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Automated analytical systems for drug development studies Part IV. A microdialysis system to study the partitioning of lomefloxacin across an erythrocyte membrane in vitro ☆

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Abstract

An automated system utilizing microdialysis sampling, intermittent dosing, and liquid chromatographic analysis was assembled in order to study the partitioning of lomefloxacin, a fluoroquinolone antimicrobial, into human erythrocytes in vitro. The apparent erythrocyte:buffer partition coefficient was found to be approximately 2.0 with this system and by a manual method. The value was concentration-dependent; lower partition coefficients were observed at lomefloxacin concentrations less than 1 $\mu g\ ml^{-1}$. At all concentrations, values obtained by microdialysis were statistically indistinguishable from those obtained by a conventional manual method. The results indicate that erythrocyte partition coefficients can be measured successfully with the microdialysis system. Furthermore, microdialysis sampling eliminates the tedious methodology associated with traditional erythrocyte partitioning studies, including sample clean-up. Due to automated intermittent dosing and on-line LC analysis, the system operates unattended.

Keywords: Fluoroquinolone; Human erythrocytes; Liquid chromatography; Lomefloxacin; Microdialysis sampling; Partition coefficient

1. Introduction

The study of partition coefficients is an important aspect of investigating the physico-chemical properties of pharmaceuticals. The partition coefficient is the ratio of the concentration of drug

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in one phase to the concentration in a second phase. Drug partition coefficients can be determined in a variety of different systems, including oil–water, polymer–buffer, micelle–buffer, and cell–buffer systems. For instance, *n*-octanol–water or hexadecane–water partition coefficients have been used to predict drug lipophilicity. In a biological system, the partition coefficient indicates the extent to which a drug will partition into the medium of interest and the intracellular and extracellular equilibrium concentrations that will result. Since most drugs are carried to their sites of action by the bloodstream, and since many drugs of clinical importance distribute into red blood cells (e.g. digoxin, cyclosporine, and fleroxacin), studies of drug partitioning into erythrocytes are important in defining the overall distribution characteristics of these compounds.

Traditionally, erythrocyte partition coefficients have been studied in vitro by a standard manual method which employs centrifugation [1–3], ultrafiltration [4], or equilibrium dialysis [5]. The manual method is successful for determining accurate partition coefficients, but can be tedious and time-consuming. This report presents a microdialysis method suitable for measuring erythrocyte partition coefficients in vitro.

Microdialysis is a non-equilibrium dynamic sampling method in which the analytes diffuse across a semipermeable membrane due to a concentration gradient and are carried away by the constantly pumping perfusion medium [6]. The semipermeable membrane acts as a filter and allows sampling of only the unbound or extracellular drug concentration. Microdialysis sampling has been used successfully to explore drug–protein binding [7–10], drug stability [11], and in dissolution testing [6]. It eliminates tedious sample preparation steps and thus is more time-conserving than the conventional manual method. Finally, microdialysis is a continuous sampling process and can, therefore, be employed on-line allowing for full automation.

In this report, the microdialysis technique was used to study lomefloxacin partitioning into human erythrocytes. Lomefloxacin is a fluoroquinolone antimicrobial (Fig. 1). It contains two functional groups that are ionizable in the physiologically relevant pH range, 4.0–10. One group is acidic and one is basic, and the molecule exists mainly as a zwitterion at physiological pH. At this pH, the fluoroquinolones are in their lowest state of aqueous solubility and in their highest state of lipophilicity. The apparent 1-octanol–water partition coefficient for lomefloxacin has been determined to be 0.14 at pH 7 [12], suggesting that this compound is not very lipophilic. The fluoroquinolones complex with metal ions, which compromises their bioavailability [13–16]. Therefore, antacid therapy, iron supplements, and dairy products are contraindicated with this class of drugs. The fluoroquinolones are not readily metabolized in the body and are excreted virtually unchanged in the urine. Lomefloxacin binds to plasma proteins with a fraction bound of approximately 0.20 [17].

An active transport system has been reported for lomefloxacin in rat erythrocytes. Evidence in that report suggests that the drug utilizes the nicotinic acid transport system to cross erythrocyte membranes [18]. The human erythrocyte partition coefficient for lomefloxacin was previously determined to be 1.9 by the standard manual method [19]. This paper will introduce a novel method for studying the erythrocyte partition coefficients of lomefloxacin utilizing a fully automated system which incorporates microdialysis sampling, intermittent drug dosing, and on-line LC analysis. A statistical comparison will be made between the standard manual method and the microdialysis system.

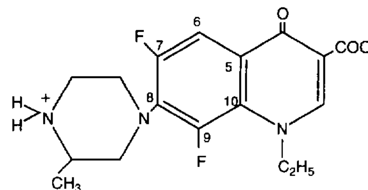

Fig. 1. Structure of the zwitterionic form of lomefloxacin.

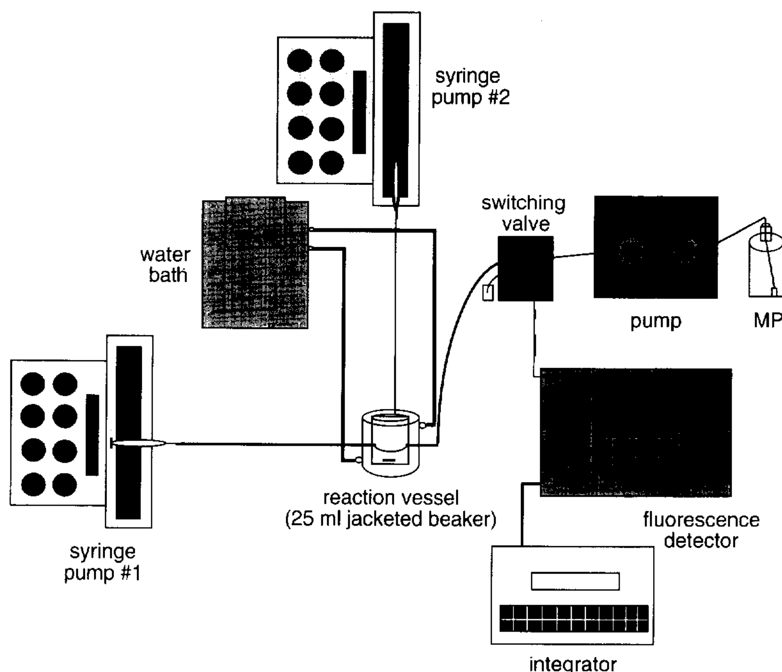

Fig. 2. Diagram of the automated system for studying erythrocyte partitioning.

2. Materials and methods

2.1. Materials

Lomefloxacin HCl was supplied by G.D. Searle and Co. (Skokie, IL). All other chemicals were of reagent grade or better and were obtained from commercial sources. Water was purified in a Milli-Q water system (Millipore Corp., Bedford, MA). All glassware was washed with nitric acid and rinsed with metal-free water before use to eliminate any trace metal contamination [20]. All samples containing lomefloxacin were wrapped in aluminum foil to protect them from light [21,22]. Measurements of pH 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. Apparatus

The automated system (Fig. 2) consisted of a microdialysis sampling system with programmed intermittent addition of a drug stock solution and a liquid chromatograph. The control buffer or erythrocyte suspension was placed in a 25 ml jacketed reaction vessel which was maintained at constant temperature by a Fisher (Pittsburgh, PA) Isotemp refrigerated circulator, model 910. Automated, intermittent dosing of the drug to the vessel was provided by a Harvard (South Natick, MA) 44 programmable infusion pump. The sampling system consisted of a DL-5 probe (5 cm) obtained from BAS (West Lafayette, IN) and a Harvard 44 programmable infusion pump for continuous perfusion of the probe. The dialysate was passed through the 2 μl injection loop mounted on a Valco Instrument (Houston, TX) Model EC6W on-line injector with electrical actuator. The injector was controlled by the Harvard syringe pump # 1 [23] (see Fig. 2). The chromatographic method was described previously by Ross et al. [14]. The LC system consisted of a Waters (Milford, MA) model 6000A pump, a Shimadzu (Kyoto, Japan) model RF-530 fluorescence detector (excitation, 286 nm; emission, 418 nm), and a Shimadzu model C-R5A integrator. An MOS Hypersil (C8) reversed-phase column (5 μm,

15 cm × 4.6 mm i.d.) and a Perisorb RP-18 guard column (P.J. Cobert, St. Louis, MO) were used. The mobile phase was tetrahydrofuran–acetonitrile–H_3PO_4 (100 mM)–triethylamine–water (10:50:10:0.03:qs 100) with a flow rate of 1.5 ml min^{-1}. All injections for the manual experiments were made in triplicate. The peak height of each sample was determined, and lomefloxacin concentrations determined by comparison with a calibration curve prepared using known standards. The calibration curve was linear in the range 0.1–20 μg ml^{-1}, with a limit of detection (LOD) of less than 1 μg ml^{-1}.

2.3. Probe characterization

The probe was characterized by measuring the relative recovery from buffer while varying the perfusion rate or the drug concentration in the vessel at 37 °C.

$$\text{Recovery} = \frac{\text{Concentration of drug in dialysate}}{\text{Concentration of drug in reaction vessel}} \quad (1)$$

A measurement of the relative recovery was taken before and after each experiment; the values were determined to be within 5% of each other. The amount of lomefloxacin adsorbing to or lost to the probe or to the reaction vessel was determined in the following manner. A stock solution of drug was prepared at a concentration of 3.0 μg ml^{-1}. A portion of the stock solution was added to the reaction vessel and a recovery experiment was performed. The stock drug concentration was compared to the concentration of drug in a sample of solution from the reaction vessel following the recovery experiment.

2.4. Erythrocyte suspension preparation

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 erythrocytes were washed three times with 0.01 M phosphate-buffered saline (pH 7.4) containing 10 mM glucose (PBS–glucose) and centrifuged, discarding the supernatant. The erythrocyte pellet was resuspended in PBS–glucose to achieve a hematocrit of 0.3.

2.5. Partition coefficient determination

2.5.1. Microdialysis studies

A 20 ml aliquot of either PBS–glucose solution or erythrocyte suspension was delivered to the reaction vessel. Lomefloxacin was intermittently added to the vessel (see Table 1) over a concentration range 0.38–9.0 μg ml^{-1}. It was previously determined that 60 min was an adequate time for the equilibration process to occur [19]. The hematocrit and pH were determined before and after the experiment, and did not deviate from hematocrit 0.3 and pH 7.4. The experiments were performed at room temperature (RT) or 37 °C. A DL-5 microdialysis probe was mounted in the reaction medium as described previously [23], and was perfused with PBS–glucose buffer at a rate of 1 μl min^{-1}. Dialysate samples were collected in the injection loop over a 5 min interval and injected over 30 s. The integrator collected the data over the entire 6 h experiment and printed the peak height responses at the end of the run. The apparent erythrocyte partition coefficient (*D*) was calculated using the following equation [2]

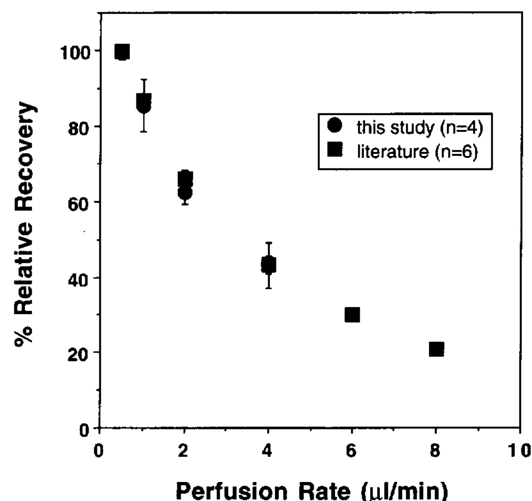

Fig. 3. Effect of syringe pump perfusion rate on relative recovery. The literature data were taken from R. Dicken [25]. The drug concentration in the reaction vessel was 3 (●) and 1 (■) μg/ml at 37 °C and pH 7.4.

$$D = \frac{C_{ref} - C_{pw}(1 - H)}{C_{pw}H} = \frac{C_{in}}{C_{out}} \quad (2)$$

where C_{ref} is the concentration of drug in the dialysate when the reaction vessel contained buffer, C_{pw} is the concentration of drug in the dialysate when the reaction vessel contained erythrocyte suspension, and H is the hematocrit. This equation gives the ratio of intracellular to extracellular drug concentrations.

The total amount of drug in the erythrocytes and surrounding buffer was measured following the microdialysis partitioning studies in order to determine drug loss to the probe, vessel and to the cell membranes. This study was performed by removing 10 ml of the erythrocyte suspension at the end of the experiment and diluting the aliquot with 10 ml of H_2O in order to lyse the cells. The sample was returned to the vessel and the concentration of drug in the dialysate was compared to that of a 1:1 dilution of the same drug concentration in buffer. This experiment was performed in triplicate.

2.5.2. Manual studies

Partition coefficients for lomefloxacin into human erythrocytes measured by the manual method [2] served as a control for the microdialysis method measurements. Lomefloxacin was added to aliquots of the erythrocyte suspension to yield a total drug concentration of 0.38–9.0 μg ml^{-1} while maintaining a hematocrit of 0.3 and a pH of 7.4. The suspension was incubated at RT or 37 °C for 60 min, including centrifugation for 3 min at 15 000g. The supernatant (C_{pw}) was analyzed for lomefloxacin concentration by LC with manual injection. For each concentration, a reference solution without erythrocytes (C_{ref}) was analyzed. Eq. (2) was again utilized to determine the apparent erythrocyte partition coefficient (D).

The total amount of drug in the erythrocytes and surrounding buffer was measured following the manual partitioning experiments in order to determine drug loss to cell membranes. This study was performed by preparing extra samples at the 9.0 μg ml^{-1} concentration for lomefloxacin and treating them in the same manner as above. The samples were then diluted with an equal amount of H_2O and agitated several times in order the lyse the erythrocytes. A 0.9 ml aliquot of each sample was delivered to a Centrifree ultrafiltration device with 10 000 MW cut-off filter (Amicon, Beverly, MA). The samples were centrifuged at 1250g for 30 min in a Dynac II centrifuge (Clay Adams, B-D). Two reference solutions were prepared in the same manner; however, one was filtered while one was not. The drug concentrations in the samples and in the reference solutions were compared. This experiment was performed in triplicate.

2.5.3. Statistical analysis

The rate by which the dialysate (C_{ref} or C_{pw}) achieved a new equilibrium value following each addition of drug (probe kinetics) was calculated using the following equation

$$A = A_{max}(1 - e^{-kt}) \quad (3)$$

where A is the peak height at time t, A_{max} was the peak height at equilibrium, and k is the probe kinetic rate constant (min^{-1}). A plot of $-\ln[1 - (A/A_{max})]$ vs. time for both the C_{ref} and the C_{pw} values was linear with a slope of k. The k values determined here for both C_{pw} and C_{ref} at varying concentrations were compared statistically by ANOVA using the program Minitab, Release 8.2 (Minitab, Inc., State College, PA). When the ANOVA indicated significant differences among the means, the T-method (Tukey's honestly significant difference method) was used to compare the means [24]. All statistical comparisons were at the 95% confidence level.

The apparent partition coefficient for lomefloxacin was related to the total drug concentration by Eq. (4) using non-linear least-squares regression (Microcal Origin, version 3.5, Microcal Software, Inc.)

$$D = D_{max}\frac{[\text{Drug}]}{[\text{Drug}] + K} \quad (4)$$

where D is the partition coefficient for a given drug concentration, and D_{max} and K are the regression constants. The model parameter values (D_{max} and K) from the non-linear regression were compared statistically using the method described above.

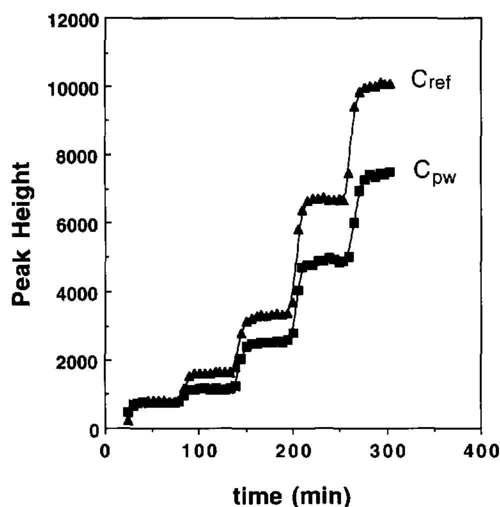

Fig. 4. Relative peak height (proportional to drug concentration) as a function of time using the automated system at RT. C_{ref} represents the drug dialysate from buffer solution, while C_{pw} is the drug dialysate from an erythrocyte suspension. The dosing profile is given in Table 1 and the kinetic rate constants (k; min^{-1}) from the curve fits are given in Table 2.

3. Results and discussion

The DL-5 probe was characterized by determining the effect of perfusion rate on relative recovery at 37 °C. Consistent with previous studies [6,11], it was observed that there was an exponential relationship between relative recovery and perfusion rate, in which an increase in perfusion rate corresponded to a decrease in recovery from the probe [25] (Fig. 3). A perfusion rate of 1 $\mu l\ min^{-1}$ was chosen for the microdialysis method in order to achieve a relatively high probe recovery (approximately 90%) for the drug without increasing the analysis time. That is, under these conditions, the LC run time was longer than the time necessary to fill the injection loop. The effect of varying lomefloxacin concentration (1–23 $\mu g\ ml^{-1}$) on the relative recovery was found to be insignificant, resulting in an average recovery of 87 ± 2% (standard deviation, $n = 7$) at a perfusion rate of 1 $\mu l\ min^{-1}$. Finally, the extent of lomefloxacin adsorption to the probe or amount of drug lost to the probe or to the reaction vessel was found to be less than 1% ($n = 3$), and was therefore determined to be insignificant also.

Fig. 4 shows the change in relative peak height for C_{ref} and C_{pw} over time using the automated system with microdialysis. Drug was added to the reaction vessel every 60 min (see Table 1). The plateaus represent the equilibrium concentrations (A_{max}) for each given dose. The rate (k; min^{-1}) for the probe to achieve equilibrium was determined (Eq. (3)) at each concentration for C_{ref} and C_{pw} (see Table 2). By comparing the mean values of k, it was observed that there was no statistical difference for the rate of probe equilibration between concentrations, between C_{ref} and C_{pw}, or between days. Therefore, it was determined that the probe behaved similarly in the presence or absence of erythrocytes in the concentration range studied. Furthermore, it was concluded that this system cannot be used to measure the kinetic rate of uptake of lomefloxacin into human erythrocytes since the rate of probe equilibration did not change when erythrocytes were added to the reaction medium. This evidence suggested that the rate of probe equilibration was the rate-determining step in this process. These results did, however, suggest that the probe behaved similarly whether the reaction medium consisted of buffer or an erythrocyte suspension, indicating that the system was stable throughout the course of the study.

To determine the amount of drug lost to the probe, vessel or to the erythrocyte cell membrane,

Table 1
The automated dosing program for syringe pump # 2 (Fig. 2), which provides intermittent delivery of a concentrated drug solution to the reaction vessel

Syringe pump computer command	Volume of drug solution added (μl)	Duration of syringe pump command
Dose	25	30 s
Pause		60 min
Dose	25	30 s
Pause		60 min
Dose	50	30 s
Pause		60 min
Dose	100	30 s
Pause		60 min
Dose	100	30 s
Stop		

Table 2
Kinetic values[a] (k; min^{-1}) from Eq. (3) and data in Fig. 4, reflecting the rate for the probe to achieve equilibrium following dosing

	Lomefloxacin concentration ($\mu g\ ml^{-1}$)					
	0.75	1.5	3.0	6.0	9.0	Ave $k^{b,c}$
For probe in buffer (C_{ref}; $n = 2$)	0.121	0.144	0.207	0.181	0.151	0.165 ± 0.041 ($n = 10$)
For probe in erythrocyte suspension (C_{pw}[b]; $n = 3$)	0.257 ± 0.078	0.229 ± 0.058	0.207 ± 0.024	0.173 ± 0.003	0.199 ± 0.028	0.213 ± 0.049 ($n = 15$)
Ave[b,d], $n = 5$	0.202 ± 0.094	0.195 ± 0.063	0.207 ± 0.027	0.184 ± 0.015	0.180 ± 0.034	0.194 ± 0.051 ($n = 25$)

[a] Reported as a function of lomefloxacin concentration ($mg\ ml^{-1}$) for the probe in buffer solution (C_{ref}) or an erythrocyte suspension (C_{pw}).
[b] $\pm$Standard deviation.
[c] Mean k value over all concentrations.
[d] Mean ka value for combined C_{ref} and C_{pw}.

the total amount of drug in the cells and surrounding buffer was measured following both the microdialysis and manual partitioning studies. It was observed that the loss of drug was $6 \pm 1\%$ (standard deviation, $n = 3$) for the microdialysis method, and $4 \pm 6\%$ (standard deviation; $n = 3$) for the manual method. These values suggested that only a small amount of drug was not accounted for following the experiments, and was, therefore, considered to be insignificant.

A comparison of the manual and microdialysis methods for determining apparent partition coefficients for lomefloxacin with increasing drug concentration is given in Figs. 5 and 6. By non-linear regression, the apparent partition coefficient values were fitted to Eq. (4). The values for D_{max} and K are shown in Table 3. The means of the D_{max} and K values were compared. There was no statistical difference between the two methods, manual vs. microdialysis, or between the two temperatures, RT vs. 37 °C. These results indicated that the microdialysis and manual methods gave similar, if not identical, partition coefficient values for lomefloxacin into human erythrocytes. Therefore, the automated system was useful for determining erythrocyte partition coefficients for lomefloxacin as a function of drug concentration.

The means of the individual apparent partition coefficient (D) values (Figs. 5 and 6) for each concentration were also compared. It was found that there was no statistical difference between D values determined by the manual vs. microdialysis methods at each concentration. However, the partition coefficient at $0.38\ \mu g\ ml^{-1}$ was statistically different from the D values observed at all of the other given concentrations. This evidence, as well as the shape of the curves in Figs. 5 and 6, suggested that there was a concentration dependence on lomefloxacin partitioning into human erythrocytes, and that partitioning was a saturable process.

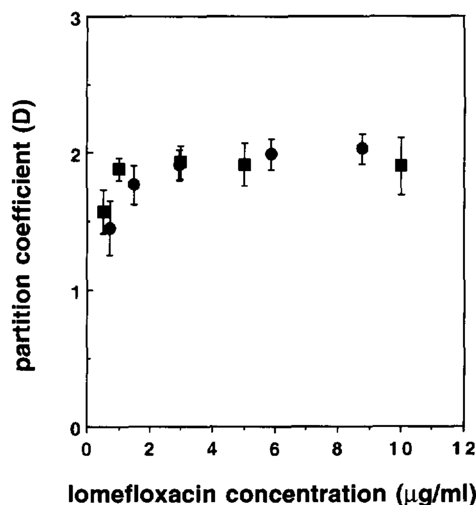

Fig. 5. Apparent partition coefficient (D) for lomefloxacin as a function of drug concentration ($\mu g\ ml^{-1}$) for the manual (■; $n = 15$) and microdialysis (●; $n = 6$) methods at RT. The results of the non-linear regression on the data are given in Table 3.

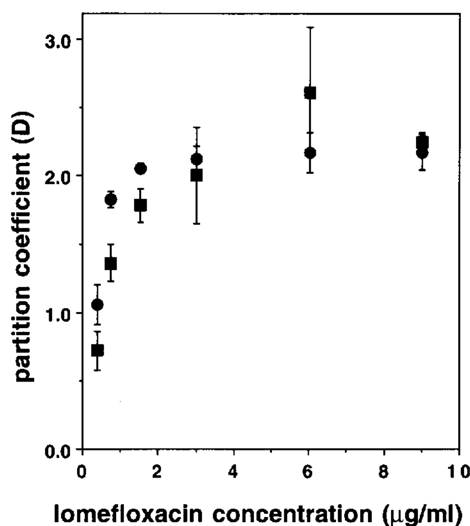

Fig. 6. Apparent partition coefficient (D) for lomefloxacin as a function of drug concentration ($\mu g\ ml^{-1}$) for the manual (■; $n = 4$) and microdialysis (●; $n = 3$) methods at 37 °C. The results of the non-linear regression on the data are given in Table 3.

The saturation value (D_{max}) for the partition coefficient, D, was approximately 2. There are several reasons why a partition coefficient of greater than one is possible. These reasons include a transport system for the drug, or binding of the drug to either an intracellular component or to the cell membrane. A transporter for lomefloxacin into rat erythrocytes had been previously reported [18]. The evidence given here supports a transport system for lomefloxacin into human erythrocytes, in that there is a concentration dependence for erythrocyte partitioning and that the partitioning process is saturable. The automated system is being used in this laboratory to further study the mechanism and the factors affecting the partitioning of lomefloxacin across the erythrocyte membrane.

Table 3
Regression constants (D_{max} and K) from Eq. (4) and data in Figs. 5 and 6 for the manual and microdialysis methods at RT and 37 °C

Method	Temperature	$D_{max} \pm SD$	$K \pm SD$	n
Manual	RT	1.97 ± 0.05	0.10 ± 0.03	15
Microdialysis	RT	2.11 ± 0.02	0.32 ± 0.02	6
Manual	37 °C	2.68 ± 0.18	0.82 ± 0.22	4
Microdialysis	37 °C	2.34 ± 0.12	0.32 ± 0.09	3

4. Conclusion

The automated system reported here is useful for studying partition coefficients in human erythrocytes, and gives results that are statistically identical to the conventional manual method. The automated system eliminates the tedious methodology associated with traditional erythrocyte partitioning studies, including sample clean-up. The system operates unattended, requiring less laboratory time by the researcher. However, the system cannot be used to study rapid partitioning kinetics since the probe is diffusion-controlled. The versatility of the system could allow for the effect of other physicochemical parameters on erythrocyte partitioning to be explored, such as changing temperature or the presence of transport inhibitors. Further studies are in progress in which this system will be used to investigate the high partition coefficient of lomefloxacin and the mechanism by which the drug enters the human erythrocyte.

Acknowledgments

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