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Monitoring diclofenac sodium in single human erythrocytes introduced by electroporation using capillary zone electrophoresis with electrochemical detection

A method for determination of the drug diclofenac sodium introduced into individual human erythrocytes by electroporation using capillary zone electrophoresis with electrochemical detection at a carbon fiber array microelectrode was developed. In this method, the whole cell was injected into the separation capillary by electromigration. Cell lysis was accomplished by injecting a plug of the separation buffer (1.25×10^{-2} mol/L $Na_2B_4O_7$–3.13×10^{-3} mol/L NaOH). The optimum conditions of separation and detection were 20 kV for the separation voltage and 1.0 V for the detection potential. The concentration of diclofenac sodium in the single cells was quantified by a calibration curve. The mean concentration of diclofenac sodium introduced into the cell was 4.21 μmol/L. The relative standard deviation of the concentration of diclofenac sodium introduced into ten cells is 10%.

Keywords: Capillary electrophoresis / Electrochemical detection / Electroporation / Diclofenac sodium / Drug / Single cell analysis / Erythrocyte EL 4467

1 Introduction

The use of whole, living animals for testing the toxicity of chemicals such as new drugs is being carefully scrutinized. Societal pressure and humane considerations are making it more and more difficult. Experiments done with cells have the potential to replace most animal testing, provided that the data acquired from cells can be made to reflect the response of the whole organism. Diclofenac sodium, sodium (*o*-(2,6-dichloroanilino) phenyl) acetate, is a relatively safe and effective nonsteroidal drug with pronounced antirheumatic, anti-inflammatory, analgesic, and antipyretic properties [1], which is widely used in the treatment of degenerative joint diseases and other arthritic conditions [2, 3]. When it is introduced into erythrocytes, monitoring its concentration in erythrocytes is very important. In routine tests, hundreds of thousands of cells are homogenized to provide a sufficient amount of analytes for quantification. The results obtained can not reflect the situation of each cell. Single-cell analysis can potentially benefit to obtain the information of individual cells. Capillary zone electrophoresis (CZE) has firmly planted itself as an important separation technique. There are several specific instances where CZE is clearly the best method-of-choice for the study of single biological cells. The study of single cells by CZE benefits from many of the inherent features of its operation. These are small sample size, high separation efficiency, high separation speed, biocompatible environment, and low cost. CZE with an electrochemical detector has been demonstrated to be a suitable method for single-cell analysis [4, 5].

Some of the first work demonstrating the ability of capillary electrophoresis (CE) to examine analytes at the single-cell level was presented by Ewing and co-workers [4]. They used electrochemical detection for CE to detect electroactive neurotransmitters in single whole snail neurons [6, 7], in cytoplasmic injection from snail neurons [7–11], and in single whole human lymphocytes [12]. Concurrent with Ewing's reports of cytoplasmic sampling using CE, Jorgenson and co-workers [13] demonstrated the capacity of CE to analyze entire cells, although they also determined amino acids and neurotransmitters in single neurons using open-tubular liquid chromatography with electrochemical detection [14, 15]. The potential to analyze even smaller cells has been demonstrated with the analysis of human erythrocytes, one of the smallest volume cells, by Yeung's group [16–21] using CE and several laser-based detection schemes. Measured analytes include the ions K^+ and Na^+ [16, 17], lactate and pyruvate [18], glutathione after derivatization with monobromobimane [16], the major proteins [19], enzymes [20], and antigens [21]. Research in the Sweedler group [22–25] was focused on individual nerve cells using CE with postcolumn radiochemical and on-column laser-induced fluorescence detection. In our laboratory, CZE has been applied

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Abbreviations: SCE, saturated calomel eletrode

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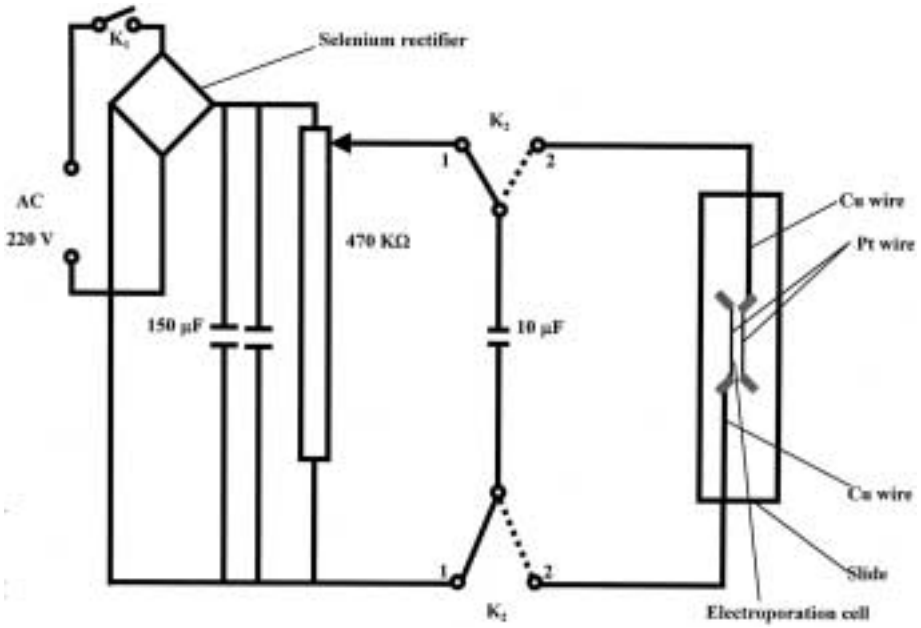

Figure 1. Circuit diagram of RC electric pulse generator.

to determination of glutathione in individual human erythrocytes [26] and mouse peritoneal macrophages [27] by using the electrochemical detection. In these schemes no derivatization is needed. In this work, we developed a method for monitoring diclofenac sodium in individual human erythrocytes introduced by electroporation using CZE with electrochemical detection.

2 Materials and methods

2.1 Apparatus

2.1.1 Electroporation equipment

Figure 1 shows the laboratory-made RC electric-pulse generator. This generator mainly included two parts: charge and discharge parts. The charge part comprised an alternating current (AC) power supply of 220 V, a selenium rectifier, two electrolyte filter capacitors of 150 μF, a potentiometer of 470 kΩ, and an electrolyte capacitor of 10 μF (which also was the power supply of the discharge part). The discharge part contained the electrolyte capacitor of 10 μF and an electroporation cell. The electroporation cell mainly included two parallel platinum wire electrodes (300 μm diameter, 20 mm length) with a distance of *ca.* 375 μm. The two platinum wires were welded with two copper wires (5 cm length, 500 μm diameter), respectively. The Pt electrodes were adhered to the bottom of a slide by ethyl α-cyanoacrylate adhesive. In Fig. 1, K_1 was the AC power switch and K_2 was the charge/discharge switch. When K_1 was turned on and K_2 was switched to the charge mode (place 1 in Fig. 1), the alternating current was rectified to the direct current (DC) by the selenium rectifier and the two electrolyte filter capacitors of 150 μF. Then, the electrolyte capacitor of 10 μF was charged to a constant voltage through the potentiometer. The voltage of the electrolyte capacitor charged could be adjusted by using the potentiometer. When the K_2 was switched to the discharge mode (place 2 in Fig. 1, dotted line), the capacitor released its charge through the electroporation cell.

2.1.2 CZE system

Details of the CZE separation system used in this work was similar to our previous description [28]. Briefly, the apparatus consisted of a fused-silica capillary with 30 μm ID and 375 μm OD (J&W Scientific Incorporated Company, Folsom, CA, USA), which was cut to a length of about 70 cm and placed between two buffer reservoirs. A high voltage was applied at the injection end, while the reservoir containing the electrochemical detection cell was held at ground potential. Separation was carried out at an applied voltage of 20 kV generated by a high-voltage power supply (Model 9323-HVPS; Beijing Institute of New Technique, Beijing, China). The electrochemical detection at a constant potential was performed using the end-column amperometric approach with an electrochemical analyzer (Model CHI800; CH Instruments, Austin, TX, USA). The detection cell and the detector were housed in a Faraday cage in order to minimize the interference from external sources of noise. Electrochemical detection was

carried out with a three-electrode system. It consisted of a carbon fiber microdisk array electrode as the working electrode, a coiled Pt wire as the auxiliary electrode (which also served as the ground for the potential drop across the capillary) and a saturated calomel electrode (SCE) as the reference electrode. The arrangement of the electrochemical detection cell was previously illustrated in detail [28]. The carbon fiber microdisk array electrode was described previously [29].

2.2 Cell preparation

About 0.4 mL of human blood from a normal adult was collected in a 1.5 mL microcentrifuge tube and centrifuged at 1000 rpm for 5 min to separate erythrocytes. Then, the supernatant liquid was removed. The remaining amount of erythrocytes was about 0.2 mL. In order to wash the erythrocytes, a sixfold physiological buffer saline (PBS) was added into the microcentrifuge tube. After vibrating lightly, the mixture was centrifuged again, and then the supernatant liquid was removed again. This step was repeated over five times until the supernatant was clear and transparent. After the supernatant liquid was removed, the concentrated erythrocytes were obtained. The concentration of the erythrocyts was about 4.0×10^6 cell/μL.

2.3 Scheme of the cell electroporation

In order to introduce diclofenac sodium into erythrocytes by electroporation, the erythrocyte suspension was prepared by diluting 2.0 μL of erythrocytes concentrated to 500 μL with the solution containing PBS and diclofenac sodium. In this erythrocyte suspension, the concentration of diclofenac sodium was 1.6×10^{-3} mol/L and the concentration of the erythrocytes was 1.6×10^4 μL. The erythrocyte suspension (*ca.* 0.5 μL) containing PBS and diclofenac sodium and about 0.5 μL of 0.5% w/v trypan blue solution were dropped into the place between the two platinum electrodes of the electroporation cell, which had been put on the platform of an inverted biological microscope. After the both solutions were mixed adequately, the colorless erythrocytes (about 8.0×10^3 cell/μL) suspended in the blue solution can be seen clearly under the microscope with a magnification of 400x. After that, the power supply switch K_1 of the electroporation generator was turned on and its charge/discharge switch K_2 was switched to the charge mode (place 1). After 5 s, K_2 was switched to the discharge mode (place 2). Such, one electric pulse was added to the erythrocytes. If several pulses were needed, K_2 could be switched between charge and discharge mode again and again until the erythrocytes were electroporated. Since the erythrocytes electroporated were blue, they could be monitored under the inverted microscope. Finally, the erythrocyte suspension was taken out from the eletroporation cell and put into a 0.5 mL microtube. The volume of the erythrocyte suspension obtained was less than 1 μL, which contained *ca.* 8.0×10^3 erythrocytes. In order to obtain enough volume (about 20 μL at least) for about ten single cell injections, the process mentioned above was repeated more than 20 times. The erythrocyte suspension of 20 μL mentioned above contained *ca.* 1.6×10^5 cells, which was equivalent to 80 nL of human blood. This volume was less than the volume for a large-scale measurement. Since the erythrocyte suspension contained diclofenac sodium during the electroporation process, this drug was still in the suspension after electroporation and it would disturb the detection of the drug in single erythrocytes. The drug in the erythrocyte suspension should be washed down before injecting one erythrocyte into the capillary. The erythrocyte suspension collected in a 0.5 mL microtube (about 20 μL) was diluted about 25 times by PBS. After being fully mixed, the suspension was allowed to stand for more than 2 h. When the cells subsided to the bottom of the tube, the supernatant liquid was removed. This process was repeated at least four times.

2.4 Injection of the whole cell

In order to measure diclofenac sodium by CZE, the cells must be transferred directly into the injection end of the separation capillary under the field of view of an optical microscope. A droplet of the erythrocyte suspension of 5 μL was placed on a clean microscope slide. After the microscope slide was placed on the inverted biological microscope with a magnification of 400×, the injection end of the capillary filled with electrophoresis buffer was gently immersed in the droplet under the guidance of a three-dimensional micromanipulator. In order to see the opening of the injection end, a *ca.* 5 mm section of the polyimide-coating at the injection end of the capillary was removed by burning before use. A platinum wire was placed in the erythrocyte suspension to serve as the electrophoresis anode. As soon as the erythrocyte was drifting towards the injection end, an injection voltage of 3.0 kV was applied to transport the whole cell into the capillary tip. The entire process of cell injection typically took $30 \sim 150$ s.

2.5 Single cell analysis

Before each run, the capillaries were flushed with 0.1 mol/L NaOH for 5 min, and then with water and the corresponding separation buffer for 5 min, respectively, by means of a syringe. The carbon fiber microdisk electrode

cemented onto a microscope slide had to be aligned with the detection end of the capillary under a microscope. Then, a voltage of 20 kV was applied across the capillary and the detection potential was applied at the work electrode. After the electroosmotic current reached a constant value (after about 10–20 min), the electromigration injection of the whole cell mentioned above was carried out. After the whole cell was injected into the capillary, the capillary was manipulated up, out of the erythrocyte suspension, and then immersed in the CZE running buffer solution as the cell lysis solution. Once the individual erythrocyte was lysed, the separation voltage of 20 kV was applied and the electropherogram was recorded.

2.6 Reagents

A 1.0×10^{-2} mol/L stock solution of diclofenac sodium or a 5% w/v trypan blue stock solution was prepared by dissolving an appropriate amount of diclofenac sodium (pharmaceutical grade, Yanzhou pharmaceutical factory, Yanzhou, China) or trypan blue (Sigma, St. Louis, MO, USA) in water. Dilute solutions were obtained by serial dilution of the stock solution with PBS. The PBS consisted of 0.135 mol/L NaCl and 0.02 mol/L NaH_2PO_4-NaOH (pH 7.4). All reagents were of analytical grade except for diclofenac sodium. All solutions were prepared with double-distilled water.

3 Results and discussion

3.1 Optimum conditions for electroporation introducing diclofenac sodium into erythrocytes

Biological membranes are polarized membranes and the electric fields exist across them. Thus, in some way, the phospholipid bilayer of a cell membrane can be compared to a capacitor where the distance between two plates is extremely thin. Excessive voltages can lead to the breakdown of a dielectric by overcoming its permitivity. Thus, the electroporation process is at a critical electric field value, the permitivity of the membrane will be exceeded, and its conductivity dramatically increased. It is assumed that the increase in conductivity will be accompanied by local disruptions of the bilayer structure, hence pore formation and much enhanced permeability to macromolecules [30, 31]. Pore formation is reversible under the proper conditions and resealing of the membrane occurs within seconds or minutes. The membrane poration (capacitor breakdown) and membrane resealing (capacitor regeneration) do occur, as one would expect in the case of a macroscopic capacitor [32]. It was observed that electrical breakdown of artificial and natural membranes occurred in the range of 0.5–2V [32]. Further, irreversible breakdown was noted when the applied external electric field exceeded 4–6 times the critical value or when the duration of electric pulse was longer than 20–100 μs. However, this lethal effect of pulse duration was found to vary quite significantly according to cell type, size, and osmoticum. It is clear that the reversible electropore is expected. In order to obtain living cells for single-cell detection, the cells should be still alive after electroporation. Therefore, the optimum reversible electroporation conditions of erythrocytes are researched, including electric pulse strength, pulse duration, and pulse number. In other words, we are finding their critical values for reversible electroporation. The pulse strength is the electric field intensity between the two platinum electrodes in the electroporation cells. The pulse duration is the whole occurrence time of the electric pulse. The pulse number is the total numbers of electric pulse needed for generating reversible electropore of erythrocytes. The electroporation efficiency is the percentage of cell occurring reversible electropore among the whole cells.

Trypan blue is a dyestuff to distinguish reversible electroporated cells. If the cells are electroporated reversibly, they can be observed with blue color under the microscope. It was founded that erythrocytes could not be dyed by trypan blue, when the cells were incubated in trypan blue solution for 24 h. This means that trypan blue could not be introduced into erythrocytes by pinocytosis or diffusion. However, erythrocytes can be stained by trypan blue after cells are reversibly electroporated, because many "pores" are generated on the cell membrane. Trypan blue can penetrate into the cells through these reversible "pores" and dye the cells. Therefore, trypan blue can be used to distinguish reversibly cells electroporated.

The concentration of diclofenac sodium may affect the erythrocyte membrane structure and lead the cells to lyse easily. In 1.6×10^{-3} mol/L diclofenac sodium, erythrocytes can not be lysed. It can be noted that all cells cause irreversible electroporation, if one electric pulse with the pulse strength of 7.0 kV/cm or 8.0 kV/cm at the pulse duration of 40 μs is applied to the cells. In other words, the electric pulse conditions have exceeded the critical values for reversible electroporation. When the pulse strength decreases, the percentage of cell electroporated irreversibly decreases. If one electric pulse with the pulse strength of 6.0 or 6.5 kV/cm is applied to the cells, the percentage of surviving cells can retain 50% or 30%. When one or two electric pulse with 5.5 kV/cm is applied to the erythrocytes, 60–70% of the cells cause reversible electropores. However, if more than two electric pulses

are applied, more than 50% of the cells membranes rupture after several minutes. The pulse strength of 4.0 or 4.5 kV/cm can lead only 20–30% of the cells to generate reversible electroporation, even if several pulses are applied. Only at the pulse strength of 5 kV/cm, nearly 90% of the erythrocytes cause reversible electropore and no rupture generates after 2 ~ 4 pulses are applied. Therefore, the optimum conditions for electroporation introducing diclofenac sodium into erythrocytes in PBS, trypan blue, and diclofenac sodium medium is 5 kV/cm for the pulse strength, 3 for the pulse number, and 40 μs for the pulse duration.

3.2 Determination of standard diclofenac sodium

It is found that diclofenac sodium can be detected by CZE-electrochemical detection at a carbon fiber microelectrode with a constant potential of 1.0 V *vs.* an SCE. The separation and detection conditions are 1.25×10^{-2} mol/L $Na_2B_4O_7$–3.13×10^{-3} mol/L NaOH for the buffer solution, 20 kV for the separation voltage, 5 kV and 10 s for the injection voltage and the injection time, respectively. Since erythrocytes are suspended in PBS, PBS is injected into the separation capillary with an erythrocyte during injecting the cell. Therefore, the behavior of PBS and standard diclofenac sodium should be observed first. The electropherograms of PBS and 5.0×10^{-4} mol/L diclofenac sodium in PBS are shown in Fig. 2 (curve 1 and 2). From curve 1 it can be found that there are two peaks on the electropherogram of PBS. One (peak 1) is high and narrow while the other (peak 2) is low and wide. The peak of diclofenac sodium appears behind the peaks of PBS. The difference between the migration time of the peaks of PBS and the migration time of the peak of diclofenac sodium (third peak in curve 2) is about 264 s. It means that the peak of diclofenac sodium and the peaks of PBS can be well separated. For diclofenac sodium, the limit of detection (according to the ratio of single-to-noise of 3) is 10^{-6} mol/L, whereas a linear dynamic range of at least three orders of magnitude ($10^{-6} \sim 10^{-3}$ mol/L) is found when a capillary with 30 μm ID, 70 cm length is used.

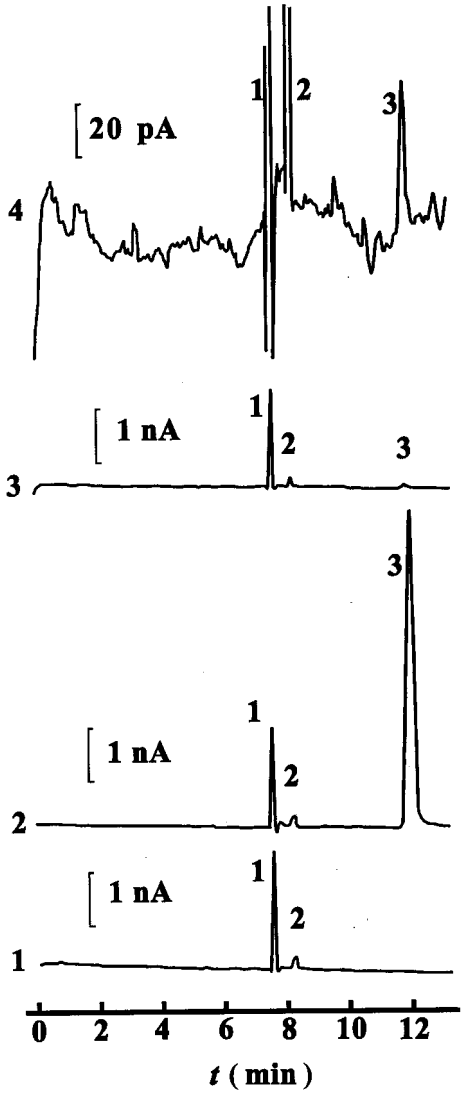

Figure 2. Electropherograms of PBS and diclofenac sodium. (1) PBS; (2) 5.0×10^{-4} mol/L diclofenac sodium in PBS; (3) diclofenac sodium in individual human erythrocyte introduced by electroporation; (4) 50 times of the curve 3. Buffer 1.25×10^{-2} mol/L $Na_2B_4O_7$–3.13×10^{-3} mol/L NaOH; capillary, 70 cm length, 30 μm ID; injection, 5 kV for 10 s; separation voltage, 20 kV; detection potential, 1.0 V.

3.3 Lysis of erythrocyte

Cell lysis is accomplished by injecting a plug of the separation buffer around the cell and allowing it to incubate. If a droplet of the separation buffer is added into a droplet of the erythrocyte suspension in PBS on the microscope slide, the erythrocytes are lysed in 10 s (observed under the microscope). However, when an erythrocyte with PBS is injected into the capillary and then the buffer is added into the separation capillary, the cell can not be lysed within 30 min. This is because, although there is the separation buffer at two ends of a plug of PBS containing an erythrocyte, the time for the buffer reaching the cell in the capillary is longer than that on the microscope slide. If a "lysis voltage" is applied to the capillary, the speed for the separation buffer reaching the cell and the lysis process itself will be accelerated. When the "lysis voltage" of 0.6 kV is used, the erythrocyte can be lysed in less than 60 s.

3.4 Identification of diclofenac sodium in erythrocytes introduced by electroporation

The electropherogram of the single erythrocyte before introducing diclofenac sodium is the same as the electropherogram of PBS shown in Fig. 2 (curve 1). This result indicates that the substances in the cells can not be detected within 13 min under these conditions. Figure 2 (curve 3) shows the electropherogram of one erythrocyte after introducing diclofenac sodium. One small new peak (peak 3) following the peaks of PBS with a migration time of *ca.* 12 min is found. In order to distinguish the electrophoretic peaks clearly, Fig. 2 (curve 3) is magnified 50 times and shown in Fig. 1 (curve 4). By comparison to Fig. 2 (curve 2) it can be found that the migration time of the peak 3 shown in Fig. 2 (curve 4) is the same as that of the peak of the standard diclofenac sodium in PBS solution shown in Fig. 2 (curve 2). Therefore, it can be concluded that this peak is just the peak of diclofenac sodium introduced into the erythrocytes by electroporation. The large difference of the migration time of diclofenac sodium and other peaks in the erythrocyte indicates that the substances in the erythrocyte do not interfere with the qualitative and quantitative determination of diclofenac sodium introduced in human erythrocytes. Figure 3 depicts the electropherograms of three erythrocytes.

3.5 Analysis of diclofenac sodium in individual erythrocytes

A total of ten cells were analyzed consecutively. Figure 4 shows a set of runs that is representative of the data collected. The run numbers refer to the chronological order of the ten cells investigated. They are plotted on a common scale to allow quantitative comparisons. Figure 4A shows the variation in migration time of diclofenac sodium in the erythrocyte. There is a clear drift in migration time. Overall ζ-potential changes, resulting from the adsorption of the substances in the cells on the surface

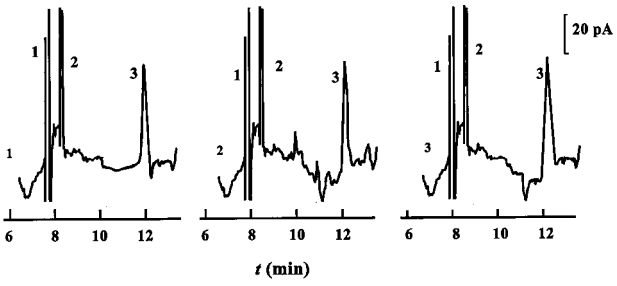

Figure 3. Electropherograms of diclofenac sodium in three individual human erythrocytes. Conditions as in Fig. 2.

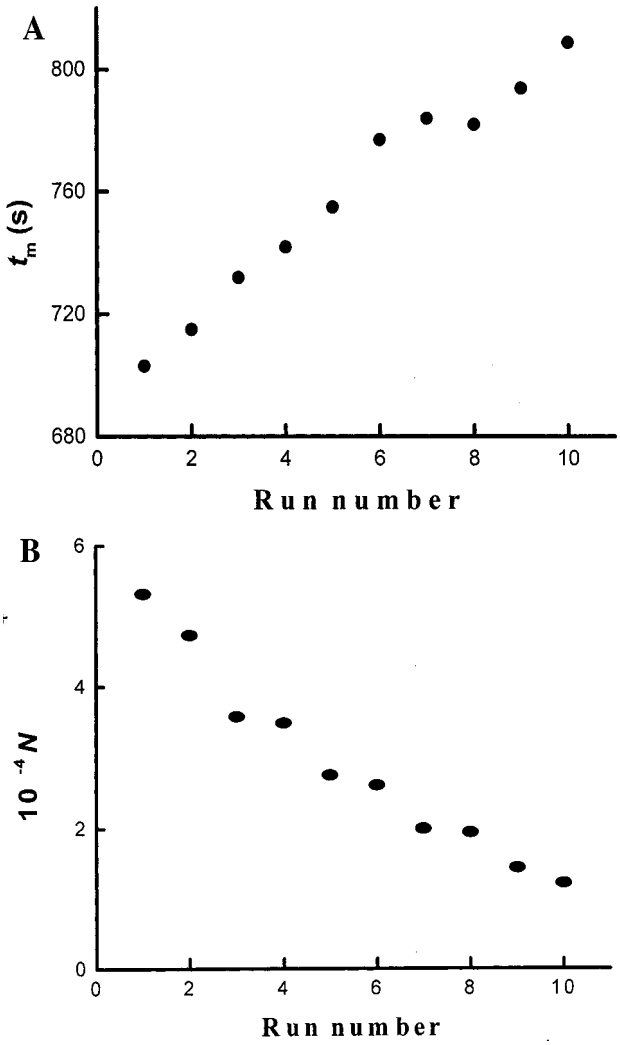

Figure 4. Changes in (A) migration time, and (B) number of theoretical plates of the electrophoretic peak of diclofenac sodium in individual erythrocytes introduced by electroporation. Conditions as in Fig. 2.

of the capillary wall, are probably responsible for this. This is verified by the correlation between the number of theoretical plates and run number shown in Fig. 4B. In the plot the number of theoretical plates changes on the small range with increasing the run number. The behavior has been observed in our previous work for determination of glutathione in individual human erythrocytes [26] and mouse peritoneal macrophages [27]. The concentration of diclofenac sodium introduced into the erythrocyte by electroporation is quantified by the calibration curve of standard diclofenac sodium. Table 1 shows a set of data of ten erythrocytes sampled. The mean concentration of diclofenac sodium introduced into erythrocytes is 4.21 μmol/L. The relative standard deviation of these data is 10%.

Table 1. Concentration of diclofenac sodium in erythrocytes

Run number	1	2	3	4	5	6	7	8	9	10	Mean	RSD
Concentration (μmol/l)	3.51	4.27	4.74	3.51	4.11	4.32	4.31	4.79	4.13	4.46	4.21	10%

4 Concluding remarks

We have found that the electric pulse can cause the reversible breakdown of the erythrocyte membranes and much enhance permeability of molecules such as trypan blue and diclofenac sodium. In PBS, trypan blue, and 1.6×10^{-3} mol/L diclofenac sodium medium, about 90% erythrocytes will be electroporated reversibly at 5 kV/cm for the pulse strength, 40 μs for the pulse duration and 3 for the pulse number and then diclofenac sodium is introduced into erythrocytes. The diclofenac sodium introduced into cells could be determined by CZE with electrochemical detection by a carbon microdisk array electrode. Single-cell analysis using CZE with electrochemical detection has the potential qualitatively and quantitatively to determine drugs introduced into cells.

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