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Investigation of Microdialysis Sampling Calibration Approaches for Lipophilic Analytes: Doxorubicin

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Abstract

Microdialysis is an important sampling technique in many pharmacokinetics and pharmacological studies. Applying microdialysis to lipophilic analytes can be difficult as low extraction efficiencies are generally obtained with these types of analytes. In this investigation, the effects of applying microdialysis to the lipophilic compound, doxorubicin are discussed. Using varying concentrations of doxorubicin (DOX) from 1 to 20 μM , in vitro probe calibrations were performed by delivery, recovery and no-net flux. Any changes in the extraction efficiencies calculated were monitored through the addition of an internal standard, antipyrine. DOX was chosen as a representative lipophilic analyte because its red color could be visibly monitored on the probe. At higher concentrations the probe membrane became redder. For delivery experiments, the inlet of the probe was more highly colored than the outlet. The opposite was true for recovery experiments, in which the outlet was more highly colored than the inlet. It was observed that while antipyrine was well-behaved in these experiments, for DOX the extraction efficiency determined by recovery was not the same as the extraction efficiency determined by delivery ($p < 0.005$, 0.05). It was further observed that for DOX the extraction efficiency determined by a no net flux experiment was in good agreement with the value determined by delivery and not that determined by recovery. However, the only point in which no DOX was present in the perfusate was not on the no-net flux line. In addition, the transport of DOX across the microdialysis membrane was considerably slower than the transport of antipyrine.

Keywords

Doxorubicin; Microdialysis; Calibration

1. Introduction

Microdialysis is a diffusion controlled process, driven by the movement of analytes across a semi-permeable membrane as a result of a concentration gradient from the external medium to the perfusate [1-4]. As the perfusate is being continuously pumped through the probe, equilibrium is not established; rather the concentration in the dialysate is some fraction of the concentration in the external medium. This is known as the extraction efficiency (EE),

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and is used to quantitatively relate the concentration of analyte in the dialysate to that in the sampled medium [5].

Probe calibration is performed by experimentally determining the EE which is defined as: $EE = (C_p - C_d)/(C_p - C_s)$, where C_p is the initial concentration in the perfusate, C_d is the concentration determined in the dialysate and C_s is the known concentration in the external medium. The EE may be determined by a recovery experiment (EE_R), where the concentration of the compound is higher in the external medium than in the perfusate ($C_p=0$), and the net transport will be into the probe. In this instance, EE is reduced to $EE_R = C_d/C_s$. Alternatively, EE may be determined by the addition of a known concentration of analyte in the perfusate, while the external medium does not contain any analyte ($C_s=0$). This is known as a delivery experiment (EE_D), and is described by: $EE_D = (C_p - C_d)/C_p$. In a delivery experiment, the net transport will be out of the probe. Under identical experimental conditions for a given microdialysis probe and analyte the extraction efficiency determined by delivery should be the same as that determined by recovery. This is important for quantification purposes because while the recovery of a microdialysis experiment can be determined *in vitro*, it is in general not possible to determine recovery *in vivo* as the tissue concentration is not known. Therefore, *in vivo* probe calibration is usually performed by delivery and the corresponding recovery is assumed to be same [5].

Other modes of probe calibration include retrodialysis and the no-net flux (NNF) method [2, 6]. In retrodialysis, an internal standard is added to the perfusate and its rate of delivery through the probe is measured throughout the microdialysis sampling experiment. The internal standard chosen must be chemically similar, have identical diffusion properties but be analytically distinguishable from the analyte of interest [6]. Examples of internal standards include radiolabeled forms of the analyte of interest, tritiated water and antipyrine [2]. However, the addition of the internal standard to the system may disrupt the experiment as it is usually added in high concentrations.

In the NNF method, the mass transport of the analyte across the membrane as a function of analyte concentration is determined. Varying concentrations of analyte, both above and below its concentration in the external medium, are successively added to the perfusate. If the concentration of analyte is higher in the perfusate than in the external medium, then the analyte will diffuse from the perfusate into the external medium. If the concentration of analyte is lower in the perfusate than in the external medium, diffusion of the analyte will occur into the perfusate. When the concentration of the analyte in the perfusate is equal to the concentration in the external medium, there is no-net flux of analyte across the membrane. The results are plotted as the change in concentration of dialysate versus the original concentration in the perfusate and the EE is determined from the slope of the resulting line [5]. As the analyte of interest is used for calibration, this is a more accurate method for probe calibration. However, a limitation of this method is the length of time it requires to collect sufficient data to provide an accurate estimate of the equilibrium state (~12 h) [6].

Although microdialysis is a commonly used sampling technique, sampling lipophilic analytes generally results in low extraction efficiencies [7-10]. The addition of binding agents, such as cyclodextrins and arachidonic acid, to the perfusate, has been shown to improve their efficiencies [3, 9, 11]. Recently, microdialysis sampling of the anti-cancer drug, docetaxel was demonstrated. Docetaxel is lipophilic and possesses poor water solubility. Using three different modes of probe calibration, (recovery, retrodialysis and NNF) it was recognized that there were differences in the EE calculated between each method, and it was also observed that with increasing concentrations of docetaxel the EE_R

decreased. Nevertheless, this study illustrated that microdialysis sampling of lipophilic compounds was possible [12].

Another example of a lipophilic compound is doxorubicin (DOX), which was one of the first identified anthracyclines and is isolated from the pigment producing *Streptomyces* species. Anthracyclines are antibiotics that are regularly used in the treatment of cancers, such as lung and ovarian cancers [13]. In this report, the microdialysis sampling of DOX, whose structure is shown in Figure 1, was systematically investigated. Varying concentrations of DOX were employed and the EE's were determined by both recovery and delivery experiments and by NNF. In addition, antipyrine was used as a known well-behaved reference which has been used as an internal standard in many microdialysis studies [14]. This study will provide information on the effects of applying microdialysis to the lipophilic compound DOX, and will help in its application for *in vivo* studies.

2. Materials and Methods

2.1 Chemicals

Antipyrine, sodium chloride, potassium chloride, magnesium chloride, calcium chloride and sodium monobasic phosphate were obtained from Sigma Aldrich (St. Louis, MO, USA). Doxorubicin hydrochloride (DOX) was obtained from Tocris Biosciences (Ellisville, MO). Acetonitrile (ACN) and *o*-phosphoric acid were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Ringer's solution consisted of 147 mM NaCl, 3 mM KCl, 1 mM MgCl₂ and 1.3 mM CaCl₂ (152.3 mM ionic strength) and filtered through a 47 mm, 0.22 µm nylon filter (Fisher Scientific, Fair Lawn, NJ, USA) prior to use. All antipyrine and DOX solutions were prepared in Ringer's solution. To help with DOX solubility, 10% ACN was added to DOX solutions [15].

2.2 Chromatographic conditions

The HPLC system consisted of a Shimadzu LC-10AD solvent delivery module, a SPD10AV UV detector module (Shimadzu, Columbia, MD, USA). Sample injections were made into a Rheodyne 7725i injection valve with a 10 µl injection loop. An injection volume of 5 µl was used for all sample analyses. The system was operated using Shimadzu EZ Start (version 7.3) software. A Phenomenex Synergi Polar RP column (2.1 × 150 mm, particle size 4 µm, Phenomenex, Torrance, CA, USA) was employed. The mobile phase consisted of sodium phosphate (25 mM, pH 2.5)/acetonitrile (75/25, v/v). All HPLC mobile phases were filtered through a 47 mm, 0.22 µm nylon filter (Fisher Scientific, Fair Lawn, NJ, USA) prior to use. An isocratic elution was employed at a flow rate of 0.3 ml/min. All sample analyses were carried out using UV detection at 254 nm with a deuterium lamp.

The selectivity of the method was determined through the analysis of six blank sample matrices (Ringer's solution) with no contaminants obtained. The method was validated with respect to elution time and peak area. Low RSD values of 3-4.5% were obtained for peak area for both antipyrine and DOX, with similar RSD values achieved for elution time reproducibility for both analytes (n=3). The responses were linear in the range of 0.5-25 µM for both antipyrine and DOX. Detection limits of 0.25 µM and 0.1 µM were obtained for antipyrine and DOX, respectively.

2.3 Microdialysis system

Linear microdialysis probes were fabricated in-house based on previously described techniques [16]. Probes were composed of a polyacrylonitrile (PAN) dialysis membrane (350 µm o.d., 250 µm i.d., MWCO 40 kDa, Hospal Industrie, Meyzleu, France). A 10 mm effective membrane length was used for all experiments. Polyimide tubing (175 µm o.d.,

122 μm i.d., Microlumen, Tampa, FL) was used for the probe inlet and outlet. A piece of tygon tubing (1520 μm o.d., 508 μm i.d., Norton Performance Plastics, Akron, OH) was used as an adaptor for the probe inlet and perfusate syringe. All probe pieces were connected by UV glue (Ultraviolet Exposure Systems, Sunnyvale, CA) by curing with an ELC-450 UV Light System (Electrolite Corporation, Bethel, CA). Before every experiment, probes were perfused with a Ringer's solution for 1 h at a flow rate of 1 $\mu\text{L}/\text{min}$ maintained by a CMA 400 Syringe Pump (CMA/Microdialysis AB, Stockholm, Sweden).

2.4 In vitro characterization of microdialysis probes

2.4.1 Delivery—For delivery experiments, a Ringer's solution was placed in a thermostated stirred plastic beaker at 37 °C. The analytes of interest, in the matching electrolyte solution, were perfused through the probe at 1 $\mu\text{L}/\text{min}$ for 2 h. Dialysate samples were collected over 20 minute intervals. For both recovery and delivery experiments, a concentration of 10 μM was employed for antipyrine, while the DOX concentration was varied in the range 1–20 μM .

2.4.2 Recovery—For recovery experiments, a standard solution of the analytes of interest in a Ringer's solution was placed in a thermostated stirred plastic beaker at 37 °C. The matching electrolyte solution was perfused through the probe at 1 $\mu\text{L}/\text{min}$ for 2 h and dialysate samples were collected over 20 minute intervals.

2.4.3 NNF—For NNF experiments, a 10 μM concentration of DOX and antipyrine in Ringer's solution was placed in a thermostated stirred plastic beaker at 37 °C. Blank, 4, 6, 8, 10, 12, 14 and 16 μM concentrations of both analytes were perfused through the probe for 1 h each. The order of perfusion solutions were chosen at random. Between each concentration, the probe was perfused with Ringer's solution for 1 h. Dialysate samples were collected over 10 minute intervals, with a total of four samples for each concentration used for the NNF plot.

2.5 Data analysis

Statistical analysis of the data was performed by OriginLab (Version 6.0) software by the *t* and ANOVA tests to determine differences between the (%) EE_D and EE_R obtained for antipyrine and DOX.

3. Results

3.1 Probe calibration by delivery and recovery

For these studies, the concentration of antipyrine remained constant (10 μM), and the concentration of DOX was varied (1, 5, 10 and 20 μM). Initially, probe calibration was performed by delivery with the probe perfused with a Ringer's solution for 1 h prior to the start of the experiment. Delivery experiments were performed as described in Section 2.4.1. Before the recovery experiment, the probe was again perfused with a Ringer's solution for 1 h. Recovery experiments were performed as discussed in Section 2.4.2. Mean EE data for delivery and recovery experiments were determined for DOX and antipyrine and the data were evaluated using the *t*-test, as shown in Table 1. From this data it was evident that there were significant differences ($p < 0.01$) between the EE_R and EE_D for DOX. Figure 2a shows a typical delivery profile for DOX (10 μM). The EE_D (87.0 ± 4.9) followed the same trend and was consistently high for three separate investigations over the course of the study (2 h). For the recovery experiments (EE_R 52.7 ± 3.5), DOX took a longer time to reach a steady-state (80 min.), as illustrated in Figure 2b. A similar EE_R trend was observed for the remaining concentrations of DOX, with a steady-state never reached with 20 μM DOX (data

not shown). This was in contrast to antipyrine where a steady-state EE_D and EE_R was achieved within 20 min.

In order to determine whether the order of the experiments performed had an effect on the EE 's, first a recovery experiment was performed followed by a delivery experiment. The EE_R for DOX was still consistently lower than the EE_D , showing that the order of the experiment did not affect the results (Table 2). A second recovery experiment (EE_{R2}) was performed after the delivery experiment. For two of the probes, the EE_{R2} was in agreement with the initial EE_R for both DOX and antipyrine but showed dramatic differences for one probe (but different probes) for each analyte. These differences were ascribed to the variation in repeating delivery and recovery experiments.

DOX is a red colored solution, and it was observed that with higher concentrations of DOX the membrane became more highly colored. The membrane remained red in color even after perfusing with Ringer's solution for 1 h between delivery and recovery experiments. Figure 3 illustrates a comparison of the probe membrane between blank, delivery and recovery experiments using the same concentration of DOX (10 μ M). These results suggest that DOX was never completely washed from the probe membrane and was absorbing to it.

3.2 No-net flux

The NNF method involves both a recovery and delivery experiment and is generally used to validate other probe calibration methods. As stated previously, the order of perfusion solutions were chosen at random. Figures 4 shows NNF plots obtained for antipyrine and DOX. Their analytical concentrations in the bulk solution were 9.8 ± 0.4 μ M for antipyrine and 11.1 ± 0.4 μ M for DOX. The sample concentrations were determined from the microdialysis experiment using the y-intercept of the NNF plot (C_{NNF}) and the slope was used to determine the EE_{NNF} (Table 3). In addition, the sample concentration was determined as a recovery experiment (C_R) using the EE_{NNF} as the recovery. For antipyrine, the EE_R and EE_{NNF} were in good agreement but resulted in calculated C_{NNF} and C_R values significantly lower than the analytical concentration. For DOX, significant differences were observed between EE_R and EE_{NNF} ; however, the C_{NNF} was in reasonable agreement with the analytical concentration.

3.3 Repeatability of EE measurements

The repeatability of the EE for antipyrine and DOX (10 μ M) was determined for both recovery and delivery experiments. Recovery experiments were performed in which the sample solution was cycled between a blank Ringer's solution and a Ringer's solution containing antipyrine and DOX (10 μ M each). A steady-state concentration was achieved for antipyrine within the second sampling interval of each recovery cycle (Figure. 5a). Similarly, antipyrine was not detected in the first dialysate sample after changing the sample solution back to blank Ringer's solution. The concentration of DOX in the dialysate never reached a steady-state and was never completely eliminated from the probe even after perfusing the probe with blank Ringer's solution for 1 h, Figure 5a. Again, the membrane was colored red from DOX absorption at the end of the experiment.

For the delivery experiment, the perfusion solution was cycled between a Ringer's solution and a Ringer's solution containing both antipyrine and DOX. Again, a steady-state concentration was not achieved for DOX, Figure 5b. With each change of perfusate to Ringer's solution the concentration of DOX initially increased, which suggests that DOX is absorbing to the membrane with some washing out during the rinse step. In comparison, antipyrine achieved a steady-state concentration in the dialysate by the second sampling

interval and was not detected in the second dialysate sample after changing the perfusate to a blank Ringer's solution.

3.4 Probe clearance of antipyrine and DOX

In order to demonstrate the differences between the clearance of antipyrine and DOX from a probe in a recovery experiment, the probe was initially placed in a thermostated stirred plastic beaker with both antipyrine and DOX (10 μ M each) and the probe was perfused with Ringer's solution for 1 h. After 1 h the bulk solution was changed to a blank Ringer's solution and the clearance of both analytes were monitored continuously. Again, antipyrine decreased and was not detectable by the second sampling interval; however, the concentration of DOX remained high in the dialysate more than 3 h after changing the bulk solution to blank Ringer's solution (Figure 6a).

For the corresponding delivery experiment, the probe was initially perfused with both antipyrine and DOX for 1 h followed by perfusion with blank Ringer's solution for 4 h. Again, antipyrine decreased rapidly and was not detectable in the dialysate by the second sampling interval; however, DOX was clearly still present in the dialysate even after perfusing with blank Ringer's solution for 4 h. (Figure 6b). In addition, at the end of this experiment the membrane was still red indicating absorbed DOX.

4. Discussion

In this investigation, microdialysis sampling was applied to the lipophilic compound, DOX. However, microdialysis sampling of lipophilic compounds generally results in low extraction efficiencies [7-10]. The microdialysis sampling of another lipophilic drug, docetaxel, was reported by Schuk et al., who observed that there was a concentration effect on the EE_R [12].

In our experiments, a steady-state concentration in the dialysate was reached for DOX within 20 minutes during a delivery experiment but it took over 80 minutes to reach steady-state during a recovery experiment (Figure 2b). Steady-state dialysate concentrations were reached within 20 minutes for antipyrine during both delivery and recovery experiments. In addition, it was observed that the EE_D was consistently higher than the EE_R for DOX, although a concentration dependence on either EE_D or EE_R was not observed, Table 1. The slow approach to steady-state for DOX relative to antipyrine is indicative of the membrane presenting a barrier to mass transport for DOX. That steady-state was achieved more rapidly for DOX during delivery than recovery was likely due to the enhanced mass transport of DOX coming into the probe via the perfusate, which is in comparison to its relative diffusion to the membrane during recovery.

The coloration of the membrane was also indicative of absorption of DOX to the membrane. Unsurprisingly, the color was most intense at the probe inlet during a delivery experiment where the highest concentrations of DOX should occur. Likewise, during a recovery experiment, the highest concentrations should occur at the outlet and this was the region most highly colored in these experiments, Figure 3. However, merely the fact that DOX absorbs to the membrane would not lead to differences in EE_D and EE_R if the absorption was saturable. Nevertheless, the observation that EE_D was significantly higher than EE_R indicates that absorption to the membrane results in a lower dialysate concentration during a recovery experiment. It was significant that the order in which the recovery and delivery experiments were performed did not change the relationship between EE_D and EE_R , Table 2. This indicates that absorption of DOX to the membrane was not significantly changing the nature of the membrane and that the differences in EE were not due to changes in membrane permeability, but rather loss of DOX into the membrane.

An alternative method of probe calibration is the NNF method which was used to validate the results of the delivery and recovery experiments. In a NNF experiment, the points above the intercept represent a recovery experiment (concentration in the sample greater than the concentration in the perfusate and net transport into the probe) while the points below the intercept represent a delivery experiment (concentration in the sample less than the concentration in the perfusate and net transport out of the probe). If a straight line is obtained then EE_R should equal EE_D . As expected, this is what was observed for antipyrine (Figure 4a). A very interesting result was observed for DOX, as shown in Figure 4b. The NNF data resulted in a straight line whose slope corresponded to an EE_{NNF} which was in agreement with the EE determined by delivery. The slope was identical on both sides of the intercept, indicating that EE_R equals EE_D . However, this was only true if the initial point where the perfusate concentration was zero was ignored. This point represents the typical recovery experiment in which analyte was present in the sample solution but not in the perfusate. Clearly the inconsistency was not with all recovery conditions but only with no analyte in the perfusate. The order in which the solutions were perfused was chosen at random and the data suggests that once the perfusate contains any DOX the EE is constant. This again illustrates the sink nature of the membrane for DOX.

The repeatability of the EE has previously been demonstrated for the hydrophilic compound, caffeine [5]. In this investigation it was evident that antipyrine follows a similar trend *i.e.*, antipyrine reached a steady-state by the second dialysate sample. In addition, antipyrine was not detected in the first dialysate sample after either changing the bulk solution or perfusate to blank Ringer's solution, suggesting that it has a fast clearance from the probe (Figures 5 and 6). DOX never reached a steady-state in either the recovery or delivery experiments, which again indicates that the membrane was preventing mass transport between the bulk solution and the perfusate, Figure 5. In the delivery experiment, the concentration of DOX initially increased with each change of perfusate to blank Ringer's solution, which indicates that DOX was absorbing to the membrane with some washing out during the rinse step. The low concentrations obtained in the recovery experiments, strongly suggests that the absorption of DOX was not saturable and that there was loss of DOX into the membrane.

5. Conclusions

In this report, the *in vitro* microdialysis sampling of the lipophilic analyte, DOX, was investigated. The concentration of DOX was varied, and probe calibrations were performed by delivery, recovery and NNF methods. While, it was demonstrated that EE_D did not equal EE_R for DOX, no concentration dependence on EE was observed. Observation of the membrane following experiments showed that DOX absorbs to the probe membrane, which supports previous studies of other lipophilic analytes. This was also seen as a slow approach to steady-state and slow clearance of DOX from the probe after each experiment relative to the well-behaved hydrophilic compound antipyrine. Using a NNF experiment, it was observed that the difference in EE_D and EE_R only occurred when the perfusate contained no DOX. These results indicate that while hydrophobic compounds may be sampled using microdialysis, the results must be interpreted carefully. Not only may the calculated sample concentration be incorrect due to miscalibration, but just as importantly, the rate of change in the concentration observed may be confounded by the uptake and washout of a lipophilic compound from the membrane (slow approach to steady-state and wash out).

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Figure 1.
Structure of doxorubicin (DOX).

Figure 2.

(a) EE stability of DOX during a delivery experiment. The probe was perfused with a Ringer's solution containing DOX and antipyrine for 2 h (10 μ M each). (b) EE stability of DOX during a recovery experiment. The bulk solution was a Ringer's solution containing DOX and antipyrine (10 μ M each).

Figure 3. Digital images of probe membranes (a) control probe, (b) perfused with antipyrine and DOX (10 μ M each) for 2 h (*i.e.* delivery experiment) and (c) placed in a bulk solution of antipyrine and DOX (10 μ M each) for 2 h (*i.e.* recovery experiment). Images at 10x magnification.

Figure 4. (a) Typical NNF plot for the determination of EE_{NNF} for antipyrine. (b) Typical NNF plot for the determination of EE_{NNF} for DOX.

Figure 5.

(a) Repeatability of the dialysate concentration for antipyrine and DOX in a recovery experiment. The bulk solution was alternately changed from a Ringer's solution (as indicated by dashed lines) to a Ringer's solution containing 10 μM of both antipyrine and DOX (as indicated by solid line).

(b) Repeatability of the dialysate concentration for antipyrine and DOX (10 μM each) during a delivery experiment. The microdialysis probe was alternately perfused with a Ringer's solution (as indicated by dashed lines) and a Ringer's solution containing 10 μM of both antipyrine and DOX (as indicated by solid lines).

Figure 6.

(a) A comparison of the normalized peak height response for antipyrine and DOX during a recovery experiment. The microdialysis probe was initially placed in a thermostated stirred beaker containing a bulk solution of 10 μM antipyrine and DOX for 1 hr and subsequently changed to a bulk solution of Ringer's solution for 4 h (dashed lines indicate when the bulk solution was changed to Ringer's).

(b) A comparison of the normalized peak height response for antipyrine and DOX during a delivery experiment. The microdialysis probe was initially perfused with a Ringer's solution containing 10 μM of both antipyrine and DOX for 1 h and subsequently perfused with a Ringer's solution for 4 h (dashed lines indicate when the bulk solution was changed to Ringer's).

Table 1Comparison of (%) EE_D and (%) EE_R for DOX and antipyrine

DOX Conc (μM)	Doxorubicin		Antipyrine	
	(%) EE _D ^a	(%) EE _R	(%) EE _D ^a	(%) EE _R
1	83.5 ± 11.5	54.7 ± 8.4 ^b	80.0 ± 2.3	84.8 ± 6.5
5	87.0 ± 4.1	60.0 ± 1.8 ^d	76.0 ± 2.3	89.5 ± 0.2 ^c
10	87.0 ± 4.9	52.7 ± 3.5 ^d	85.6 ± 4.2	90.4 ± 2.7
20	82.5 ± 2.7	38.7 ± 9.9 ^b	73.3 ± 2.9	81.5 ± 3.1 ^d
Mean	85.5 ± 5.8	51.5 ± 10.5 ^c	78.7 ± 5.0	86.6 ± 5.5 ^d

^aResults are mean ± SD, n=3^bEE_R is significantly different from EE_D at p < 0.005^cEE_R is significantly different from EE_D at p < 0.01^dEE_R is significantly different from EE_D at p < 0.05

Table 2

A comparison of between (%) EE_D and (%) EE_R for DOX and antipyrene (10 μ M each), where a recovery experiment was performed first (EE_{R1}), then delivery EE_D and a final recovery experiment (EE_{R2})

Probe no.	Doxorubicin			Antipyrene		
	EE_{R1} ^a	EE_D	EE_{R2}	EE_{R1}	EE_D	EE_{R2}
1	48.3 $\pm$ 2.9	67.5 $\pm$ 3.5 ^c	50.5 $\pm$ 1.9	81.9 $\pm$ 0.9	78.7 $\pm$ 0.9	79.4 $\pm$ 1.0
2	41.9 $\pm$ 2.2	58.8 $\pm$ 6.2 ^c	38.9 $\pm$ 3.1 ^d	77.6 $\pm$ 4.6	79.8 $\pm$ 0.7	69.2 $\pm$ 0.3
3	31.1 $\pm$ 4.0	79.4 $\pm$ 9.1 ^b	45.2 $\pm$ 9.3	75.8 $\pm$ 4.4	73.1 $\pm$ 3.7	71.5 $\pm$ 4.7
Mean	40.5 $\pm$ 8.7	68.6 $\pm$ 10.3	44.9 $\pm$ 5.9	78.4 $\pm$ 3.2	73.4 $\pm$ 5.4	75.4 $\pm$ 3.6

^aResults are mean $\pm$ SD

^b EE_D is significantly different from EE_{R1} at $p < 0.01$

^c EE_D is significantly different from EE_{R1} at $p < 0.05$

^d EE_{R2} is significantly different from EE_{R1} at $p < 0.05$

Table 3Comparison of (%) EE_R and (%) EE_{NNF}, the analytical concentration and C_R for DOX and antipyrine

Analyte	(%)EE _R ^a	(%) EE _{NNF}	Anal. conc. (μM)	C _R (μM)	C _{NNF} (μM)
DOX	26.0 ± 12.5 ^b	79.5 ± 22.3	11.1 ± 0.4	2.9 ± 1.4 ^{c,d}	12.1 ± 0.9
Antipyrine	85.7 ± 1.0	87.5 ± 1.9	9.8 ± 0.4	8.4 ± 0.2 ^c	8.8 ± 0.1

^aResults are mean ± SD, n=3^bEE_R is significantly different from EE_{NNF} at p < 0.05^cC_R is significantly different from the analytical concentration at p < 0.05^dC_R is significantly different from the C_{NNF} at p < 0.005