracy %	%Coefficient of variance	%Yield
			C1	C2	C3					
1	100	12479403	98	97	95	1.6	96.67±1.528	96.67	1.58	96.67
2	50	6237015	49.2	47.8	46.2	1.6	47.73±1.501	95.46	3.14	95.46
3	25	4158010	23.5	24.2	23.2	1.6	23.63±0.5132	94.52	2.17	94.52
4	12.5	2120902	11.2	10.9	12.1	1.6	11.40±0.6245	91.2	5.48	91.2
5	6.25	1178278	6.2	6.3	6.1	1.6	6.2±0.1000	95.38	1.61	95.38

Roxithromycin standards over the same concentration range showed mean concentrations from 6.20 ± 0.10 mg/L to 96.77 ± 1.55 mg/L, with accuracy exceeding 91% in all cases (**Table 4**).

Table 4. Assay of Standard Roxithromycin dilutions.

Sr#	Actual Concentration (mg/L)	AUC	Expected Concentration (mg/L)			RT (min)	Mean±SD	Accuracy %	%Coefficient of variance	%Yield
			C1	C2	C3					
1	100	3495672	98.20	97.00	95.12	18	96.77±1.552	96.77	1.60	96.77
2	50	1589442	49.2	47.8	46.2	18	47.73±1.501	95.46	1.34	95.46
3	25	836285	23.5	24.2	23.2	18	23.63±0.5132	94.52	2.17	94.52
4	12.5	398231	11.2	10.9	12.1	18	11.40±0.6245	91.2	5.48	91.2
5	6.25	76738	6.2	6.3	6.1	18	6.20±0.10	99.2	1.61	99.2

The intracellular and extracellular concentrations were determined by using peak area and height data from the standard calibration curve from each of the drugs respectively. Doxycycline demonstrated very high levels of intracellular accumulation. The intracellular concentration was between 18.10 mg /L and 18.96 mg /L and the extracellular concentration was between 2.50mg /L and 2.81mg /L giving C/E ratios that were between 6.72 and 7.54 thus indicating significant cellular penetration (Table 1) with an average C/E of 7.040 ± 0.4386 (**Table 5**).

Table 5. Cellular/ Extracellular concentration Ratio of Doxycyline.

Sr#	AUC INT	Height INT	Intracellular Concentration mg/L	AUC EXT	Height EXT	Extracellular Concentration mg/L	C/E Ratio
1	9057495	131440	18.840	1202044	9137	2.8056	6.72
2	8954308	102587	18.102	1181701	8764	2.639	6.86
3	9265834	255232	18.960	1969653	9723	2.501	7.54

Enoxacin also showed substantial intracellular accumulation however there was less of a difference between the two concentrations compared to doxycycline where intracellular levels were between 9.16 mg /L and 9.97 mg /L versus extracellular concentrations which were between 0.98 mg /L and 1.11 mg /L, therefore C/E ratios ranged from 8.97 to 9.40 with a mean C/E ratio of 9.146 ± 0.2245 (**Table 6**). Consequently enoxacin has the highest level of intracellular accumulation compared to the three antibiotics.

Table 6. Cellular/ Extracellular concentration Ratio of Enoxacin.

Sr#	AUC INT	Height INT	Intracellular Concentration mg/L	AUC EXT	Height EXT	Extracellular Concentration mg/L	C/E Ratio
1	1239823	85528	9.9679	198558	14140	1.1115	8.97
2	149856	86543	9.2672	176544	16342	1.0212	9.07
3	139876	83465	9.1645	129874	13986	0.975	9.399

In contrast, roxithromycin showed significantly lower levels of intracellular accumulation compared to both doxycycline and enoxacin with intracellular concentration ranging between 1.47 mg /L and 1.97 mg /L and extracellular concentrations ranging between 0.82 mg /L and 0.86 mg /L; producing a range of C/E ratios (1.71 to 2.36) and an average C/E ratio of 2.006 ± 0.3303 (**Table 7**).

Table 7. Cellular/ Extracellular concentration Ratio of Roxithromycin.

Sr#	AUC INT	Height INT	Intracellular Concentration mg/L	AUC EXT	Height EXT	Extracellular Concentration mg/L	C/E Ratio
1	42075	1702	1.4706	20275	860	0.8601	1.709
2	54064	2301	1.9651	19145	845	0.8321	2.3616
3	45134	1692	1.5982	23965	898	0.8209	1.9468

The results from a comparative study of C/E ratios indicate pronounced differences in the intracellular levels of the various antibiotics. Enoxacin had the greatest amount of cellular accumulation compared to doxycycline and roxithromycin, which had the lowest C/E ratios. These observations demonstrate that there are drug-specific differences in cellular uptake and retention, and thus may impact their intracellular antimicrobial activity.

DISCUSSION

Intracellular pathogens are great source of prolonged lethal and recurrent infections because of their capability of coping environmental challenges and surviving even after phagocytic ingestion. Optimal therapy approach to deal with this situation is, the use of particularly those antibiotics which are capable of intracellular uptake and accumulation inside the granulocytic phagocytes specially neutrophils to inactivate and kill those microorganisms. However, this relationship is not always directly proportional.

C/E ratio is an imperative parameter to determine the drug concentration inside and outside the relevant cell. Different method approaches were used for quantitative analysis of drug to determine cell concentration, which were Radio assay, HPLC and flourometric techniques. HPLC has replaced radioassay and flourmetric techniques to avoid the risks linked with radiolabelled compounds and restriction of fluorescence associated structure detection, respectively. Three main drugs Doxycycline, Enoxacin and Roxithromycin were chose from different classes of antibiotics to give us a major idea for the potential of that class and specifically that drug to treat and cure cellular pathogens. Cmax and Tmax are chief parameters of pharmacokinetics that ultimately determine and affect bioavailability, were reviewed from literature for each drug.

HPLC method for doxycycline was developed and validated and the peaks of drug and internal standard diazepam were well resolved. Retention time of doxycycline was found to be on 11.6mins and diazepam on 5.6mins. Calibration curve was plotted Area under curve against known concentration of doxycycline(100mg-6.25mg/L) and regression equation was r=0.96. This suggests the reproducibility of results. Samples were prepared from the above-mentioned procedure for cellular and extracellular concentrations and were run on HPLC. C/E ratio of doxycycline was calculated from the calculated values of Cellular and extracellular concentration, and it was found to be C/E Ratio of DOX= 7.040±0.4386 (Mean ± Standard deviation). This result fair comply with results of research done on Minocycline transport into Human neutrophils and suggests 7 times more intracellular penetration of DOX than extracellular fluid concentration which made it more preferable for killing of intracellular microorganisms (24).

Fluoroquinolones, have outstanding ability to penetrate more in phagocytic cells and eradicate microorganism more effectively that resist phagocytic killing (25). Many researches have been done up till now for finding C/E ratio of different drugs of fluoroquinolones and showed worthy results (26-28). For enoxacin, method was developed and calibration curve was plotted manually in software Graph Pad Prism. Standard curve was plotted by area under curve against known concentration of enoxacin (100mg-6.25mg/L) and regression equation was r=0.99 which suggested it reproducible. C/E ratio of enoxacin is found to be 9.146±0.2245 (Mean ± Standard deviation). This value depicts that enoxacin concentrate 9 times more inside the neutrophils than extracellular. It's a significant value which allows making a comparison with other fluoroquinolones and making more favorable to use against intracellular bacteria like Str. pneumoniae and Staphylococcus aureus (29).

Roxithromycin belong to group macrolids, and this class also found to possess high ability to accumulate inside the phagocytes than non-phagocytes (30). Calibration curve was plotted manually in Graph Pad Prism software against concentration(100mg-6.25mg/L) and regression equation was found to be r=0.99. C/E ratio of roxithromycin is found to be C/E Ratio= 2.006±0.3303 (Mean ± Standard deviation). It illustrates the accumulation of drug twice inside the neutrophils than to extracellular. This can be high upto 7 to 8 times depending upon the conditions provide to them.

CONCLUSION

The present study concludes that doxycycline, enoxacin and roxithromycin have different rates of accumulation inside polymorphonuclear neutrophils, indicated by the ratios of drug concentration inside cells compared to outside. Of the three tested antibiotics, the drug with the most accumulation inside neutrophils was enoxacin (C/E = approx 9), followed by doxycycline (C/E = approx 7), while roxithromycin (C/E = approx 2) showed much less accumulation within neutrophils. Therefore, the physicochemical properties of specific drugs and their pharmacokinetics are responsible for differences in their ability to be taken up and retained inside neutrophils. Doxycycline and enoxacin exhibited substantial concentrations within neutrophils, suggesting a greater efficacy against intracellular or phagocyte-associated pathogens, which can cause prolonged and recurrent infections that are difficult to treat. Although roxithromycin exhibited less accumulation than doxycycline and enoxacin under the conditions used here, the localization of roxithromycin in immune cells provides evidence of its therapeutic relevance; intracellular accumulation may vary according to the condition of the specific immune cell and experimental conditions. Therefore, these findings will aid in selecting appropriate therapy when considering infections caused by intracellular organisms and will support additional research looking at antimicrobial efficacy and using larger sample sizes and testing other antibiotics for functional activity to better define the clinical relevance of drug accumulation within immune cells.

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