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A simple and selective fluorometric assay for dopamine using a calcein blue–Fe^{2+} complex fluorophore

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ABSTRACT

A novel fluorimetric assay for dopamine using calcein blue (CB) complexed with Fe^{2+} ion as a chemical sensor is described. The fluorescence arising from CB of the CB–Fe^{2+} complex is quenched by the Fe^{2+} ion. When dopamine is added to a solution of the CB–Fe^{2+} complex, a dopamine–Fe^{2+} complex is formed as the result of a ligand exchange reaction between CB and dopamine which permits the fluorescence from CB to be recovered. The fluorescence intensity at the wavelength of 440 nm (at the excitation wavelength of 340 nm) was found to be proportional to the concentration of the dopamine added to the CB–Fe^{2+} complex solution, which permits dopamine to be quantitatively determined. The selectivity for dopamine in the presence of other catecholamines and related compounds was good. The calibration curve for dopamine, determined using experimental data was successfully simulated based on the equilibrium of the ligand exchange reaction between CB and dopamine. The working range is from 50 μM to 1 mM and the limit of detection and limit of quantization are ca 10 μM and 50 μM, respectively. The assay is simple and economical, compared with conventional methods such as an enzyme-linked immunosorbent assay (ELISA).

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1. Introduction

Dopamine, a member of the catecholamine class of compounds, plays a crucial role as a neurotransmitter in normal homeostasis [1]. The level of dopamine in the human brain is an important parameter in various diseases. For example, dopamine is one of the markers used in the diagnosis of a number of diseases related to neurotransmitters, including Parkinson's disease. Because of its importance, rapid method for its selective determination would be highly desirable [2,3]. In neurochemical studies involving the central nervous system, methods for the determination of distribution and dynamics of dopamine at the tissue level in and living cells is also required. In such cases, fluorescence probes for dopamine would be expected to be useful because it would be possible to visualize the distribution and dynamics of dopamine in vivo and in real time.

There are a number of methods currently available for the determination of dopamine, including high-performance liquid chromatographic analysis with derivatization reagents, an enzyme-linked immunosorbent assay and, electrochemical and spectroscopic assays [4–9]. For example, with concerning to the fluorometric determinations of dopamine, including chatecholamines with the similar chemical structure to dopamine, Feming et al. have reported the simultaneous fluorometric determination of dopamine, norepinephyrine and serotonin in brain tissues by using the native fluorescence properties of the analytes [10]. Wang et al. have reported the fluorometric determination of dopamine with use of enhancement of its native fluorescence property by using ethanol as a sensitization reagent of flurorescence intensity [11]. However the both methods are not selective so that a separation technique such as ion exchange separation and a thin layer chromatographic separation was utilized for the selective determination of dopamine. While fluorescence quenching phenomena due to a reaction product of tyrosine with hydrogen peroxide in the presence of horseradish peroxidase [12] and due to a reaction with holes generated from quantum dot [13] have been applied to the selective determination of dopamine. In addition fluorometric determination methods by using derivatization reactions such as ethylenediamine after oxidation by mercury(II) nitrate [14] and 1,2-diphenylethylendiamine [15] have been proposed. These fluorometric derivatization techniques have been applied to simultaneous determination of dopamine including chatecholamines with use of separation techniques such as HPLC [16,17]. These assays have mainly been employed for in vitro measurements but monitoring dopamine in vivo has been capable by using both a capillary electrophoresis and a microdialysis technique [18,19].

Abbreviations: CB, calcein blue; Cys, cysteine; L-DOPA, 3-(3,4-dihydroxyphenyl)-L-alanine; DOPAC3,4-, dihydroxyphenylacetic acid; GABA4-, aminobutyric acid; GluL-, glutamic acid; Gly, glycine; HisDL-, histidine; HVA, homovanilic acid; 3-MT3-, methoxytyramine; TyrL-, tyrosine.

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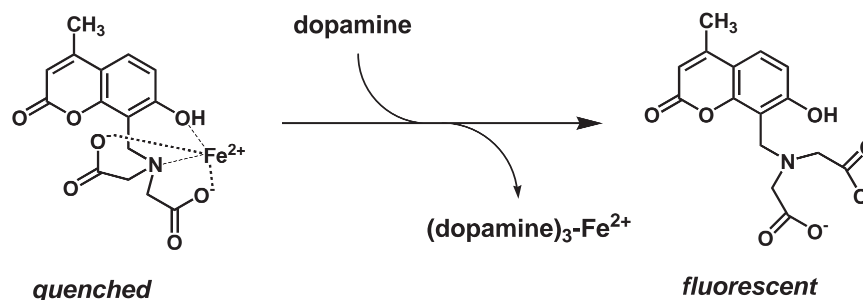

Fig. 1. Scheme for the fluorescence detection for dopamine.

On the other hand, a fluorescent assay using a probe that specifically recognizes dopamine, would be expected to be very promising for real time, in vivo monitoring. In fact, fluorescent supramolecular probes have been considered for use in in vivo measurements of dopamine. For example, several chemical probes constructed from phenylboronic acid derivatives for the detection of dopamine and analogs thereof have been reported [20–22]. Although probes containing a boronic acid moiety exhibit a selective response to dopamine via the specific recognition of its diol group, they are subject to considerable interference from analogs of dopamine that also contain a diol group.

We recently reported on some fluorescent assays that utilize a chemosensor for biogenic substances such as histamine based on a ligand exchange mechanism. In these assays, the fluorescence probes consist of a fluorescent dye moiety and a metal-ligand moiety, the latter of which recognizes the target molecule [23,24]. The probes show fluorescence when the fluorescent dye moiety is in a free form, and not complexed with a metal ion but when the target molecules cause the metal ion to dissociate from the metal-ligand moiety by a ligand exchange reaction, fluorescence occurs.

In this paper, we propose a new assay for dopamine that involves the use of a fluorescent probe in which a Fe^{2+} ion is complexed with calcein blue (CB). CB, a fluorescent dye, is a coumarin derivative that contains an iminodiacetic acid structure [25–27]. Fig. 1 shows the scheme for the detection of dopamine based on the ligand exchange mechanism using the CB–Fe^{2+} complex. The fluorescence of CB is quenched by the Fe^{2+} ion in the CB–Fe^{2+} complex. When dopamine is added to the solution of the CB–Fe^{2+} complex, a dopamine–Fe^{2+} complex is formed and the Fe^{2+} ion is released from the CB–Fe^{2+} complex due to a ligand exchange reaction. The uncomplexed CB now undergoes fluorescence. It is noteworthy that that the CB–Fe^{2+} complex had an excellent selectivity for dopamine over other catecholamines and related compounds.

2. Experimental

2.1. Apparatus

UV–vis spectra and fluorescence spectra were obtained with a UV–vis spectrophotometer (V-560, JASCO) and fluorescence spectrophotometers (F-7000, Hitachi and RF-5300, Shimadzu), respectively. A microtiter plate reader (ARVO SX, PerkinElmer) was used for measuring the fluorescence response of the CB–Fe^{2+} complex for dopamine and other catecholamines and related compounds.

2.2. Reagents

Calcein blue (CB) was purchased from Dojindo Laboratories Co. (Kumamoto, Japan). Cadaverine dihydrochloride, L-cysteine (Cys), dopamine hydrochloride, 4-aminobutyric acid (GABA), L-glutamic acid (Glu), glutathione (reduced form), glycine (Gly), histamine, DL-histidine (His), 1,4-butanediammonium dichloride (putrescine), 5-hydroxytryptamine (serotonin), 3-(3,4-dihydroxyphenyl)-L-alanine (L-DOPA), 3,4-dihydroxyphenylacetic acid (DOPAC), L-adrenaline, homovanilic acid (HVA), L-(-)-phenylalanine, 4-hydroxyphenethylamine hydrochloride (*p*-tyramine) and L-tyrosine (Tyr) were obtained from Wako Pure Chemical Co. (Osaka, Japan). 3-Hydroxyphenethylamine hydrochloride (*m*-tyramine) and 3-methoxytyramine hydrochloride (3-MT) were supplied by Oakwood Products, Inc. (South Carolina, USA) and Sigma Aldrich Co. (St. Louis, USA), respectively. L-Noradrenaline bitartrate was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). Other reagents and solvents were purchased from Kishida Chemical Co. (Osaka, Japan), and used without further purification unless indicated otherwise.

2.3. Preparation of solutions

A 5 mM calcein blue (CB) stock solution was prepared by dissolving CB in a 0.1 M KOH solution, followed by neutralization with a 0.1 N HCl solution. CB–metal ion complex (CB–M^{2+}; $M^{2+} = Co^{2+}$, Cu^{2+}, Fe^{2+}, Ni^{2+}) solutions were prepared by mixing equimolar concentrations of the stock solution of CB and MCl_2 in Dulbecco's phosphate-buffered saline (DPBS). 100 mM of the stock solutions of L-DOPA and adrenaline were prepared by dissolving their hydrochloric acid salts in a 0.1 N HCl solution. A 50 mM stock solution of Tyr and a 100 mM stock solution of HVA were prepared with a 0.1 N HCl solution and ethanol, respectively. Stock solutions of the other amines were prepared as 100 mM solutions in deionized water. All sample solutions of amines were prepared by dilution with DPBS at appropriate concentrations from the above stock solutions. A dopamine solution and other amine solutions were added to the CB–metal ion complex solution and fluorescence and excitation spectra were taken for the resulting mixed solutions by using the fluorescence spectrophotometers.

2.4. Determination of dopamine contents in pharmaceutical injection

Commercial dopamine injections, "Dopamine hydrochloride Inj Kit 100", which contains 100 mg of dopamine hydrochloride and 10 g glucose and 60 mg of $NaHSO_3$ in 5 mL of a solution, and "Kakodin injection 100 mg", which contains 100 mg of dopamine hydrochloride and 2.5 mg of $NaHSO_3$ in 5 mL of a solution, were purchased from I'rom Pharmaceutical Co. Ltd (Tokyo, Japan) and Takeda Pharmaceutical Co. Ltd. (Osaka, Japan), respectively. The commercial injections were diluted to 1/50–1/15 with deionized water and 10 μL aliquot of the each diluted injection sample was added to 990 μL aliquot of a 0.5 μM CB–Fe(II) complex solution in 0.1 M DPBS buffer solution. The fluorescence intensity of the resulting mixed solution at 430 nm was measured with the fluorescence spectrophotometer, where the excitation wavelength was set at 330 nm. The 105 mM stock dopamine solution was prepared by

dissolving 0.0995 g of dopamine hydrochrode and 2.7 g of $NaHSO_3$ with deionized water and mess-up to a 5.0 mL-volumetric flask. The series of standard solutions (0–14 mM) were prepared by diluting the stock dopamine solution. The calibration curve for dopamine was obtained by mixing 10 μL aliquot of the standard solution with 990 μL aliquot of the 0.5 mM CB–Fe(II) complex solution and by measurement of fluorescence intensity at the wavelength of 430 nm. The validation of the proposed method was conducted by measurement of the dopamine content of the injection samples according to the spectrophotometic method recommended by Japanese pharmacopoeia [28]. The calibration curve was obtained for a series of standard dopamine solution (0–500 μM) prepared from the stock solution by measurement of absorbance at the wavelength of 280 nm. The contents of dopamine in the injection samples were obtained from the apparent molar absorptivity coefficient of the diluted injection samples (5–10 times dilution) by comparing the molar absorptivity coefficient of the standard solutions.

3. Results and discussion

3.1. Fluorescence response of CB–metal complexes to dopamine and other amines

Fig. 2 shows excitation and fluorescence spectra of solutions of CB and various metal complexes (CB–M^{2+}, M^{2+}: Co^{2+}, Cu^{2+}, Fe^{2+}, Ni^{2+}), which were prepared by mixing equimolar amounts of a CB solution and each of the metal solutions. As can be seen from Fig. 2, the light intensities for the excitation and fluorescence of the CB–M^{2+} complex are much lower than those of CB, which is due to the quenching effect by metal ions [26]. Cu^{2+}, Fe^{2+} and Ni^{2+} are particularly strong quenchers to the fluorescence of CB. As described in the introduction section, since the proposed assay is based on a ligand exchange mechanism, the fluorescence intensity depends on the amount of metal ions that can be dissociated from the CB–M^{2+} complex by dopamine. The selectivities of the CB–M^{2+} complex for dopamine against various amines were examined by adding a dopamine solution as well as solutions of various amines to the CB–M^{2+} complex solution. The chemical structures of the amines examined in this study are shown in Fig. 3(A). Fig. 3(B) shows the fluorescence intensity of the CB–M^{2+} solution at a wavelength of 460 nm, as measured using a microtiter plate reader, when an amine solution was added to the solution of the CB–M^{2+} complex. The fluorescence intensity of the CB–Co^{2+} and the CB–Ni^{2+} complexes increases by about 3 to 10-fold after the addition of Cys, histamine or His, compared with the initial intensity. The fluorescence intensity of the CB–Cu^{2+} complex also increases upon the addition of not only Cys, histamine or His but also dopamine, Glu, glutathione or Gly. The CB–Cu^{2+} complex shows relatively higher fluorescence responses to His, but does not have an outstanding selectivity for dopamine, among the other amines. In contrast, the CB–Fe^{2+} complex showed a selective fluorescence response to dopamine. The fluorescence intensity of the CB–Fe^{2+} complex increased by 12 fold, when dopamine was added to the CB–Fe^{2+} solution, compared with the fluorescence intensity before the addition. When the other amines were added to the CB–Fe^{2+} complex solution, the fluorescence intensities remained nearly the same as the initial value. The difference in fluorescence responses among the CB–metal complexes to various amines appear to be dependent on the metal ion being used. The CB–Fe^{2+} complex exhibited a remarkably high selectivity to dopamine among the amines examined in this study. Thus, we selected the CB–Fe^{2+} complex as the most promising probe for the dopamine assay and conducted the following experiments using the CB–Fe^{2+} complex.

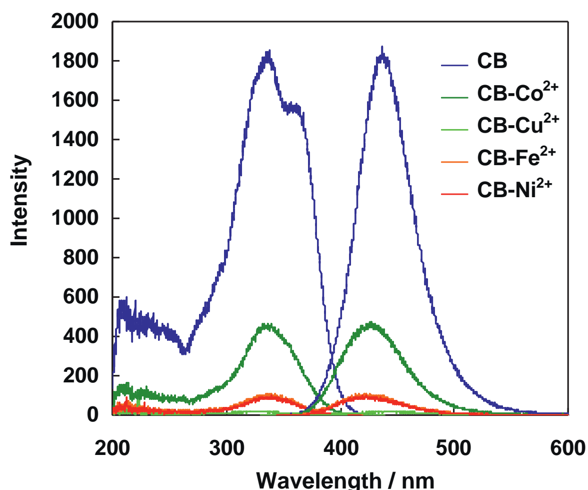

Fig. 2. Excitation and emission spectra of CB–M^{2+} (M^{2+} = Co^{2+}, Cu^{2+}, Fe^{2+}, Ni^{2+}) complex solutions (5 μM) in DPBS.

In order to confirm the stoichiometry of the CB–Fe^{2+} complex, fluorescence intensity of a series of 0.5 μM CB solutions containing Fe^{2+} at various concentrations (0–1.5 μM) at the wavelength of 440 nm was measured. The relationship between the concentration ratio of Fe^{2+} to CB and the relative fluorescence intensity normalized by the fluorescence intensity of the CB solution without Fe^{2+} is shown in Supplementary Fig. 1. As can be seen from Supplementary Fig. 1, a break point is clearly observed at the concentration ratio of unity, indicating that the stoichiometry of the CB–Fe^{2+} complex is 1:1 for CB and Fe^{2+}. Since Fe^{2+} is known to be easily oxidized to Fe^{3+}, the quenching effect by Fe^{3+} is evaluated and the relationship between the concentration ratio of Fe^{3+} to CB is also examined in the same manner to the case of Fe^{2+}. As can be seen from Supplementary Fig. 2, the quenching effect by Fe^{3+} is much smaller than by Fe^{2+} and the fluorescence intensity is gradually decrease with increase in the concentration of Fe^{3+}, as shown in Supplementary Fig. 1. This indicates that oxidation valence of an iron ion, which initially prepared the CB–Fe^{2+} complex, can be kept as the initial oxidation valence of Fe^{2+}.

In addition, in order to support that the present response mechanism is the ligand-exchange mechanism, we have confirmed that the fluorescence spectrum of the CB–Fe^{2+} complex, where the fluorescence of CB is quenched by Fe^{2+}, is returned nearly to the initial fluorescence spectrum of CB with somewhat shift of maximum wavelength, when an EDTA solution is added to the CB–Fe^{2+} complex solution. The fluorescence spectra of the CB–Fe^{2+} complex solution before and after an addition of an EDTA solution, together with the fluorescence spectrum of CB solution are shown in Supplementary Fig. 3.

3.2. Selective fluorescence response of the CB–Fe^{2+} complex to dopamine

In order to evaluate the selectivity of the CB–Fe^{2+} complex for dopamine compared to other catecholamines and related compounds (Fig. 4(A)) that can be present in biological samples, the fluorescence response of the CB–Fe^{2+} complex was recorded after adding a variety of compounds. The results are shown in Fig. 4(B) together with the chemical structures of the compounds. As can be seen from Fig. 4(B), interestingly, a remarkable enhancement in the fluorescence intensity of the CB–Fe^{2+} complex is observed only when a solution of dopamine is added to the CB–Fe^{2+} complex. Adrenaline and noradrenaline showed 4.8 fold and 2.4 fold increases in fluorescence intensity, respectively, compared with the initial fluorescence intensity of the CB–Fe^{2+} complex. However, the addition of other compounds did not cause a significant change in

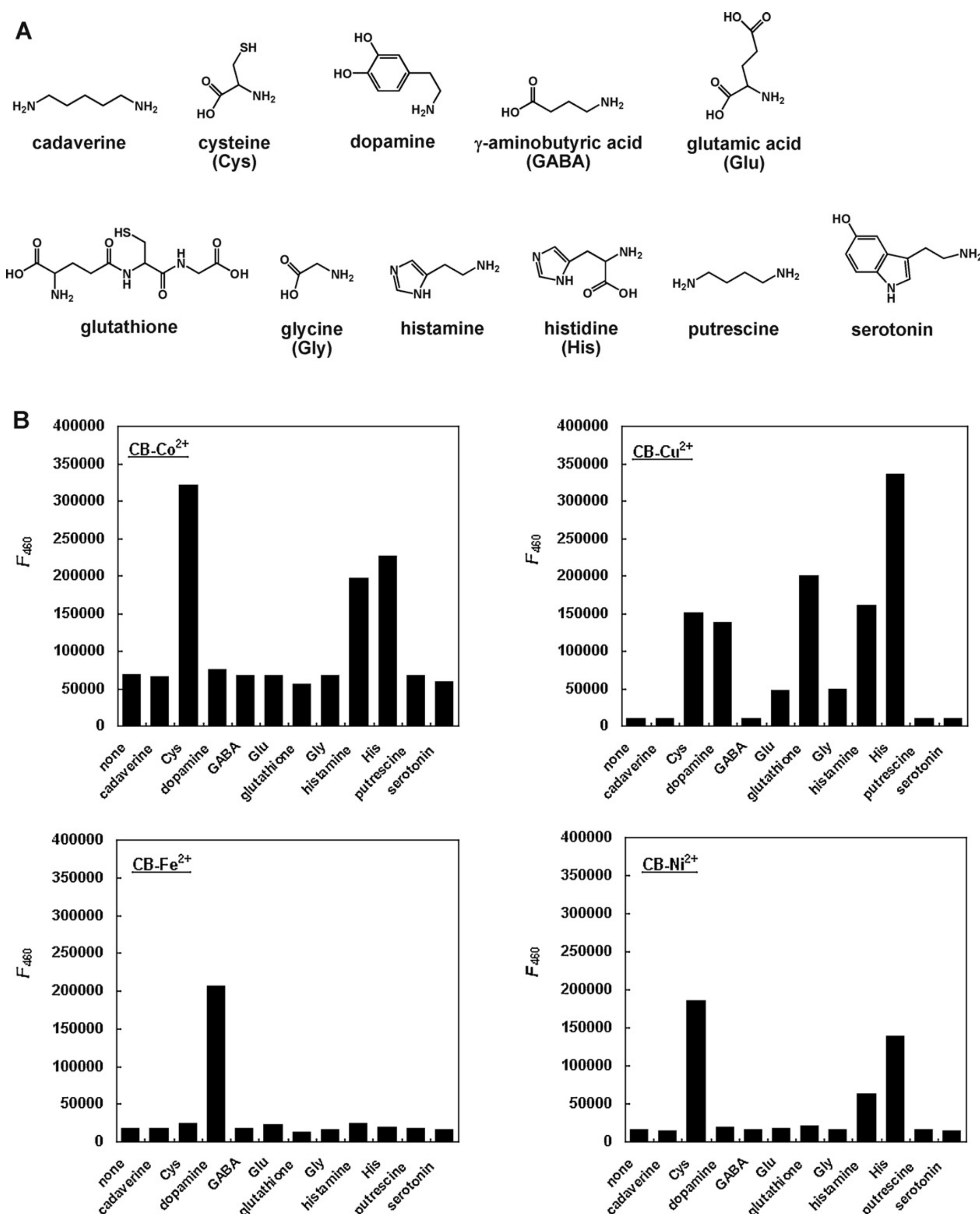

Fig. 3. Fluorescence response of CB–M^{2+} complexes to various amines. (A) Structures of the biogenic amines tested. (B) Fluorescence intensity of a CB–M^{2+} solution (5 μM) after the addition of several amines (3 mM) in DPBS. The concentrations in parenthesis are the final values after mixing the two solutions. The wavelength of excitation was set at 355 nm.

the fluorescence intensity. These results indicate that the CB–Fe^{2+} complex can be used to detect dopamine with a high degree of selectivity in the presence of catecholamines and related compounds. As described previously, the response mechanism of the present assay is based on a ligand exchange mechanism between CB and dopamine, as shown in Fig. 1. This means that the selectivity of the CB–Fe^{2+} complex for dopamine against other catecholamines and related compounds is likely dependent on difference in the formation constant of the dopamine–Fe^{2+} complex and that of the Fe^{2+}, when complexed with other compounds. It is interesting that the CB–Fe^{2+} complex showed a highly selective response to dopamine among the catecholamines examined, which have a similar chemical structure consisting of a catechol moiety and an alkyl amine moiety. Indeed, adrenaline and noradrenaline, whose structures are the most similar to dopamine, show a somewhat high fluorescence response to the CB–Fe^{2+} complex by ca 40% and

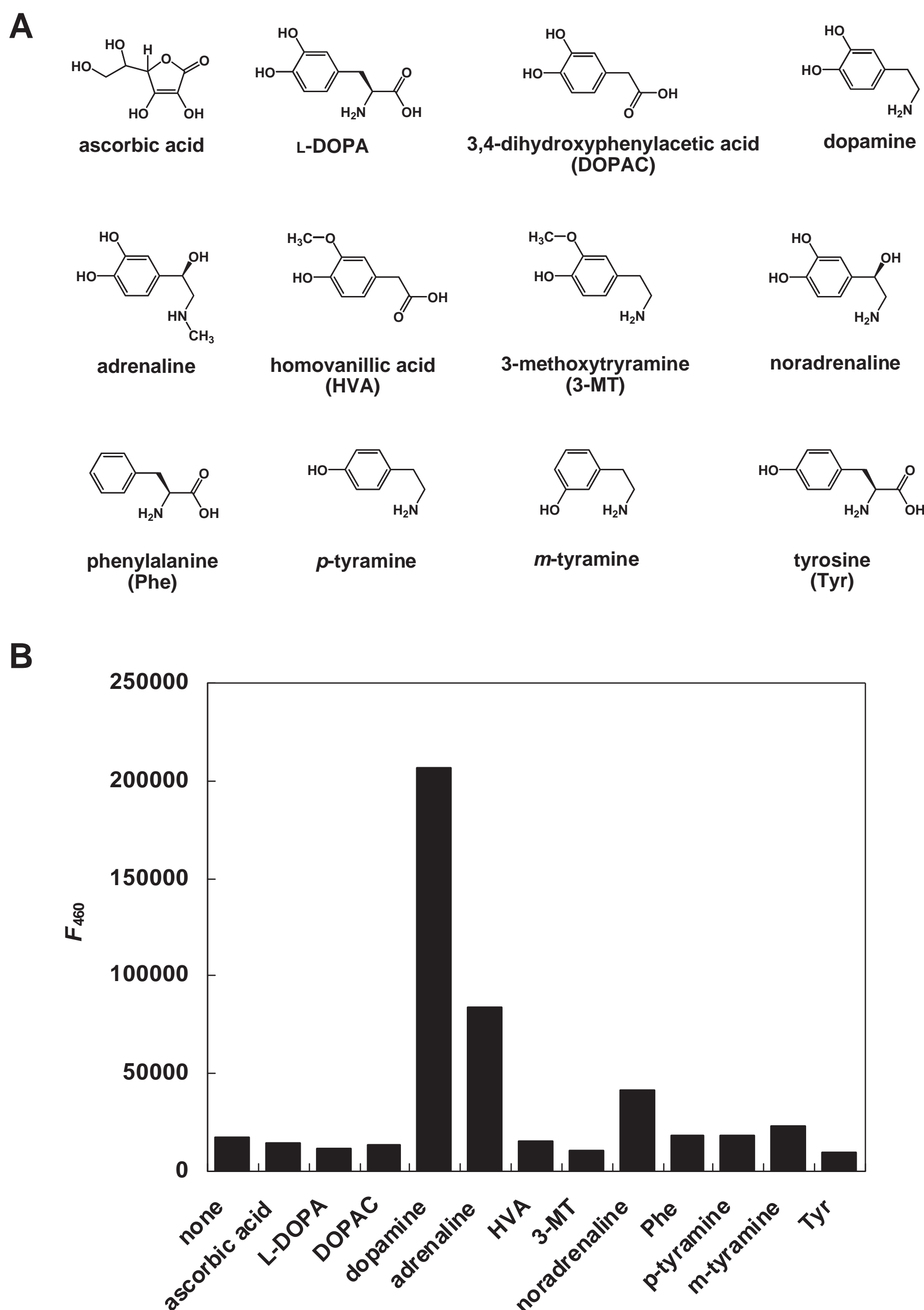

Fig. 4. Selectivity of the CB–Fe^{2+} complex for dopamine. (A) Chemical structures of the catecholamines and related compounds used in this study. (B) Fluorescence response of the CB–Fe^{2+} complex (5 μM) at a wavelength of 460 nm to various catecholamines and related compounds (3 mM) in DPBS. The intensities were obtained 10 h after the addition of the sample solutions to the CB–Fe^{2+} complex solution. The wavelength of excitation was set at 355 nm.

ca 20%, respectively, compared with the fluorescence intensity for dopamine. While the responses of the CB–Fe^{2+} complex to L-DOPA and DOPAC, which contain a catechol moiety but a carboxyl moiety instead of an alkyl amine, are very small and almost the same as the initial fluorescence intensity, as shown in Fig. 4(B). This indicates that the carboxyl moiety clearly does not contribute to the formation of Fe^{2+} complexes with L-DOPA and DOPAC by preventing the Fe^{2+} ion from forming a CB–Fe^{2+} complex. The difference in the chemical structure of 3-MT from dopamine is the fact that a hydroxyl group, at the 3rd position of dopamine is substituted with a methoxy group. The much larger response of the CB–Fe^{2+} complex to dopamine than to 3-MT suggests that the catechol moiety shows a strong affinity for forming a dopamine–Fe^{2+} complex. Indeed, *p*-tyramine and *m*-tyramine, whose chemical structures are similar to dopamine except for the catechol moiety, namely, that both compounds have a mono-hydroxyl group on the benzene ring, show a very small fluorescence response, as shown in Fig. 4(B). Therefore, complex formation between dopamine and the Fe^{2+} ion was

examined in the following section, including a theoretical simulation of the experimental results based on the complex formation equilibrium.

3.3. Complex formation of the dopamine–Fe^{2+} complex

To verify the fact that dopamine forms a complex with the Fe^{2+} ion and prevents Fe^{2+} ions from forming a CB–Fe^{2+} complex by a ligand exchange reaction, an analysis of the coordination number of dopamine to the Fe^{2+} ion was conducted by employing UV–vis spectroscopic methods. Fig. 5(A) shows a Job's plot for complex formation between the Fe^{2+} ion and dopamine in DPBS, where the total concentration of dopamine and the Fe^{2+} ion is maintained constant at 1.0 mM and the ratio of the concentration of dopamine is varied. A break point can be seen at a mole fraction of 0.75 for dopamine, indicating that dopamine coordinates with the Fe^{2+} ion in a 3:1 stoichiometry.

In order to simulate the curve in Fig. 5(A) to estimate the stoichiometry of the dopamine–Fe^{2+} complex and its formation constant, the following chemical equilibrium of the complex formation reaction is considered. Namely, in a solution containing Fe^{2+} ions (abbreviated by M) and dopamine (abbreviated by L′), three kinds of complexes, ML′, ML'_2 and ML'_3 are assumed to be formed, as estimated from the coordination chemistry of the Fe^{2+} ion and dopamine as a bidentate ligand. Since dopamine has three dissociative protons, two phenolic protons and protonated amine group, the abbreviation of dopamine should be $L'H_3$, however, in order to simplify the equation, protons and charges of the complexes are omitted in this case. The formation reactions of ML′, ML'_2 and ML'_3 and their conditional successive complex formation constants, K_1, K_2 and K_3, are expressed by Eqs. (1)–(3).

$$M + L' \rightarrow ML'; K_1 = [ML']/[M][L'] \quad (1)$$

$$ML' + L' \rightarrow ML'_2; K_2 = [ML'_2]/[ML'][L'] \quad (2)$$

$$ML'_2 + L' \rightarrow ML'_3; K_3 = [ML'_3]/[ML'_2][L'] \quad (3)$$

where the bracket indicates the molar concentration of each chemical species.

When the initial concentrations of Fe^{2+} ions and dopamine in the solution are assumed to be expressed as $C_M{}^T$ and $C_L{}'^T$, respectively, the mass balances of the Fe^{2+} ion and dopamine are expressed by Eqs. (4) and (5), respectively.

$$C_M{}^T = [M] + [ML'] + [ML'_2] + [ML'_3] \quad (4)$$

$$C_L{}'^T = [L'] + [ML'] + 2[ML'_2] + 3[ML'_3] \quad (5)$$

When [ML′], $[ML'_2]$ and $[ML'_3]$ in Eqs. (4) and (5) are substituted by those obtained by arranging Eqs. (1)–(3), Eqs. (6) and (7) are obtained.

$$[M] = C_M{}^T/(1 + K_1[L'] + K_1K_2[L']^2 + K_1K_2K_3[L']^3) \quad (6)$$

$$C_L{}'^T = [L'] + [M](K_1[L'] + 2K_1K_2[L']^2 + 3K_1K_2K_3[L']^3) \quad (7)$$

When [M] in Eq. (7) is substituted by Eq. (6) and the resulting equation is rearranged with respect to [L′], the following quadric equation is derived.

$$K_1K_2K_3[L']^4 + (K_1K_2 + K_1K_2K_3(3C_M{}^T - C_L{}'^T))[L']^3 + (K_1 + K_1K_2(2C_M{}^T - C_L{}'^T))[L']^2 + (1 + K_1(C_M{}^T - C_L{}'^T))[L'] - C_L{}'^T = 0 \quad (8)$$

A suitable root for [L′] can be obtained by numerical calculation using Eq. (8) by assuming the appropriate conditional successive formation constants K_1, K_2 and K_3 and assuming that the values of the initial concentrations of the Fe^{2+} ion and dopamine ($C_M{}^T$ and $C_L{}'^T$) are the same as those used in the experiments in Fig. 5(A). Since the absorbance in Fig. 5(A) corresponds to the Fe^{2+}–dopamine$_3$ complex, the concentrations of ML'_3 can be calculated from Eqs. (6) and (3) by using suitable roots, [L′] obtained by numerical calculation. Fig. 5(B) shows the calculated curves for the simulation of Fig. 5(A) by plotting $[ML'_3]$ against the concentration of ratio of $C_L{}'^T$ to the total concentration of $C_L{}'^T$ and $C_M{}^T$. As can be seen from Fig. 5(B), the concentration of ML'_3 increases with an increase in the conditional formation constant K_3 and a maximum of $[ML'_3]$ clearly appears at the concentration ratio, $C_L{}'^T/(C_L{}'^T + C_M{}^T)$, of 0.75, when the conditional formation constant K_3 is larger than 10^5. The shape of the calculated calibration curve is in good agreement with the experimental result shown in Fig. 5(A). This confirms that the stoichiometry of the Fe^{2+}–dopamine complex is 1:3 for Fe^{2+} ion: dopamine. From the present simulation, the overall conditional formation constant for the Fe^{2+}–dopamine$_3$ complex, $K_1 \times K_2 \times K_3$, is estimated to be around 10^{10}–10^{11} orders of magnitude, which is larger by 10^2–10^3 fold than the conditional formation constant of the CB–Fe^{2+} complex, the value of which was estimated by our preliminary titration experiment, 4×10^8.

3.4. Calibration curve for dopamine based on the ligand exchange mechanism

As shown in Fig. 6(A), the assay method is applicable to the determination of dopamine over a wide concentrations range (6×10^{-5} M to 1×10^{-3} M) and a good linear relationship ($R^2 = 0.995$) exists between the concentration and fluorescence intensity. However, a decrease in fluorescence intensity was confirmed for dopamine in the concentration range from 0 to 6×10^{-5} M (see inset of Fig. 6(A)). This may be attributed to the quenching effect by dopamine [21]. Actually, we found that the fluorescence intensity of CB decreases in the presence of dopamine (data not shown). Similar findings have been reported based on investigations of interactions between some fluorophores and amino acids [29]. Therefore, the decrease in the florescence intensity of the CB–Fe^{2+} complex in a lower concentration range of dopamine (~ μM) can be attributed to a quenching effect caused by dopamine.

In order to evaluate the applicability of the proposed method to real samples, we measured the dopamine contents in pharmaceutical dopamine injection samples. The analytical results are listed in Table 1, together with those obtained by the spectrophotometric method recommended by the Japanese Pharmacopeia. The analytical results obtained by the proposed method are in good agreement with those obtained by the spectrophotometric method as well as the labeled values from pharmaceutical companies within 5% deviation. The stability of the CB–Fe(II) complex solution was confirmed to have initial performance at least for 3 weeks by keeping at 4 °C in a refrigerator.

For simulation of the calibration curve for dopamine obtained by the present assay, shown in Fig. 6(A), the concentration of the free form of CB, which is liberated from the CB–Fe^{2+} complex by a ligand exchange reaction with dopamine, was calculated from the following chemical equilibrium because the fluorescence intensity of Fig. 6(A) is proportional to the concentration of CB, if the presence of dopamine in the solution does not affect the fluorescence intensity of CB. When dopamine (abbreviated by L′) is added to a

Table 1
Determination of dopamine contents in pharmaceutical injection samples.

Samples	Contents of dopamine as hydrochloride in 5 mL		
	Contents in label	Proposed method	Conventional method[a]
1	100 mg	99 mg	103 mg
2	100 mg	105 mg	104 mg

[a] Spectrophotometry at the wavelength of 280 nm.

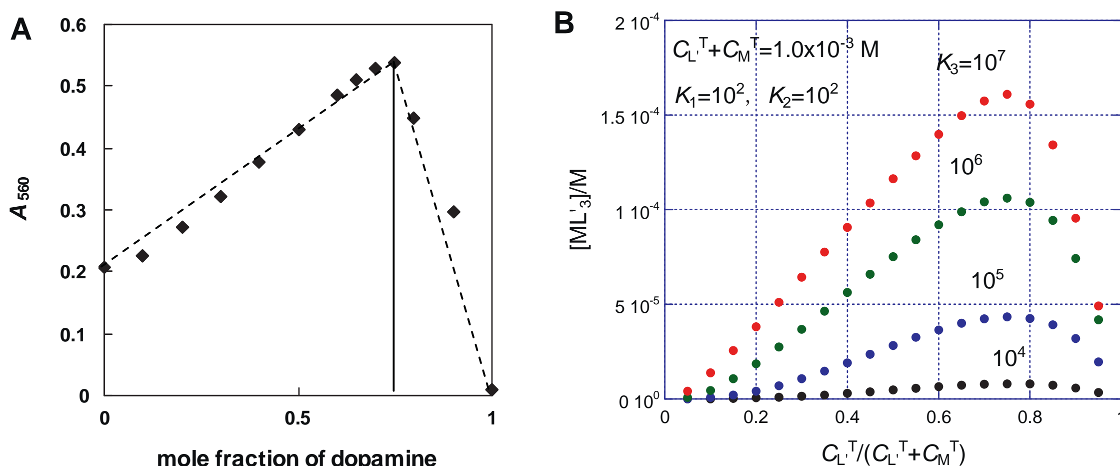

Fig. 5. (A) A Job's plot for the formation of dopamine–Fe^{2+} complex determined by UV–vis spectroscopy (Absorbance at a wavelength of 560 nm was measured. $[Fe^{2+}]$ + [dopamine] = 1 mM, DPBS). (B) Simulation of the Job's plot for the complex formation of dopamine–Fe^{2+} complex by assuming the successive formation constants, $K_1 = K_2 = 10^2$, $K_3 = 10^4$–10^7. (L′: dopamine, M: Fe^{2+}).

solution of the CB–Fe^{2+} complex (abbreviated by ML), the ligand exchange reaction between dopamine and CB (abbreviated by L) is expressed by Eq. (9) and its equilibrium constant, K, is expressed by Eq. (10).

$$ML + 3L' \rightarrow ML'_3 + L \quad (9)$$

$$K = [ML'_3][L]/[ML][L']^3 \quad (10)$$

where the bracket means the molar concentration of chemical species, the same as in Eqs. (1)–(3).

The mass balance for Fe^{2+} ions, CB and dopamine are expressed by Eqs. (11)–(12), assuming that the initial concentration of the Fe^{2+} ion, CB and dopamine are $C_M{}^T$, $C_L{}^T$ and $C_L{}'^T$, respectively.

$$C_M{}^T = [M] + [ML] + [ML'_3] = [ML] + [ML'_3] \quad (11)$$

$$C_L{}^T = [L] + [ML] \quad (12)$$

$$C_L{}'^T = [L'] + 3[ML'_3] \quad (13)$$

In this case the concentration of free Fe^{2+} ions in a solution containing the CB–Fe^{2+} complex and dopamine is assumed to be negligibly low. This assumption may be valid because the concentration of dopamine added to the CB–Fe^{2+} complex solution is much higher than the complex under the present experimental conditions.

When [ML] and $[ML'_3]$ in Eq. (10) and (13) are substituted with those obtained by arranging Eqs. (11) and (12), the following equations are derived.

$$K = 1/3(C_L{}'^T - [L'])[L]/(C_L{}^T - [L])[L']^3 \quad (14)$$

$$C_M{}^T = C_L{}^T - [L] + 1/3(C_L{}'^T - [L']) \quad (15)$$

By eliminating [L] from Eqs. (14) and (15), we obtain the following quadric equation concerned with [L′] as a parameter of K.

$$1/3K[L']^4 + K(C_L{}^T - 1/3\,C_L{}'^T)[L']^3 - 1/9[L']^2 + 2/9\,C_L{}'^T[L'] - 1/9(C_L{}'^T)^2 = 0 \quad (16)$$

A suitable root for [L′] can be obtained by numerical calculation using Eq. (16) by assuming $C_M{}^T = C_L{}^T = 5 \times 10^{-7}$ M, which are the same as the present experimental condition of Fig. 6(A). Since the fluorescence intensity in Fig. 6(A) corresponds to the concentration of CB, i.e. [L], the value of [L] for the each dopamine concentration, $C_L{}'^T$, is calculated from Eq. (15) using [L′] obtained by the numerical calculation and the initial concentrations of the CB–Fe^{2+} complex (5×10^{-7} M). The calculated calibration curve plotted [L] against

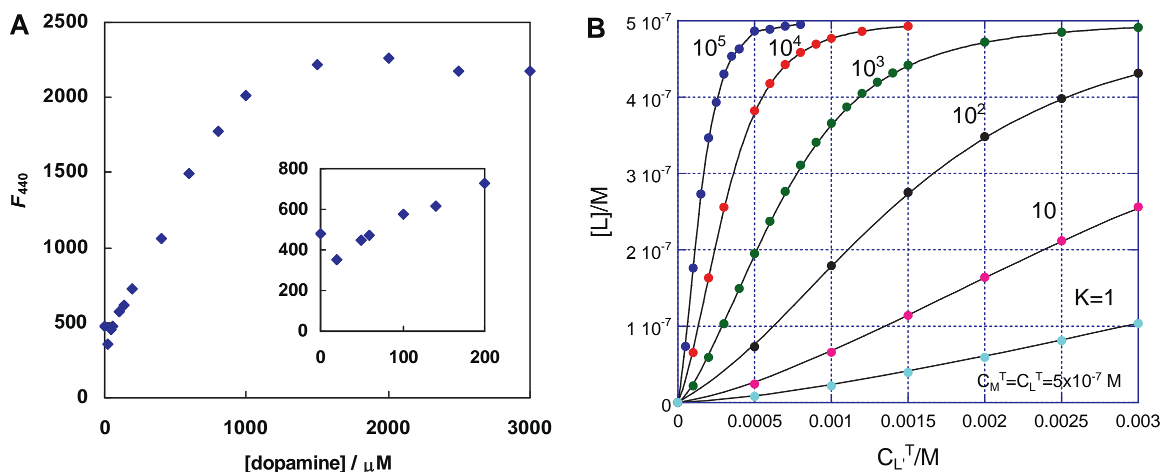

Fig. 6. (A) Fluorescence intensities of the CB–Fe^{2+} complex (0.5 μM) upon the addition of dopamine (0–3000 μM) in DPBS. The wavelength for excitation was set at 340 nm. The concentration of the CB–Fe^{2+} complex and dopamine are the final concentrations after mixing solutions of both compounds. (B) Theoretical curve for the relationship between the concentration of CB exchanged by dopamine and the concentration of dopamine added as a parameter the ligand exchange reaction constant, K, by using Eqs. (15) and (16) for simulation of the curve in Fig. 6(A). (L: CB, M: Fe^{2+}).

the concentration of dopamine, $C_L{}'^T$, is obtained as parameter of the equilibrium constant of the ligand exchange reaction, K and is shown in Fig. 6(B). As can be seen from Fig. 6(B), the concentration of CB ([L]) exchanged with dopamine is proportional to the concentration of dopamine ($C_L{}'^T$) when the equilibrium constant, K, is up to 10^3 in the concentration range of dopamine from 0 to 3 mM. In the case where the equilibrium constant is higher than 10^3, the concentration of CB exchanged with dopamine steeply increases with an increase in the lower concentration range of dopamine added to the complex solution and finally approaches the initial concentration of the CB–Fe^{2+} complex (5×10^{-7} M), which means that CB in the CB–Fe^{2+} complex almost exchanges with dopamine. By comparing the calculated calibration curve with the experimental one, the equilibrium constant of Eq. (10) is estimated to be in the order of 10^3. Since the equilibrium constant K is the ratio of the conditional formation constant of the $(dopamine)_3$–Fe^{2+} complex and that of the CB–Fe^{2+} complex, the estimated equilibrium constant of Eq. (10) may be reasonable, taking into account the fact that the conditional formation constant of the CB–Fe^{2+} complex, 4×10^8, obtained in our preliminary experiments and the conditional overall formation constant, $K_1 \times K_2 \times K_3$, of the $(dopamine)_3$–Fe^{2+} complex, 10^{10}–10^{11}, as estimated in the previous section.

4. Conclusion

In conclusion, a fluorescence assay for dopamine using a CB–Fe^{2+} complex as a fluorophore based on a ligand exchange mechanism, where the Fe^{2+} ion in the complex is deprived by dopamine, is presented. The fluorescence response of the CB–Fe^{2+} complex to dopamine was successfully simulated by equilibrium calculations based on a ligand exchange reaction. In the present assay, the CB–Fe^{2+} complex was found to be an excellent probe for dopamine among the other CB–metal complexes with respect to its selectivity to dopamine. Interestingly, the CB–Fe^{2+} complex showed a high selectivity for dopamine among the catecholamines and related compounds examined in this study. The higher selectivity of the CB–Fe^{2+} complex may be due to the fact that the conditional formation constant of a $(dopamine)_3$–Fe^{2+} complex is higher than that of the Fe^{2+} complex with other catecholamines and related compounds. Indeed the conditional formation constant of the $dopamine_3$–Fe^{2+} complex was estimated using a UV–vis spectrophotometric method as well as by a theoretical simulation. However, we were not able to quantitatively evaluate the interactions of Fe^{2+} ions with other catecholamines and related compounds and, therefore, are unable to explain the response selectivity of the CB–Fe^{2+} complex to those compounds at this stage.

The present assay is very simple, in that the procedure is involves the simple addition of a solution of the CB–Fe^{2+} complex to samples containing dopamine and then measuring the fluorescence. We have confirmed that our method is applicable for the determination of dopamine contents of pharmaceutical injections. However at this stage we have not applied the assay to the in vivo imaging of living cells, since it is not clear whether the complex can penetrate the cell membrane and enter the cell or not yet. One of great concerns is about the effect of calcium and magnesium ions, because the concentration of these ions are relatively higher in the living cells compared with the level of dopamine. Because calcium and magnesium ions form complexes with CB, as reported by several researchers [27,30]. In our preliminary investigation about such metal ions for the determination of dopamine, about ca 5% interference was observed for the determination of 250 μM dopamine in the presence of two times higher concentration of calcium and magnesium ions, as shown in Supplementary Fig. 5.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2012.02.025.

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