Rapid determination of multidrug resistance-associated protein in cancer cells by capillary electrophoresis immunoassay

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ABSTRACT

The adenosine triphosphate (ATP) binding-cassette (ABC) transporters are a superfamily of cellular proteins that have been partly implicated as a cause of multidrug resistance (MDR) in cancer cells. The ABC superfamily consists of P-glycoprotein, multidrug resistance-associated proteins (MRP) and breast cancer-related proteins, of which MRP is of particular interest because of its ability to efflux a broader range of substrates. Since MRP1 is the most prominent member of the MRP family, a simple technique is needed for its quantification. We developed a simple, fast (total analysis time of 3 h) capillary electrophoresis immunoassay (CEIA) for the quantification of MRP1 in cancer cells. MRP1 antibody was labeled with fluorescein isothiocyanate. The labeled antibody was incubated with the cell lysate for a fixed interval (1 h), after which the cell lysate mixture was directly injected into the capillary to separate the complex of MRP1 and its antibody from free antibody. The noncompetitive CEIA method had a limit of detection of ~~0.1~~ 0.2 nM and a good linear range (1.7 - 14.9 x 10^4 cells), and was fairly reproducible (RSD < 10%). The results showed that two cell lines, A549 and RDES, expressed MRP1 in the absence of doxorubicin (DOX), with A549 registering a higher expression. ~~The amount of MRP1 increased after treatment with DOX for 12 h and was constant until 24 h~~. ~~The intracellular accumulation of DOX in cells decreased as the expression of MRP1 increased due to exposure of the cells to DOX, suggesting that the accelerated expression of MRP1 is responsible for the decrease of DOX in the cells.~~ Compared to DOX-free cancer cells, there was an acceleration of MRP1 expression during the 12 h-exposure to DOX, after which the level of expression remained nearly constant as the intracellular accumulation of DOX decreased. The results obtained in this work indicate that the

developed CEIA method is useful for relative quantification of MRPs in ~~the study on MDR in of~~ cancer cells.

1. Introduction

Chemotherapy treatment of many types of cancers is rendered ineffective due to intrinsic or acquired multidrug resistance (MDR), which is partly induced by multidrug transporter proteins such as the adenosine triphosphate **(**ATP) binding-cassette (ABC) and lung resistance-related proteins [1-3]. These multidrug transporter proteins actively efflux drugs out of the cells, thereby reducing their intracellular concentration and leading to multidrug resistance. The ABC superfamily constitutes the bulk of the multidrug transporter proteins, and consists of three main families: multidrug resistance-associated protein (MRP), P-glycoprotein, and breast cancer-related proteins [1]. Although it has been less thoroughly investigated than P-glycoprotein, MRP can efflux not only cationic and neutral hydrophobic compounds, but also anionic conjugates of sulfates, glutathione, and glucuronic acid. MRP is made up of several subfamilies including MRP1, MRP2, MRP3, MRP4, and MRP5. Because of the role of MPR1 in conferring MDR in tumors [4], along with its wide occurrence in the human body, ~~its~~ quantification of MRP1 is extremely important.

Absolute and relative quantification of the protein transporters has been reported. While absolute quantification of these transporter proteins is most useful, it is difficult, time consuming, and expensive, primarily because standards must be synthesized, purified, and identified prior to quantification by one of the analytical methods, *e.g.*, HPLC [5,6]. Most methods, however, are based on relative quantification, in which the

proteins are analyzed by various techniques without using standards. Methods of transporter protein quantification that have been studied include PCR [7-9] (RT-PCR, real time RT-PCR), Western blotting [10,11], flow cytometry [12,13], and electrochemical immunoassay [14]. Western blotting is not only semi-quantitative~~,~~ but also time consuming~~,~~ and requires large sample sizes. The main disadvantages of flow cytometry are its expensive instrumentation and difficulty in the determination of transporter proteins localized at cell organelles, since flow cytometry only measures the transporter proteins located at the cell surface. PCR techniques require a longer analysis time for separation, detection, and accurate quantification, and may suffer from contamination of the probe, which may lead to false positives [15].

Although ABC transporter proteins are generally thought to mediate drug efflux at the plasma membrane [16-18], some studies have shown that these proteins are localized in cell organelles like the nucleus [19,20]. Because the transporter proteins could be localized anywhere in the cells, it is more useful to determine the total intracellular amount of the transporter protein after carrying out cell lysis. Such determinations are more suitably carried out by capillary electrophoresis immunoassay (CEIA). Indeed CEIA may address some of the shortcomings of the established methods ~~requires antibody, like other assays~~ such as ELISA, Western blotting, and flow cytometry because it is easy to automate, requires smaller sample sizes and shorter analysis time, has simple procedures, and is capable of multi-analyte analysis [21]. CEIA in either competitive [22, 23] or noncompetitive [24] formats, may utilize antibody [22], enzymes [25] or aptamers [26,27] as ligand to interact with antigens to form complexes in highly complicated matrices. ~~address some of the aforementioned shortcomings of these established methods~~.

~~While~~ Since the pioneering works by Nielsen *et al.* [28], CEIA has found application in the determination of wide range of analytes including toxins [29], drugs and metabolites [30], hormones [31], peptides [32], and proteins [33]. While most CEIA investigations of proteins have focused on lower molecular weight proteins (10 - 80 kD), reports on the determination of higher molecular weight proteins, like ABC transporter proteins (170-190 kD) in cells are few. It is worth noting that even CEIA reports of the most extensively studied ABC transporter-protein, P-glycoprotein, are rare.

In the present study, a simple, non-competitive CEIA method for the relative quantification of MRP1 was developed. Laser-induced fluorescence (LIF) was used for detection of the transporter protein in order to solve the problem of low sensitivity inherent in the capillary electrophoresis (CE) technique. Since baseline resolution of complex and antibody is necessary for this method, antibody instead of enzymes or aptamers was employed because the smaller size of the two ligands will lead to poor resolution between the complex and free ligand for bulky proteins such as MRP1. The method involved reacting cell lysate with an excess of the labeled anti-MRP1 antibody and adding an internal standard, followed by immediate injection of the unincubated mixture into the CE system to obtain the antibody peak before the immunological reaction. After two or three swift, consecutive runs, the cell lysate mixture was incubated, after which more CE runs were made to obtain peaks for the free antibody and formed immune complex. The amount of the formed immune complex was used to determine the amount of protein contained in the cell lysate. It should be noted that no purification of the antibody was necessary, as quantification of the protein is based on the immune

complex and not the post-incubation amount of the antibody. This method was used to compare the levels of MRP1 expressed in cancer cells A549 and RDES.

2. Materials and methods

2.1. Materials

Sodium tetraborate decahydrate, glycine, tricine, doxorubicin (DOX, in hydrochloride form), absolute ethanol, rhodamine B, hydrochloric acid, sodium fluorescein, and Tris were purchased from Wako Pure Chemicals (Osaka, Japan). Monoclonal anti-MRP1 (Clone QCRL-4, Purified Mouse Immunoglobulin, Product Number M9192), sodium dodecylsulfate (SDS - electrophoresis grade), sodium taurodeoxycholate (STDC) hydrate, and (2-hydroxypropyl)-γ-cyclodextrin, were obtained from Sigma Aldrich (St. Louis, MO, USA). A Fluorescein Labeling Kit-NH_2 and EDTA were obtained from Dojindo (Kumamoto, Japan). Sodium chloride was obtained from Chameleon Reagents (Osaka, Japan). A bicinchoninic acid (BCA) protein assay kit was purchased from Pierce Biotechnology (Rockford, IL, USA). Lung cancer cells, A549, were purchased from the Health Science Research Resources Bank (Osaka, Japan). Human Ewing's family tumor cell line (RDES) was obtained from the American Type Culture Collection (VA, USA).

Stock solutions of ~~Tripsin~~trypsin-EDTA (0.05%), RPMI and DMEM media, and DPBS (1X) were purchased from Invitrogen (Grand Island, NY, USA). All solutions were prepared in pure 18-MΩ MilliQ water (Millipore SA, Molsheim, France). A stock solution of DOX (200 μM) was prepared in MilliQ water, stored in opaque containers and kept refrigerated at 4 ^{o}C. The migration~~ng~~ solution consisted of sodium tetraborate (120 mM of borate), glycine (50 mM), and tricine (50 mM) adjusted to pH 8.9. The

145 preparation of the migrationng solution for DOX measurement and the cell lysis buffer
146 has been described elsewhere [34].
147
148 *2.2. Treatment of cells with DOX*
149 Prior to treatment with DOX for a fixed time interval (12 h or 24 h), the cells (A549 or
150 RDES) were washed thrice with DPBS and separated into 3.5-cm petri dishes. The cells
151 in the dishes were cultured until the population they covered 90-100% of the bottom
152 surface area of the dish. Thereafter, fresh culture media with and without DOX were
153 added to the dishes to prepare DOX-free and DOX-treated (500 nM) cells. After addition
154 of the appropriate culture medium, the cells were incubated at 37 ^{o}C in 5% CO_2 for either
155 12 h or 24 h. Subsequently, the cells were lifted by adding 200 µL of Tripsintrypsin-
156 EDTA, suspended by adding 800 µL of DPBS, and then transferred into a microvial,
157 where they were washed (twice or thrice) with DPBS, before addition of the cell lysis
158 buffer (400 µL). The cell lysis buffer contains 100 mM NaCl, 20 mM EDTA, 1%(w/v)
159 SDS and 50 mM Tris-HCl (pH 8). The treatment of cells to obtain lysate and
160 measurement of the total protein content were described earlier [34]. Briefly, the lysis
161 buffer was added to the cells in the microvial. The solution was vortexed to enhance lysis
162 and to make the cell lysate uniform. After complete dissolution of the cells, the cell
163 lysate was sonicated for about 15 minutes to assist in breaking the long DNA strands,
164 which results in a uniform cell lysate of lower viscosity. The obtained cell lysate was
165 used for antibody binding and protein determination experiments.
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167

2.3 Reaction of cell lysate with antibody

The antibody was labeled with flourescein according to the labeling kit manufacturer's instructions (Dojindo, Kumamoto). The concentration of the labeled antibody was then determined by spectroscopic measurement at 280 and 500 nm. The number of fluorescein molecules tagged with antibody was also calculated according to the labeling kit manufacturer's instructions, using absorbance at 280 and 500 nm. The number was calculated to be 5~7 depending on the concentration ratio of the labeling reagent to the antibody. However, the antibodies tagged with different numbers of fluorescein molecule did not show any difference in the immunological reaction. Therefore, the labeled antibody tagged with 5~7 fluorescein molecules were directly employed for the immunoassay.

In the immunological reaction, a ~~A~~ known excess amount of the labeled antibody (30 nM) was added to 60 μL of the sample, followed by the sodium fluorescein (0.125 μM) as internal standard and enough 1x PBS buffer to make 100 μL. Two or three CE-LIF runs were made quickly, before the cell lysate mixture was incubated at 37 ^{o}C for 1 h, after which the mixture was directly injected into the capillary for separation by CE-LIF measurement.

2.4. CE-LIF measurement

The CE-LIF system used was described previously [34]. Briefly, a custom-made system was assembled in a room with a constant temperature (25 ^{o}C). Ordinary fused silica capillaries (50 μm i.d.; 356 μm o.d.; effective length, 30 cm; total length, 40 cm; GL Sciences, Tokyo, Japan) were used in the CE-LIF system. Samples were

hydrodynamically injected into the capillary for 10 s by siphoning (the sample vial raised 5 cm above the outlet vial), and a separating voltage (10 kV or 15 kV) was applied using a high voltage power supply (HCZE-30PN0.25, Matsusada Precision Inc, Shiga, Japan). The LIF detection was done using a 488 nm line of an argon ion laser (Stabilite 2017, Spectra-Physics, Inc., CA, USA) as the excitation source. The generated fluorescence was filtered with a notch filter (Edmund Optics Japan, 46564-K, Tokyo, Japan) and collected by a photomultiplier tube (model R3896, Hamamatsu, Shizuoka, Japan) biased at 650 V. The data generated were processed using an in-house Labview program (National Instruments, Austin, TX, USA). The capillary was flushed after every two runs with NaOH (0.1 M) and migration~~ng~~ solution for ~~four minutes~~ 4 min each.

2.5. Data processing

Pre-incubation electrophoretic measurements were made to determine the peak area corresponding to the initial amount of the antibody ($A_{ab,0}$) and the internal standard ($A_{IS\text{-}pre}$). Post-incubation electrophoretic measurements yielded the peak area corresponding to the complex (A_{comp}) and the internal standard ($A_{IS\text{-}post}$). The peak areas were proportional to the concentrations of the corresponding species. Thus, ~~(eq 1), as follows~~:

$$\frac{C_{ab,0}}{A_{ab,0}/A_{IS\text{-}pre}} = \frac{C_{comp}}{A_{comp}/A_{IS\text{-}post}} \qquad (1)$$

where $C_{ab,0}$ and C_{comp} represented the initial concentration of antibody and the concentration of complex produced, respectively.

~~Assuming that~~ Under the condition where excess amounts of antibody was added, the complex ~~was~~ formed consisted of ~~by~~ one antibody and ~~two~~ one antigen ~~molecules~~ and the concentration of MRP1 was directly calculated according to eq 2 ~~as follows~~:

$$C_{\mathrm{MRP1}} = \frac{C_{\mathrm{ab,0}}}{A_{\mathrm{ab,0}} / A_{\mathrm{IS\text{-}pre}}} \times A_{\mathrm{comp}} / A_{\mathrm{IS\text{-}post}} \qquad (2)$$

To correct the concentration of C_{MRP1} for the number of cells, C_{MRP1} was divided by concentration of protein C_{Protein} denoted by the amount of total protein P (mg mL^{-1}) ~~(eq 3)~~, as follows:

$$\frac{C_{MRP1}}{C_{\mathrm{Protein}}} = \frac{\dfrac{C_{\mathrm{ab}}}{A_{\mathrm{ab}} / A_{\mathrm{IS\text{-}pre}}} \times A_{\mathrm{comp}} / A_{\mathrm{IS\text{-}post}}}{P} \qquad (3)$$

~~Using eq 3 allowed for direct comparison of the MRP1 expressions in the cell lysate.~~ Using eq 3, simple, direct comparison of MRP1 expression in cell lysate is readily accomplished as compared to the more difficult and expensive determination of absolute amounts.

3. Results and discussion

3.1 Method development and kinetics of the complex formation

Noncompetitive CEIA was adopted because of the ~~lack~~ scarcity of transporter proteins standards (commercial or synthesized) ~~for the~~. Cell lysates of A549 were employed as samples for optimization of the separation conditions, since it is known that A549 inherently expresses MRP1 [35]. Several migration~~ng~~ buffers were tested, including

borate (pH 9), MES (pH 7), HEPES (pH 8), CAPS (pH 9.5), and Tris (pH 8.1), but the borate buffer showed the best separation of the antibody and its complex. To control adsorption of both the antibody and the complex on ordinary silica capillary walls, Zwitter ionic additives (glycine, tricine) were examined. Borate-glycine (pH 9.0) produced inferior resolution of the two peaks, while borate-tricine exhibited improved peak resolution but suffered peak tailing. Thus, the two Zwitter ions were combined to make the migration~~ng~~ solution of 50 mM glycine and 50 mM tricine in 120 mM borate buffer (pH 8.9). Variable concentrations (40 mM-150mM) of the borate buffer were examined and the optimum concentration was found to be 120 mM. The applied voltage was optimized to 10 kV to simultaneously maintain the current below 50 μA and the resolution between the antibody and the complex.

The incubation time for antibody-MRP1 complexation was determined by injecting the mixture of A549 cell lysate and anti-MRP1 at 10 min intervals for a total duration of 73 min. During this period, the mixture was incubated at 37 ^{o}C and sample was directly injected into the capillary. ~~The mixture was incubated at 37 ^{o}C after the first injection of the mixture into the capillary (0 min)~~. Fig. 1 shows the progression of complex formation as the complex peak became increasingly prominent. As seen in Fig. 1, the complex peak appeared only when the cell lysate was mixed with anti-MRP1 followed by incubation. Therefore, the new peak was definitely assigned to the complex. Fig. 2 ~~shows~~ illustrates the relationship between reaction time and the relative peak area of the complex. The curve in Fig. 2 shows that complex formation was rapid during the first 10 - 15 min and was nearly complete after about 50 min. This method can, therefore, be used for kinetic investigation of antibody-antigen interaction, as it is possible to directly inject the sample

253 into the capillary at fixed time intervals ($\Delta t > 10$ min) as incubation proceeds. Based on
254 the results in Fig. 2, an incubation time of 60 min was adopted, as the peak area remained
255 nearly constant after 60 min. Although Wang *et al.* [36] reported improved stability of
256 the complex upon addition of BSA into the cell lysate before adding the antibody, no
257 effect on the stability of either the complex or antibody was observed in this work.
258 Although the incubation time of 60 min seems to be long for a reaction in a free
259 solution, the kinetics of an immunological reaction is not necessarily fast even in the free
260 solution and is dependent on the type of a target protein. For example, the incubation
261 time of insulin antibody was only 5min which is a short incubation time [37] whereas
262 protein G needed 30 min of incubation [38] and carcinoembryonic antigen was incubated
263 for 45 min and 60 min with primary and secondary antibodies, respectively [39].
264 Table 1 summarizes some of the analytical parameters of this CEIA method for MRP1
265 determination. Compared to the Western blot determination of P-glycoprotein in human
266 colon adenocaranoma cells LS-180 [40], intraday variation in this work was comparable
267 (7.1%), while the interday variation was better than the reported value (17.4%). ~~while the~~
268 ~~linear range was better than that of competitive CEIA [31]~~. The LOD obtained was
269 similar to 0.9 nM obtained by competitive ~~CIEA~~ CEIA [23], but higher than 5×10^{-12} M
270 determined by noncompetitive IEF [24]. The lower LOD is observed in noncompetitive
271 IEF because the method incorporates a concentration step. It is worth noting that
272 selectivity against other closely related MRPs like MRP2, MRP3 was not tested since the
273 manufacturer of anti MRP1 antibody indicated that no cross reaction against other MRPs
274 was expected.

Like A549 cells [4, 35], RDES cells would be expected to express MRP1, since MRP1 expression has been detected in myeloma samples [41]. Therefore, RDES cell lysates were reacted with labeled anti-MRP1. Fig. 3 shows a typical separation of the antibody and its complex when using an RDES cell lysate as a sample. Thus, similar to A549, RDES cells ~~like A549~~, ~~result indicates that,~~ are capable of expressing ~~can express~~ MRP1. ~~as well as~~.

3.2. Determination of relative amounts of MRP1 in RDES and A549 cell lysates

The developed CEIA method was used to determine the relative amounts of MRP1 in A549 and RDES cancer cells. As shown in Table 2, the relative amounts of MRP1 in the cells were measured after incubating the cells in DOX-free, DOX, and DOX/probenecid culture media for either 12 or 24 hours. Probenecid, which is known to inhibit MRP1 [42], was employed since it has been reported to enhanced the accumulation of anthracyclines in A549 and RDES cells [43]. The results show that both cell lines expressed MRP1, even in the absence of DOX, and that A549 contained more MRP1 than RDES. Lung tissues express several ABC proteins in order to prevent the accumulation of harmful xenobiotics from inhaled air [44]. MRP1, which is known to cause MDR in many lung tumors [4], is localized in the basolateral surface, where it protects the lung tissues against airborne xenobiotics. Thus, even in the absence of DOX, A549 cells are expected to show relatively higher levels of MRP1 expression than RDES.

After treatment of cells with DOX for 12 h, the expression of MRP1 increased in both cell types, but to ~~a~~ different extents: RDES showed a greater increase (57%) than A549 (29%), although the total amount was less than A549. The levels of expression of MRP1

298 did not differ between exposures of 12 and 24 h to DOX in either A549 or RDES. A
299 nearly constant expression of MRP1 between the 12 h and 24 h incubation accompanied
300 by decrease in DOX accumulation suggest that drug efflux can still occur provided that
301 MRP1 has attained a certain level of expression. Generally, these results are in
302 agreement with previous works [45,46], in which anthracyclines, including DOX and
303 epirubicin, were reported to induce MRP1 expression in lung cancer cells. The MRP1
304 expression of the cells treated with DOX was similar to that of the cells treated with
305 DOX/probenecid with for 24 h incubation. It is interesting to note that the cells treated
306 with DOX/probenecid for 12 h showed a higher expression of MRP1 in 12 h than 24 h-
307 incubation for both A549 and RDES. This implies that MRP1 expression is also affected
308 by inhibitors, although the reason for the observed down-regulation after 24 h treatment
309 with DOX/probenecid is unknown. Similar down-regulation of P-glycoprotein was
310 observed in rat astrocytes with protracted treatment at a high concentration of DOX (500
311 ng mL^{-1}, 48 h) [47]. Therefore, a high concentration of a substrate for an ABC protein
312 may induce up-regulation and subsequent down-regulation, although further investigation
313 is necessary to clarify the mechanism involved.
314 To further evaluate the method, the relative amounts of MRP1 were compared with
315 intracellular DOX concentration, in which the amount of DOX was determined using the
316 same CE-LIF system and employing a previously developed method [34]. Several
317 studies have shown that the expression of MRP1 lowers the sensitivity of the cells
318 towards DOX [17 20, 48]. The lowered sensitivity to DOX would be induced by efflux
319 of DOX through over-expressed MRP1. Therefore, Tthe results of the present study are
320 consistent with the aforementioned findings [17 20,48] since increase of MPR1

expression and reduction of DOX concentration were observed simultaneously when either A549 or RDES was treated with DOX upon incubation for 12 or 24 h (Table 2). However, the amounts of DOX that accumulated in the presence of probenecid, in both A549 and RDES, did not reflect an increase in MRP1 expression. A similar observation was made by Rajagopal *et al*. [49] when they examined MRP1 activity using transient expression of fluorescently tagged MRP1. This observation may be ascribed to probenecid being an MRP1 substrate, which is therefore effluxed at the expense of DOX. Thus, an increase in MRP1 causes a higher efflux in probenecid than in DOX, leading to a modest increase in the intracellular DOX concentration.

4. Concluding remarks

A CEIA-LIF method for relative quantification of MRP1 was developed. The method is useful as a quick analytical tool for relative quantification of MRP1 by virtue of its simplicity~~,~~ and shorter analysis time~~and multianalytevariate analysis capability~~. The method's reliability has been demonstrated by the similarity of its results to those obtained by other established methods. The present study also demonstrates that CEIA-LIF can be used to separate higher-mass proteins (> 170 kDa), and, hence, can be used to investigate ABC and other superfamilies of proteins, which play crucial roles in cell activities. Because of the method's ability to measure the kinetics of complex formation, more comprehensive investigations of the rate of complexation can be designed to gain further understanding of how to control the functioning of transporter proteins.

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436 **Figure Captions**

437 **Figure 1.** Formation of the immune complex at different incubation times: (a) 0 min, (b)
438 11 min, (c) 22 min, (d) 42 min, and (e) 62 min. 1, anti MRP1; 2, Immuno-complex; 3,
439 fluorescein. Sample: A549 cell lysate treated with DOX for 12 h, incubation temperature
440 37 ^{o}C. Conditions for electrophoresis are given in the text.

441

442 **Figure 2.** Kinetic curve of the immune complex formation. Conditions are the same as
443 for Figure 1.

444

445 **Figure 3.** The separation of anti-MRP1 and its immune complex. 1, anti-MRP1; 2,
446 Immuno-complex; 3, fluorescein. Sample: RDES cell lysate treated with DOX for 12 h,
447 reaction time 60 min. Other conditions are the same as in Figure 1.

Table 1

Analytical parameters of the CEIA-LIF method for MRP1 quantification.

Cells/L (x 10^4)	Precision (RSD, %)	
	Intraday	Interday
3.3	6.2	8.18
6.6	5.9	7.16
13.2	5.6	6.61

n = 7, LOD (estimated ~~from~~ at S/N=3) = ~~0.1~~ 0.2 nM , Linear range; 1.7 - 14.9 x 10^4 Cells

Table 2

The levels of MRP1 expression and the amount of accumulated DOX in cancer cells.

Cell type	Treatment	Relative amount of MRP1/ protein content (nmoles/ mg)	Amount of DOX/protein content (μmoles/ mg)
A549	F	~~38.2±1.2~~ 76.4±2.4	0
	A-12	~~49.4±2.0~~ 98.8±4.0	0.42
	A-24	~~47.0±1.6~~ 94.0±3.2	0.26
	AI-12	~~71.8±2.9~~ 144±5.8	0.99
	AI-24	~~45.0±1.6~~ 90.0±3.2	-*
RDES	F	~~21.6±0.1~~ 43.2±0.2	0
	A-12	~~34.0±1.5~~ 68.0±3.0	1.15
	A-24	~~35.0±0.1~~ 70.0±0.2	0.99
	AI-12	~~49.0±2.2~~ 98.0±4.4	1.56
	AI-24	~~36.8±1.3~~ 73.6±2.6	-*

F, DOX free; A-12, 12 h incubation with DOX; A-24, 24 h incubation with DOX; AI-12, 12 h incubation with DOX and probenecid; AI-24, 24 h incubation with DOX and probenecid.

*Amounts of DOX were not determined for AI-24.

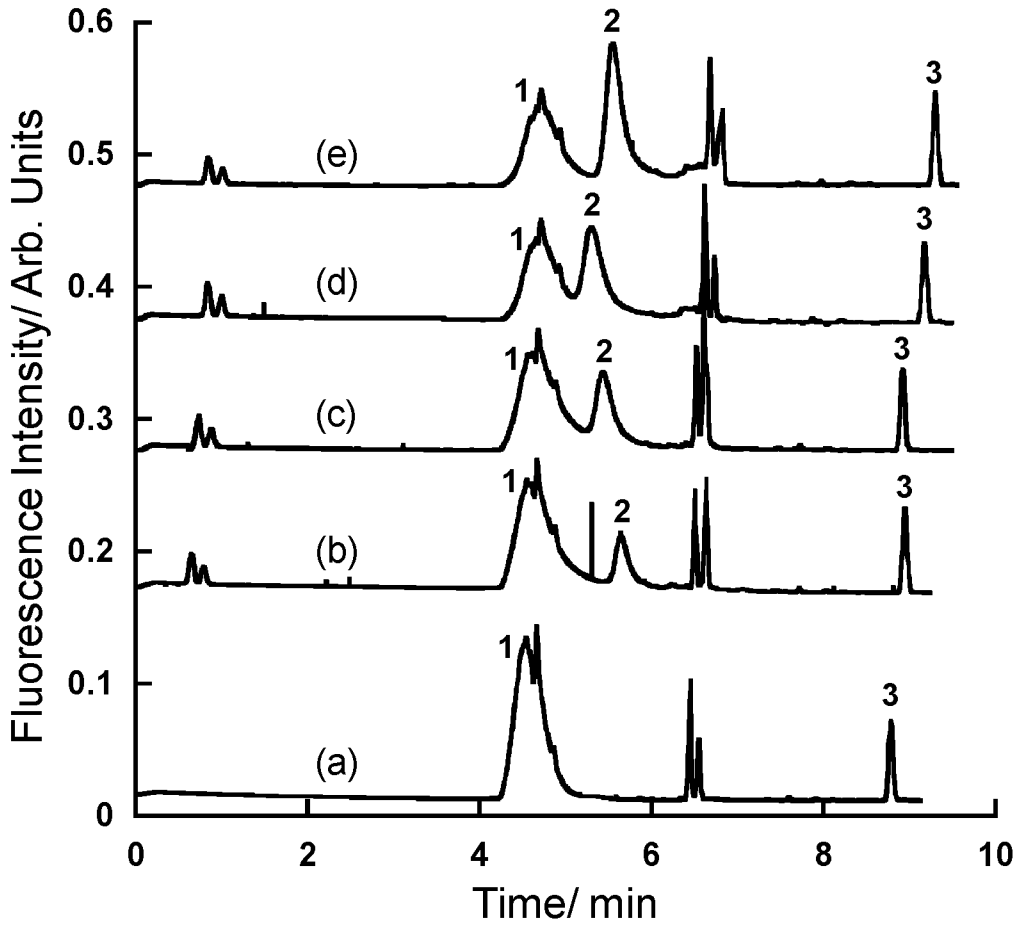

Figure 1 J. Mbuna et al.

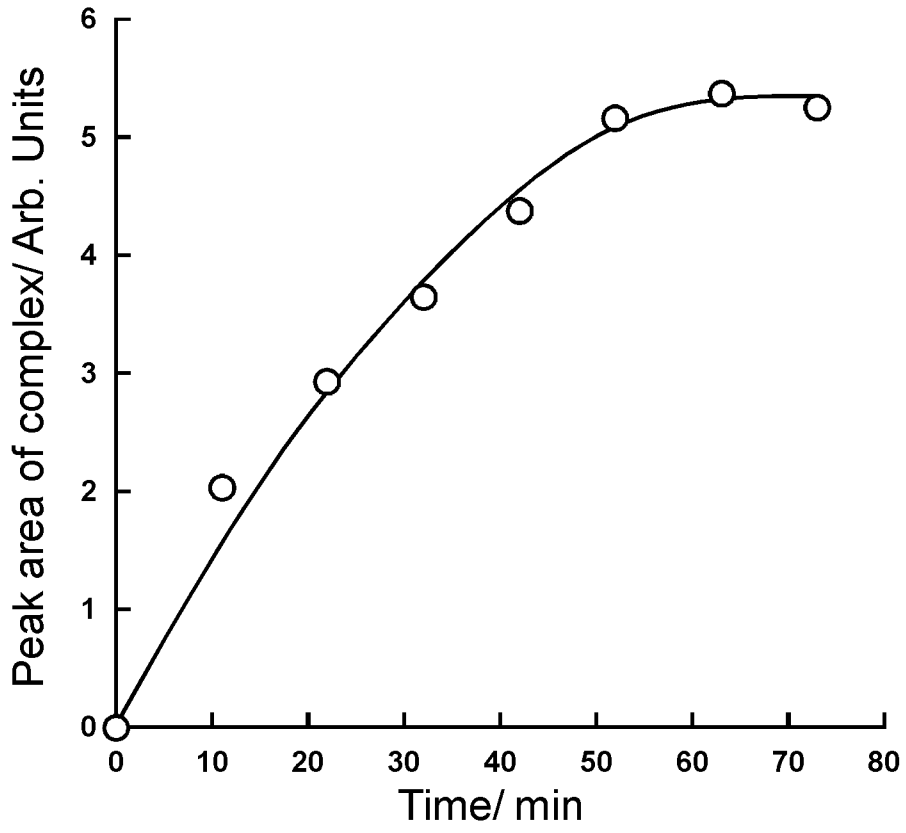

Figure 2 J. Mbuna et al.

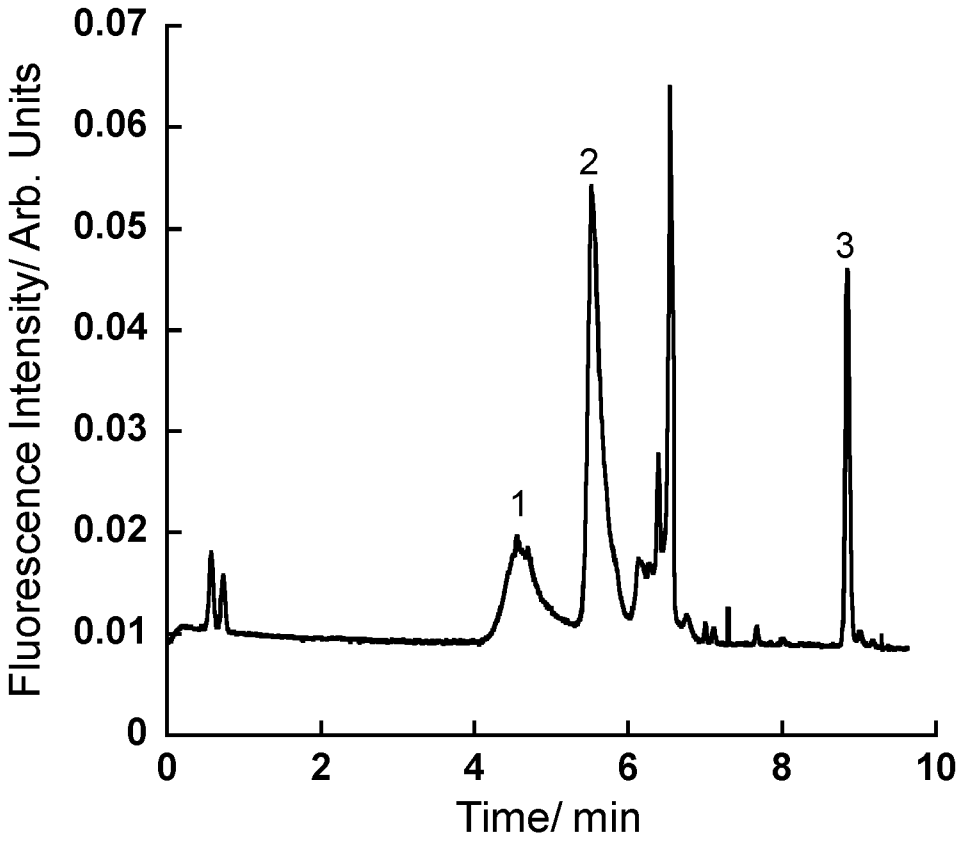

Figure 3 J. Mbuna et al.