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Fluorescence spectroscopic determination of dipyridamole binding on pancreas-1 tumor cell membrane

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Abstract

Background: The traditional method to determine the binding sites of inhibitors bound to nucleoside transporters on cell membrane is the radioactive assay. This method suffers from radiolabel instability and the need to dispose of reactive materials. Fluorescence spectroscopy has been increasingly applied to biochemical analysis due to its high sensitivity and selectivity. We describe fluorescence spectroscopy for the determination of the binding sites of dipyridamole bound to nucleoside transporters on pancreas-1 tumor cell membrane. *Methods*: Pancreas-1 tumor cell was cultured in Dulbecco's modified Eagle medium under appropriate conditions. The cell plasma membrane was separated from the ultrasonically dissolved cell mixture by gradient centrifugation. After dipyridamole was bound to the cell membrane, the test sample was obtained by ultrasonically dissolving the labeled membrane in 10 mmol/l tris–HCl membrane lysis solution. Under selected experimental conditions, the fluorescence intensity of dipyridamole was determined. *Results*: At λ_{em} of 295 nm and λ_{em} of 485 nm, dipyridamole was proportional to its concentration ranging from 1.0×10^{-12} to 5.0×10^{-11} mol/l with a detection limit of 2.8×10^{-13} mol/l (0.14 pg/ml) at 3σ. The value of affinity constant of the cell membrane to dipyridamole was $4.7(\pm 0.5) \times 10^{10}$ l/mol and the average total number of binding sites of a pancreas-1 tumor cell was $1.9(\pm 0.2) \times 10^{6}$. *Conclusions*: The proposed method can be used in the study of binding characters of dipyridamole on the cell membrane.

Keywords: Fluorescence spectrometry; Dipyridamole; Cell membrane; Nucleosides transfer

1. Introduction

Dipyridamole (2,6-bis-(diethanolamino)-4,8-dipiperinopyrimidine) is a well-known vasodilator drug that has been widely used for treatment of coronary heart diseases. This drug has an inhibitory effect on nucleosides transfer across cell plasma membranes. By inhibiting nucleosides transfer, dipyridamole may enhance the intracellular accumulation of nucleoside analogue drugs and inhibit the nucleosides salvage that might potentiate the effects of anti-metabolite drugs [1–3].

In the study of the nucleoside transport system on cell membrane, the determination of the binding sites of inhibitors bound to nucleoside transporters on cell membrane is very important. By quantifying the binding sites of inhibitors, the inhibitory characteristics on the cell membrane to nucleosides can be acquired. However, current methods for the

Abbreviations: NBTI, nitrobenzylthioinosine; DMEM, Dulbecco's modified Eagle medium; PBS, physiological buffer solution.

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measuring binding sites have not been adequate. Generally, the binding sites of inhibitors to nucleoside transporters are roughly in the range of 10^4–10^6 per cell and the number of cells in cultured medium is about 10^5–10^6/ml, thus, the amount of inhibitors bound on cell membrane is in the range of 10^{-12}–10^{-8} mol/l, which is relatively insufficient to be determined with previously developed analytical systems. For example, several analytical methods, including chromatography [4–7], spectrophotometry [8–11], fluorimetry [12,13], phosphorimetry [14], and voltammetry [15] have been used for the determination of dipyridamole in different samples except on cell membrane. The limits of detection of these methods were from 3.45×10^{-10} to 6.0×10^{-7} mol/l, which cannot be directly used in the assay of dipyridamole binding on cell membrane.

Up to date, the only method used to determine the binding sites of inhibitor, nitrobenzylthioinosine (NBTI), bound on cell membrane is based on radioactivity [16,17]. Although the radioactivity assay can achieve a high sensitivity for NBTI determination, this method has inevitable problems of environment waste hazards, harmfulness to analysts, and instability of the labels. Moreover, as an important inhibitor like NBTI, the quantitative determination of dipyridamole binding on cell membrane has not been reported yet. We present a fluorescence spectrophotometry that overcomes the drawbacks of radioactive assay for the determination of dipyridamole binding sites.

2. Materials and methods

2.1. Materials and reagents

Dipyridamole, acetonitrile, L-glutamine and GF/B filter were from Sigma (St. Louis, MO). Dulbecco's modified Eagle medium (DMEM) and fetal calf serum were from Gibco (Burlington, Ontario, Canada). All other chemicals were from Shanghai Chemical (China). Pancreas-1 tumor cell was from the Shanghai Institute of Cell and Biochemistry (China). All the reagents were of analytical grade. A dipyridamole stock solution of 1.0×10^{-5} mol/l was prepared in acetonitrile–water (2:1, v/v).

2.2. Instrumentation

Spectral recording and fluorescence measurements were performed on a Varian Eclipse spectrofluorimeter with a 150-W xenon lamp and a 1×1-cm quartz cell. Both the excitation and the emission monochromator slits were set at 10 nm, the voltage of photomultiplier detector tube was set at medium position (600 V).

2.3. Cell culture and preparation of cell membrane

Pancreas-1 tumor cells were grown in DMEM containing 15% fetal calf serum, 4.5 g/l glucose and 2 mmol/l L-glutamine at 37 °C in CO_2/humidified air (5%/95%) atmosphere. Cellar culture was performed in a dish of 15-cm diameter to a final density of $(3–5) \times 10^5$ cells/ml. After the culture medium was aspirated, the cells were washed three times with physiological buffer (PBS). The PBS was prepared with 20 mmol/l Tris, 3 mmol/l K_2HPO_4, 120 mmol/l NaCl, 1.0 mmol/l $MgCl_2$, 1.2 mmol/l $CaCl_2$ and 10 mmol/l glucose and was adjusted to pH 7.4 with HCl. The cells were scraped out from the dish, harvested to a test tube, and dissolved ultrasonically in 10 ml PBS for 4 h. Purified plasma membrane was obtained by gradient centrifugation. The obtained mixture was centrifuged at $1000 \times g$ for 10 min and the supernatant was further at $15{,}000 \times g$ for 30 min. After washing the membrane fractions with PBS 3 times, the purified plasma membrane was obtained.

2.4. Dipyridamole binding to membrane

The plasma cell membrane of pancreas-1 tumor cell was incubated with 10–400 nmol/l dipyridamole for 30 min, sufficient to allow equilibrium binding to be achieved, at 37 °C in PBS solution to attain steady-state binding. After filtered through Whatman GF/B filters, the labeled membrane was washed with PBS three times and then dissolved in 10 ml of 10 mmol/l tris–HCl solution to obtain the test sample.

2.5. Procedure

Acetonitrile–water (2:1, v/v) was used to prepare the stock solution of dipyridamole. Working solutions

were prepared by further dilution with acetonitrile–water (2:1, v/v) and then adjusted to pH 9.2 with 1.0 mol/l sodium tetraborate buffer solution. These solutions were kept in dark bottles immediately after preparation, and were stable for ≥ 1 month.

Cells (1×10^7) were used for the preparation of plasma membrane and then the binding of dipyridamole. After ultrasonic lysis of the plasma membrane bound with dipyridamole with 10 mmol/l tris–HCl, 1.0 ml of the obtained test sample was added to 9.0 ml acetonitrile–water (2:1, v/v) solution and adjusted to pH 9.2 with 1.0 mol/l sodium tetraborate buffer solution. The fluorescence intensity of labeled dipyridamole was immediately determined at 20 °C. This work set both excitation monochromatic slit width and emission monochromatic slit width at 10 nm, the voltage of photomultiplier detector tube at medium position (600 V). All these optimal instrumental parameters were kept constant except for special declaration.

3. Results and discussion

3.1. Spectral characteristics of dipyridamole and effects of pH, laying time and temperature

Fig. 1 shows the fluorescence spectrum of dipyridamole in acetonitrile–water (2:1, v/v) at pH 9.2

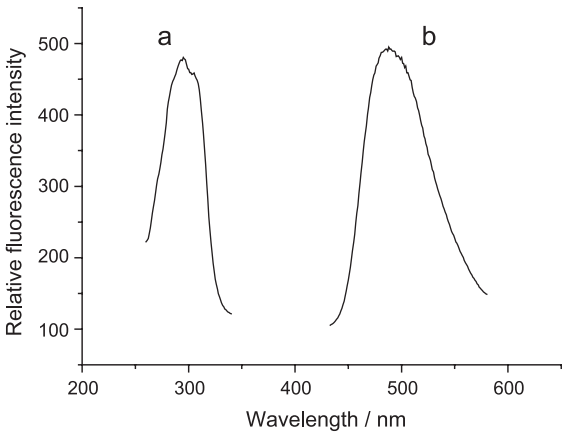

Fig. 1. Florescence excitation (a) and emission (b) spectra of 3.0×10^{-11} mol/l dipyridamole in pH 9.2 acetonitrile–water (2:1, v/v) solution.

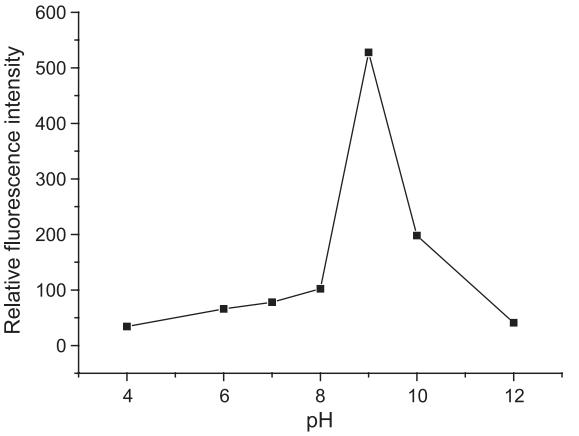

Fig. 2. Effect of pH on fluorescence intensity of 3.0×10^{-11} mol/l dipyridamole in pH 9.2 acetonitrile–water (2:1, v/v) solution.

sodium tetraborate buffer solution. The excitation and emission peaks of dipyridamole in acetonitrile–water were 295 and 485 nm, respectively. The fluorescence intensity of dipyridamole was affected by the pH of sample solution. The structure of dipyridamole includes three different nitrogen atoms in the aliphatic constituents, diethanolamine and piperidine groups. The aromatic pyrimido-pyrimidine nucleuses are responsible for the transition of the dipyridamole molecules from protonated to nonprotonated. In conjugated system, a binding of single proton promotes effective deexcitation that decreases sharply the fluorescence emission and affects drastically the optical absorption. Thus, dipyridamole emits weak fluorescence signal in strong acidic solution. While in strong basic solution, ring opening of dipyridamole may occur and the fluorescence signal is also weak. The influence of pH on the fluorescence intensity of dipyridamole was studied by adding different amounts of HCl and NaOH. As shown in Fig. 2, dipyridamole produced intense fluorescence in weak alkali solution. The optimum pH for analysis of dipyridamole was at pH 9.2.

The stability of dipyridamole was examined in a range of laying time between 0 to 60 min. With an increasing laying time, the fluorescence intensity of dipyridamole decreased by only 1.6% per 10 min. Therefore, the effect of laying time could be neglected when the data was immediately collected in the experimental procedure.

Generally, an increase in temperature results in the decreases of both the fluorescence quantum yield and the lifetime [18]. The fluorescence intensity of dipyridamole decreased by an average value of 2.0% per 10 °C between 0 and 60 °C. In this work, the determination temperature was set at 20 °C.

3.2. Calibration curve and sensitivity for fluorescence determination of dipyridamole

Under the optimal conditions, a calibration graph was obtained by plotting the fluorescence intensity of dipyridamole vs. dipyridamole concentration. In the concentration range of 1.0×10^{-12} to 5.0×10^{-11} mol/l, the plot showed a linearity ($r = 0.997$). The calibration equation was $F = 1.37 + 17.3C$, where F was the relative fluorescence intensity and C was the concentration ($\times 10^{-12}$ mol/l) of dipyridamole. The limit of detection was 2.8×10^{-13} mol/l (1.4×10^{-4} ng/ml) at 3σ (σ was the standard deviation of the blank), indicating a very high sensitivity at which the amount of dipyridample bound on cell membrane can be determined.

The reasons for obtaining such a high sensitivity were attributed to both the structure of dipyridamole and the experimental conditions. The aromatic structure and pi-system were the responsible of the high absorption and fluorescence. The sensitivity was also related to the experimental conditions, such as solvent, pH value, temperature and external heavy atoms. The fluorescence quantum yield was about 100% for the nonprotonated form. Briefly, we chose acetonitrile–water (2:1, v/v) as that solvent, pH at 9.2 and temperature at 20 °C which were the optimum conditions for obtaining high fluorescence intensity. Extra care was to avoid introducing external heavy atoms that would seriously quench fluorescence of the analyte.

3.3. Determination of dipyridamole bound on cell membrane

The dipyridamole bound on cell membrane showed the same fluorescent spectrum as that of its free state in solution after ultrasonic lysis of the cell membrane with 10 mmol/l tris–HCl. The fluorescence signal of the unlabeled membrane sample was <5.0, which was the same as that of the working solution in absence of dipyridamole, indicating no interference was observed in the sample solution. The effect of incubation time on the fluorescence intensity of the bound dipyridamole was showed in Fig. 3. With the increasing incubation time, the fluorescence intensity increased and then tended to a maximum value at 30 min, which was chosen as an optimal incubation time. After the cell membrane was incubated in PBS solution containing dipyridamole at 37 °C for 30 min and the dipyridamole bound cell membrane was ultrasonically dissolved with 10 mmol/l tris–HCl, the fluorescence intensity of the bound dipyridamole could be used for determinations of the dipyridamole bound on cell membrane and the number of binding sites on cell surface. The sample obtained from the cell membrane of 1×10^7 cells incubated in 200 nmol/l dipyridamole solution displayed a fluorescence intensity of 493 ± 41 ($n = 8$). Thus, the concentration of dipyridamole bound on the membrane was calculated to be $2.9(\pm 0.2) \times 10^{-11}$ mol/l according to the calibration equation. Consequently, at such a concentration of dipyridamole, the bound sites on the membrane of one Pancreas-1 tumor cell were deduced to be $1.7(\pm 0.1) \times 10^5$.

In order to investigate the analytical performance of this method, the recovery of dipyridamole in cell membrane sample solution was determined. Table 1 shows the recoveries obtained at three concentrations of dipyridamole. The recovery was >95%. Thus, the

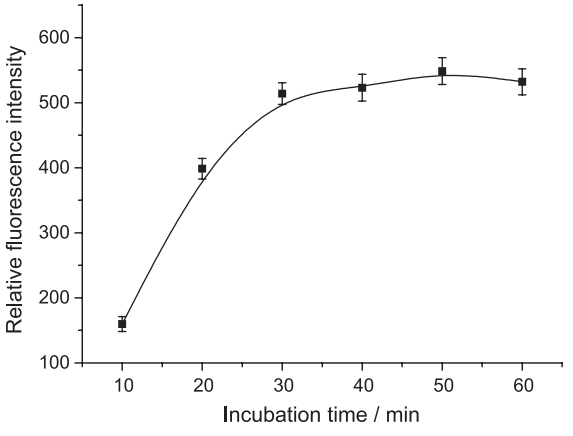

Fig. 3. Plot of fluorescence intensity of bound dipyridamole vs. incubation time of pancreas-1 tumor cell membrane in PBS solution containing 200 nmol/l dipyridamole.

Table 1
Recoveries of dipyridamole in cell membrane sample solution

Dipyridamole added (10^{-11} mol/l)	Dipyridamole found (10^{-11} mol/l)	Recoveries (%)
0.50	0.48 ± 0.05[a]	96 ± 10
1.0	0.97 ± 0.08	97 ± 8
4.0	3.8 ± 0.3	95 ± 8

[a] Standard deviation (six determinations).

lysis efficiency of cell membrane in tris–HCl solution was high, and this method was reliable for the measurement of the binding sites of dipyridamole on pancreas-1 tumor cell membrane.

3.4. Binding characteristics of dipyridamole on pancreas-1 tumor cell membrane

To investigate the binding characteristics of dipyridamole with pancreas-1 tumor cell membrane, six different concentrations of dipyridamole were used to incubate with cell membrane at 37 °C in PBS solutions for 30 min. Fig. 4 shows the dependence of the fluorescence intensity of the dipyridamole bound on the cell membrane on the concentration of dipyridamole used for incubation. The fluorescence intensity of the bound dipyridamole increased sharply with the increasing concentration of dipyridamole from 10 to 200 nmol/l. Afterwards the fluorescence intensity trended towards an equilibrium value, indicating a saturated binding of dipyridamole to the pancreas-1 tumor cell membrane.

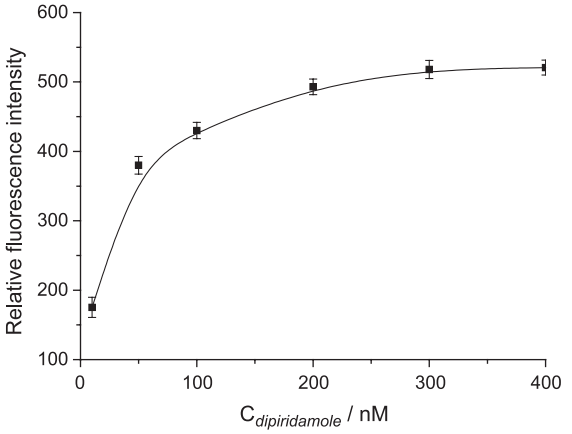

Fig. 4. Plot of fluorescence intensity of bound dipyridamole vs. concentration of dipyridamole used in incubation process.

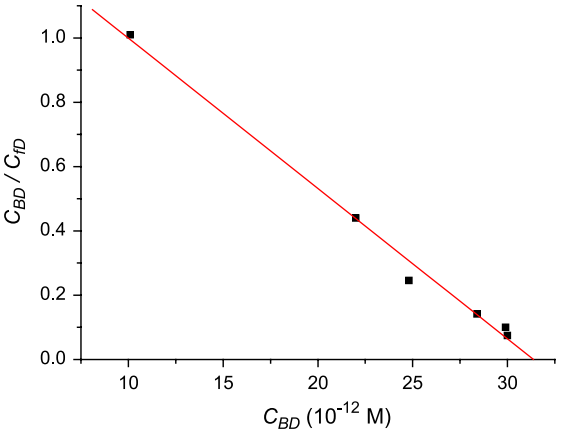

Fig. 5. Data analysis of fluorescence intensity of bound dipyridamole.

The binding reaction of dipyridamole to pancreas-1 tumor cell membrane can be described as Eq. (1).

$$B + D = BD, \quad (1)$$

where B is the binding site on pancreas-1 tumor cell membrane, D is dipyridamole and BD is the complex of B and D. When the reaction reaches equilibrium, the Eq. (2) is abided.

$$C_{BD}/C_{fD} = K_A(C_B - C_{BD}), \quad (2)$$

where C_{BD} is the concentration of dipyridamole bound to the binding site on pancreas-1 tumor cell membrane, C_{fD} is the free concentration of dipyridamole in incubation solution, K_A is the equilibrium constant of the complex reaction and C_B is the concentration of total binding sites on pancreas-1 tumor cell membrane. Fig. 5 shows the relationship between C_{BD}/C_{fD} and C_{BD} when the membrane of 1×10^7 cells was incubated in different concentrations of dipyridamole ranging from 10 to 400 nmol/l at 37 °C. From the slope and intercept, K_A and C_B can be obtained. The value of K_A was $4.7(\pm 0.5) \times 10^{10}$ l/mol. The average total number of binding sites on a pancreas-1 tumor cell was calculated to be $1.9\ (\pm 0.2) \times 10^6$.

4. Conclusions

We present a new method for the determination of dipyridamole bound to pancreas -1 tumor cell mem-

brane. The conjugation of dipyridamole on cell membrane is studied. Based on the proposed method, the binding sites of dipyridamole on pancreas-1 tumor cell membrane and the affinity constant of the cell membrane to dipyridamole are determined to be 1.9 ($\pm$ 0.2) $\times 10^6$ and 4.7($\pm$ 0.5) $\times 10^{10}$ l/mol, respectively. Compared to traditional radioactivity assay, this method is simple, rapid and harmless. It is significant for further study on the inhibition of nucleosides transfer.

Acknowledgements

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