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# Fluorescent ternary complexes of some biogenic amines and their metabolites with europium and oxytetracycline for applications in the chemical analysis

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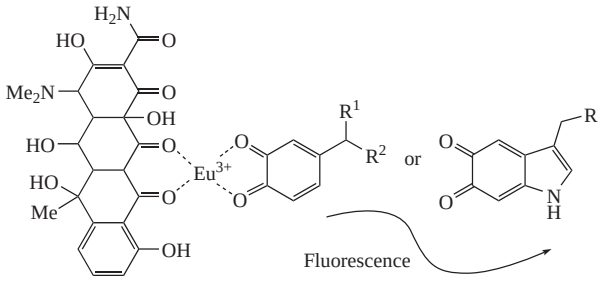
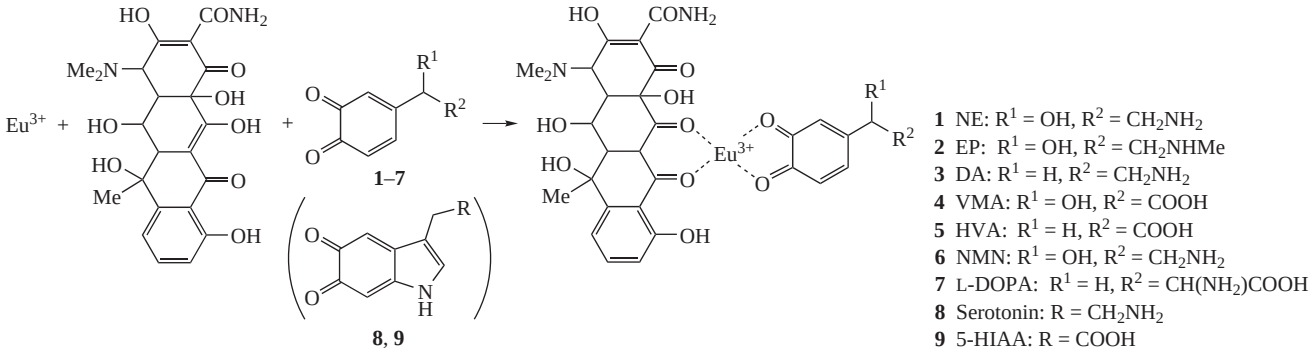
**New ternary complexes of europium with oxytetracycline possessing the intense fluorescence have been obtained for prospective applications in the high-sensitive detection of biogenic amines and their metabolites in biological fluids. Their stability constants have been determined using the Foster–Hammick–Wardley method based on the fluorescent spectroscopy data. The dependence between structure differences, stability constants of ternary complexes of europium–oxytetracycline with biogenic amines, and their metabolites, and sensitivity of their determination has been established.**




The pathogenesis of many socially important diseases is caused by processes of neuromediator exchange violation.[1–7] The need for reliable diagnostics of neuroendocrinal (neuroblastoma, pheochromocytoma, paraganglioma, *etc*.) and neurodegenerative (Alzheimer's, Parkinson's, *etc*.) diseases at early stages accounts for the interest in the development of new methods for determining neuromediator exchange markers, *viz*., biogenic amines (BA) [L-dihydroxyphenylalanine (L-DOPA), serotonin, catecholamines (CA), dopamine (DA), norepinephrine (NE), epinephrine (EP)], and their metabolites [normetanephrine (NMN), homovanillic acid (HVA), vanillylmandelic acid (VMA), and 5-hydroxyindoleacetic acid (5-HIAA)] in biological liquids at 1 pM level, which would ensure the stability of analytes in a sample during the analysis.[8–11] Fluorescent methods, particularly those based on the use of metal complex systems, are the most promising ones for reaching these goals.[12–15] BA and their metabolites weakly fluoresce in the short wavelength region of UV spectra. Since they are oxidized and decomposed into the free state, to perform the correct and reliable analysis, they should be stabilized in a sample by formation of durable compound that would manifest intense fluorescence in the long wavelength spectral region. The currently available fluorescent techniques for BA determination fail to provide the required detection levels[11,13] or do not prevent the oxidation and destruction of neuromediators during analysis in biological objects due to the analysis duration or imperfection of its conditions.[16,17] Therefore, to develop an ultra-sensitive determination of these compounds (at the level of single molecules) in biological liquids, one has to create the new indicator reactions based on compounds with intense fluorescence that improve the stability of BA and their metabolites.

In the present work, we proposed a new system based on intensely fluorescing ternary complexes of BA and their metabolites (**1**–**9**) with europium and oxytetracycline (OTC) (hereinafter, the $Eu^{3+}$–OTC–BA/metabolite ternary complex) (Scheme 1).† This is a very promising approach since it allows the fluorescence intensity of BA and their metabolites to be 200–400 fold increased in comparison with their own fluorescence and bathochromically shifts the maximum in the fluorescence spectrum, thus minimizing the interfering effect of the matrices of biological objects. To confirm the feasibility of using the developed system




**Scheme 1**

for determination of BA and their metabolites in biological liquids, it was necessary to estimate the stability of above complexes.

Therefore, the main purposes of this study were to determine the stability constants of $Eu^{3+}$–OTC–BA/metabolite complexes by means of fluorescent spectroscopy and to find the dependence between the stability constants of complexes and the sensitivity of neuromediators determination.

The absorption spectra of individual solutions of a europium salt, OTC, DA, and an $Eu^{3+}$–OTC–DA complex (Figure 1) revealed that the maxima in the spectra of single components and the complex lie at different wavelengths [$\lambda_{max}$(OTC) = 355 nm, $\lambda_{max}$(DA) = 226, 263 nm, and $\lambda_{max}$($Eu^{3+}$–OTC–DA) = 416 nm], which confirms the formation of new product, *viz*., the $Eu^{3+}$–OTC–DA complex. Similar data were also acquired on other BA and their metabolites. Taking into account the known data,[18,19] we assumed the mechanism of binding of neuromediators with the $Eu^{3+}$–OTC complex as the similar one to the binding of hydrogen peroxide or lipoproteins with this complex. The assumed structure of complexes is shown in Scheme 1. The excitation ($\lambda_{ex}$ = 416 nm) and fluorescence emission spectra ($\lambda_{em}$ = 617 nm) of the complexes obtained were also recorded. Note that the absorption and excitation spectra of all the fluorophores nearly coincide, as shown for DA as the example (Figure S1, Online Supplementary Materials). The emission spectra and the calculated relative fluorescence quantum yields do not depend on the exciting light wavelength (Table S1).[20] The observed quantum yields indicate that they do not depend on the molecular structure of BA and their metabolites.

There is a number of methods for stability constant calculation that do not require a preliminary determination of composition of the complex compound, *e.g*., the Foster–Hammick–Wardley method that we selected.[21] The constant determination was based on the model adapted for the formation of $Eu^{3+}$–OTC–BA/metabolite complex. The detection sensitivities of biogenic amines and their metabolites were differrent, while the limits of detection (LOD) for some model compounds reached ultra-low femtomolar concentrations (Table 1). This is apparently due to the different stability of $Eu^{3+}$–OTC–BA/metabolite complexes, which consequently arises from differences in the structures of analyte molecules (see Table 1) that affect the strength of their bonding with the $Eu^{3+}$–OTC complex. We assumed that the higher the determination sensitivity of a certain BA or metabolite (*i.e*., the lower the LOD), the higher the stability constant of its complex with europium and OTC. Hence, the series of decreasing stability constants should be: NMN > NE > HVA/5-HIAA > serotonin > > EP > DA/VMA > L-DOPA.

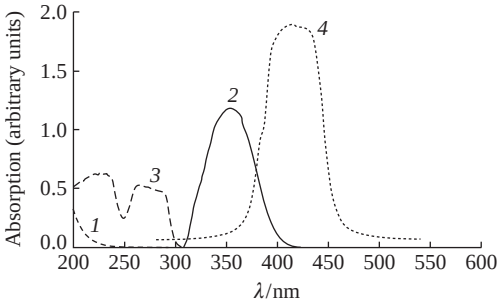



**Figure 1** Absorption spectra of solutions of (*1*) $Eu^{3+}$ salt, (*2*) OTC, (*3*) DA, and (*4*) $Eu^{3+}$–OTC–DA complex [$c$($Eu^{3+}$) = $c$(OTC) = 0.1 mM, $c$(DA) = = 1.0 nM].

† The optimal conditions for the complexation reaction and for recording the fluorescent signal of $Eu^{3+}$–OTC–BA/metabolite ternary complex in a 96-well polystyrene microplate were as follows: 0.01 M MOPS buffer solution, pH 7.5, $c$($Eu^{3+}$) = $c$(OTC) = 0.1 mM, $c$(Tween 80) = 10 mM, reaction time of 10 min, $\lambda_{ex}$ = 416 nm, and $\lambda_{em}$ = 617 nm.

**Table 1** Limits of detection (LOD) for biogenic amines and their metabolites.

| Analyzed compound | LOD/nM | Analyzed compound | LOD/nM | Analyzed compound | LOD/nM |
|---|---|---|---|---|---|
| DA | 0.03 | HVA | 0.0003 | 5-HIAA | 0.0003 |
| NE | 0.0002 | VMA | 0.04 | L-DOPA | 0.3 |
| EP | 0.02 | Serotonin | 0.001 | NMN | 0.00005 |

At the first step, the fluorescence intensity of the $Eu^{3+}$–OTC at the constant OTC concentration of 0.1 mM was measured, while the europium concentration was varied within the interval of 10–40 mM. The analysis of experimental results and data reported previously[19,22] revealed that the $Eu^{3+}$–OTC complex is formed with a metal : ligand ratio of 1 : 1.[19,22] In the reaction

$$Eu^{3+} + OTC \xrightarrow{\beta} Eu^{3+}\text{–}OTC$$

under the equilibrium conditions, the stability constant of this complex can be expressed as:

$$\beta = \frac{[Eu^{3+}\text{–}OTC]}{([Eu^{3+}]_0 - [Eu^{3+}\text{–}OTC])\,([OTC]_0 - [Eu^{3+}\text{–}OTC])} \quad (1)$$

At the large excess of $Eu^{3+}$, ($[Eu^{3+}]_0 \gg [OTC]_0$), we would obtain:

$$[Eu^{3+}]_0 - [Eu^{3+}\text{–}OTC] \approx [Eu^{3+}]_0 \quad (2)$$

Substitution of expression (2) into equation (1) affords:

$$\beta \cong \frac{[Eu^{3+}\text{–}OTC]}{[Eu^{3+}]_0\,([OTC]_0 - [Eu^{3+}\text{–}OTC])} \quad (3)$$

$$\beta[Eu^{3+}]_0 \cong \frac{[Eu^{3+}\text{–}OTC]}{[OTC]_0 - [Eu^{3+}\text{–}OTC]} \quad (4)$$

$$\frac{[OTC]_0 - [Eu^{3+}\text{–}OTC]}{[Eu^{3+}\text{–}OTC]} \cong \frac{1}{\beta[Eu^{3+}]_0} \quad (5)$$

$$\frac{[OTC]_0}{[Eu^{3+}\text{–}OTC]} - 1 \cong \frac{1}{\beta[Eu^{3+}]_0} \quad (6)$$

The fluorescence intensity of compound can be quantitatively expressed as:

$$I = k'c, \quad (7)$$

where $k' = 2.303\phi_q I_0 k$ [assuming that the fraction of excitation radiation absorbed by the fluorophore is low ($klc < 0.05$), $I_0$ is the intensity of excitation radiation, $k$ is the absorption coefficient of fluorophore at the excitation wavelength, and $\phi_q$ is the quantum yield].[23]

Combination of equations (6) and (7) gives:

$$\frac{[OTC]_0 k'}{I} \cong \frac{1}{\beta[Eu^{3+}]_0} + 1 \quad (8)$$

or

$$\frac{[OTC]_0}{I} \cong \frac{1}{k'\beta}\,\frac{1}{[Eu^{3+}]_0} + \frac{1}{k'} \quad (9)$$

Equation (9) is the linear expression of form $y = kx + b$, where

$$\begin{cases} y = [OTC]_0/I \\ x = 1/[Eu^{3+}]_0 \end{cases}, \quad \begin{cases} k = 1/k'\beta \\ b = 1/k' \end{cases} \quad (10)$$

The resulting stability constant of $Eu^{3+}$–OTC complex ($\beta$ = = 335.6, $\log\beta$ = 2.53) was used in the calculations of equilibrium concentration of the complex in a solution. The latter value was consequently used to calculate the stability constants of $Eu^{3+}$–OTC–BA/metabolite complexes with various biogenic amines and their metabolites.

Then, the fluorescence intensity of $Eu^{3+}$–OTC–BA/metabolite complexes were recorded. The BA or metabolite concentrations were maintained at the constant level (1.0 nM), whereas the concentrations of europium and OTC were changed symbatically in the range of 0.1–0.4 mM in order to proportionally vary the equilibrium concentration of $Eu^{3+}$–OTC complex. The plots in $[BA/metabolite]_0/I - 1/[Eu^{3+}–OTC]_0$ coordinates were built on the basis of acquired data and used to estimate the stability constants of each complex as equal to $b/k$. The values of $b$ and $k$ were calculated by the least squares method.[24]

Taking into account another reported data concerning the complex of europium with OTC and other analytes, the $Eu^{3+}$–OTC–BA/metabolite was assumed as formed at the ratio of 1 : 1 : 1.[25]

For the reaction

$$Eu^{3+}–OTC + BA/metabolite \xrightarrow{\beta} Eu^{3+}–OTC–BA/metabolite,$$

the following equation was derived in a similar manner with the assumptions and calculations in equations (1)–(10):

$$\frac{[BA/metabolite]_0}{I} \cong \frac{1}{k'\beta} \frac{1}{[Eu^{3+}–OTC]_0} + \frac{1}{k'} \quad (11)$$

Equation (11) is a linear expression of the form $y = kx + b$, where

$$\begin{cases} y = [BA/metabolite]_0/I \\ x = 1/[Eu^{3+}–OTC]_0 \end{cases}, \quad \begin{cases} k = 1/k'\beta \\ b = 1/k' \end{cases}. \quad (12)$$

The acquired data (Table 2) revealed the stability of $Eu^{3+}$–OTC–BA/metabolite complexes as being 1–2 orders of magnitude higher than that of copper complexes with creatinine and amino acids,[26] and 1–3 orders of magnitude higher than that of some BA complexes with calcium and lanthanum that have long been successfully applied for BA determination in biosamples (Table S2).[27–29] Thus, the high stability of prepared ternary complexes allows one to employ them for the determination of BA and their metabolites in biological fluids without consideration for any competing interactions with the matrix components of samples. Though the stability constants of complexes are similar (see Table 2), there is an obvious relationship between the determination sensitivity of BA and their metabolites, on the one hand, and the stability constants of their complexes with europium and OTC (Figure S2), on the other hand: the higher the stability constant of complex ($\beta$/log$\beta$), the lower the LOD (nM) of BA and their metabolites. Even the minor differences in the structure of these analytes allow one to distinguish them with different sensitivities.

In conclusion, we have proposed the new indicator system based on the intensely fluorescing europium–oxytetracycline–biogenic amine/metabolite ternary complexes. The stability constants of complexes with norepinephrine, epinephrine, dopamine, serotonin, and their metabolites were determined using the fluorescent spectroscopy coupled with the Foster–Hammick–Wardley method. The investigated complexes are rather stable ($\beta \sim 10^6$). The relationship between the structural features of analyte molecules, the stability constants of the complexes, and the determination sensitivity with respect to the latter was found. The proposed system is quite promising and can be applied for the determination of BA and their metabolites in biosamples since it ensures the stability of neuromediators during the analysis in biological liquids, while the high fluorescence intensity of complexes allows their detection to be performed with the high sensitivity (down to 50 fM).

**Table 2** Stability constants of $Eu^{3+}$–OTC–BA/metabolite complexes.

| BA/metabolite | $\beta/10^{-6}$ | log$\beta$ | BA/metabolite | $\beta/10^{-6}$ | log$\beta$ |
|---|---|---|---|---|---|
| NMN | 1.92 | 6.28 | EP | 1.39 | 6.14 |
| NE | 1.83 | 6.26 | DA | 1.29 | 6.11 |
| HVA | 1.82 | 6.26 | VMA | 1.11 | 6.05 |
| 5-HIAA | 1.69 | 6.23 | L-DOPA | 1.07 | 6.03 |
| Serotonin | 1.61 | 6.21 | | | |

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*Online Supplementary Materials*

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2018.09.036.

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