Photometric Detection of Host-Guest Complexation Based on Biochemical Indicating Reaction

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A novel method for rapid and sensitive determination of 1,3-disubstituted calix[4]arenes in the concentration range of 10-520 µM based on the measurement of their inhibitory effect on cholinesterase has been proposed. The enzyme was implemented in the soluble films of polysaccharides and the initial rate of indophenyl acetate hydrolysis was measured photometrically as an analytical signal. Stabilized enzyme preparations are placed in the cell of a standard plate for immunoassay and can be stored in dry conditions for 4-10 months. Guest molecules able to form complexes with the above calixarenes, i.e. amino and hydroxy acids and metal cations, suppress the inhibitory effect. This makes it possible to detect a wide spectrum of biologically active compounds. The sensitivity of detection depends on the substituents at the lower rim of calix[4]arenes and reaction conditions.

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Host-guest complexation is widely used for the detection of various biologically active compounds due to the variety of steric structure of polycyclic compounds, the diversity of functioning and relative availability. This is particularly true for [1,4]metacyclophanes with common name calix[4]arenes. The calixarene conformation is rigid enough for fixing desirable coordination of functional groups at the lower and/or upper rims and providing for the multi-point binding of the guest molecules.

Substituted calix[4]- and calix[6]arenes have been mostly used for the selective binding of metal ions included in their cavity[14] and organic compounds coordinated both inside and outside the cavity[8,15]. They find application in the optical and electrochemical methods of analytical determination of appropriate guest species[15-18] and their extraction from water media into organic solvents[19,20].

The further progress in the use of host-guest interactions for the determination of biologically active compounds as well as the extension of the spectrum of guest molecules to be detected call for the improvement of the sensitivity of the detection of complexation and for molecular design of the structure of potential host molecules. As was shown previously, 1,3-disubstituted tert-butylcalix[4]arenes form stable complexes with α-amino and α-hydroxy acids which are implemented in between planar substituents of the lower rim of calixarene cavity[21,22]. Some of these compounds were examined as carriers for the transport across lipophilic membranes. The interaction of the above calix[4]arenes with indophenyl acetate used as cholinesterase resulted in the decay of the enzyme activity caused by cooperative interaction of the reagents without any covalent or electrostatic binding[23]. The structure of host-guest complexes as well as mechanism of interaction were investigated using IR and 1H-NMR spectroscopy.

In this work, the phenomenon of non-covalent binding of host-guest complex and cholinesterase was examined for the sensitive determination of 1,3-substituted calix[4]arenes as well as of the potential guest molecules.

Experimental

Reagents and chemicals

26,28-Disubstituted derivatives of 5,11,17,23-tetra-p-tetrahydroxycalix[4]arenes (Fig.1) were synthesized by selective alkylation with alkyl halides in acetonitrile in the presence of anhydrous potassium carbonate as described elsewhere[23,24]. Nitro derivative 8 was obtained by nitration with HNO3 in dichloromethane in the presence of glacial acetic acid[23]. All the calix[4]arenes examined were synthesized at the Department of Organic Chemistry of Kazan State University and kindly provided for investigation by Prof. I.S. Antipin.

![Calixarene Structure]

1 R = -9-CH3-Fluorenyl 2 R = -4-Ch3C6H4NO2
3 R = -4-Ch3C6H4CN 4 R = -tr-Ch3CH=CNPh
5 R = -Ch3Ph 6 R = -4-Ch3C6H4OOCF3
7 R = -2-Ch3Naphthyl 8 R = -NO2, R = -Ch3Ph
9 R = -CH3COOEt 10 R = -CH3CH2NH2
11 R = CH3C(O)NHCH3 12 R = CH3CH2NHCOOEt

Fig. 1. The structure of 1,3-substituted calix[4]arenes investigated (Compound 8 contains two nitro groups instead of tert-buty radicals at the upper rim)
The structure of calix[4]arenes was proved by $^1$H-
spectroscopy, mass-spectrometry and elemental analysis.

Butyrylcholinesterase from horse serum (ChE, EC 3.1.1.8),
specific activity 500 U mg$^{-1}$, and indophenol acetate were
purchased from Sigma Chemical Company (St. Louis, USA).
N-
phthalaldehyde was synthesized by treatment of chitozanz from
squid (fish-processing plant Ust-Khairguzno, Russia, average
molecular weight 10,000) with potassium phthalimide. Polyglucin
and β-dextran were purchased from JSC "Orgsynthesis
Enterprise" (Kazan, Russia). All the other reagents used were
of analytical grade (Reakhim, Russia, and Fluka, Neu-Ulm,
Switzerland).

**Apparatus**

Measurement of enzymatic activity was performed using
portable miniphotometer AK1-C-01 (JSC "Bioanalysishber", Russia) at 530 nm. All the reagents were placed into the cell of a
standard plate for immunochemical assay and then the change in
optical density was monitored and the slope of initial linear piece
of kinetic curve or the half-reaction time were calculated as a
measure of the rate of the ChE hydrolysis of indophenol acetate.

**Procedures**

To obtain stabilized enzyme preparation, 20 μL of the ChE
solution in 0.002 M Tris (tris(hydroxymethyl)aminomethane)
buffer solution corresponding to the final loading 0.1 U was
placed in the cell of a standard plate for an immunochemical
assay. Then 20 μL of stabilizer solution (N-phthalaldehyde,
polyglucin or β-dextran) containing 0.1 % of ammonia was
added and the plate was left to dry at room temperature. The plate with stabilized ChE preparation can be stored in dry
conditions either at 4°C or at room temperature.

Before measurement, 30 μL of Tris buffer solution was added
in the cell and the solution was left for 10-15 min for
solubilization of the enzyme film. Then 30 μL of the
indophenol acetate solution in 10% aqueous ethanol and 30 μL of
1.7×10$^{-4}$ M indophenol acetate were injected into the same cell
and the initial rate of the enzymatic reaction was determined as
described earlier. Typical kinetic curves prior to and after the
contact of the ChE and calix[4]arene solutions are presented in
Fig.2.

![Graph](image)

**Fig. 2.** Kinetic curves of indophenol acetate hydrolysis prior to
(1) and after (2) the contact of ChE (0.1 U) with calix[4]arene. D
optic density.

To avoid the problems of reproducibility related to the
heterogeneity of calix[4]arene solutions after their dilution, all
the solutions were equalized for at least 24 hours before their
contact with ChE. No opalescence or changes in inhibitory effect
were observed for the working solutions during the whole period
of their use in kinetic measurements.

For the determination of guest species, i.e. heavy metal ions,
hydroxy and amino acids, the appropriate solutions were first
mixed with calix[4]arene solution, then after conditioning the
mixture was added to the cell of the plate and the rate of
enzymatic reaction was measured as described earlier.

**Results and Discussion**

**Measurement of enzyme activity**

The changes of optic density of solution are related to the
formation of colored products in the ChE hydrolysis of
indophenol acetate (Eq.1).

\[
\text{O} = \text{N} - \text{N} - \text{OCOC}_3H_7 + H_2O \rightarrow \text{ChE} \rightarrow \text{CH}_3COOH
\]

\[
\text{O} = \text{N} - \text{N} - \text{OH} \rightarrow \text{brown products}
\]

The presence of 5-10% ethanol necessary for calix[4]arene
dissolution does not alter the ChE activity if the contact duration
does not exceed 20 min. The addition of polysaccharides used as
stabilizers results in 10-15% decrease of the response although
the value of Michaelis constant calculated from kinetic curves
remains the same (see the influence of N-phthalaldehyde on the
ChE activity in Fig.3 as an example).

![Graph](image)

**Fig. 3.** The dependence of the ChE activity on the concentration of indophenol acetate in the absence (1) and in the presence of
N-phthalaldehyde (2). $t_{1/2}$ - half-reaction time.

Meanwhile, the addition of stabilizers to the final
concentration 0.05-0.5% w/v increases dramatically the storage
stability of the ChE preparations (Fig.4). The storage time is
limited by the cracking of enzymatic layer film and by the decay
of the solubility of the enzyme containing polysaccharide film.

The protecting effect of stabilizers is observed only in dry
conditions. When wetted, ChE preparations lose their activity in
several days. Although β-dextran was found most effective for
ChE stabilization, the films obtained were less soluble. Polyglucin addition results in the formation of nonhomogenious
films so that the reproducibility of the results was about twice
less than in the case of other stabilizers. Thus, relative deviation
of the half-reaction time was found to be equal to 7, 4 and 3% for
polyglucin, β-dextran and N-phthalaldehyde, respectively.
(for 6 measurements with the cells of the same plate). For these reasons, the investigation of inhibition kinetics was performed using N-phytlylchitozan as the ChE stabilizer.

\[ t_{1/2}, s \]
\[ 0 \quad 4 \quad 8 \quad 12 \quad 16 \]
\[ 0 \quad 30 \quad 60 \quad 90 \]

Storage time, months

1 - N-phytlylchitozan, 2 - polyglycine, 3 - β-dextran. \( t_{1/2} \) - half-reaction time.

**Calix[4]arene determination**

All the investigated calix[4]arenes showed a reversible inhibitory effect on ChE. The dependence of the decay of the initial rate of enzymatic reaction on calix[4]arene concentration is linearized in the plots of \( \frac{v_f}{v_i} \) vs. \( C_i \). The \( v_f/v_i \) value was calculated from the changes in the slope of linear piece of appropriate kinetic curves. The half-reaction time was found less sensitive toward the calix[4]arene concentration. The variation of incubation time up to 20 min does not affect the inhibitory effect although the standard deviation of the \( v_f/v_i \) value is increasing with decrease of incubation period. The dependence of the inhibitory effect on the concentrations of indophenyl acetate and calix[4]arene corresponds to the kinetics of competitive inhibition, i.e. the substrate and inhibitor interact with the site of ChE globule\(^{26}\). The values of inhibition constant \( K_i \) calculated from the dependence of Michaelis constant on the inhibitor concentration\(^{29}\) as well as the analytical characteristics of calix[4]arene determination are presented in Table 1.

**Table 1. Analytical characteristics of calix[4]arene determination**

<table>
<thead>
<tr>
<th>No</th>
<th>Sensitivity, ((\mu M)^{-1})</th>
<th>Concentration range, (C_i, \mu M)</th>
<th>Detection limit, (\mu M)</th>
<th>(K_i, \mu M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.016</td>
<td>12-100</td>
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<td>5</td>
</tr>
<tr>
<td>2</td>
<td>0.0093</td>
<td>20-320</td>
<td>10</td>
<td>55</td>
</tr>
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<td>3</td>
<td>0.0083</td>
<td>80-180</td>
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<td>85</td>
</tr>
<tr>
<td>4</td>
<td>0.0084</td>
<td>60-280</td>
<td>30</td>
<td>110</td>
</tr>
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<td>0.014</td>
<td>13-120</td>
<td>9</td>
<td>2.5</td>
</tr>
<tr>
<td>6</td>
<td>0.021</td>
<td>10-46</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>7</td>
<td>0.015</td>
<td>10-55</td>
<td>6</td>
<td>20</td>
</tr>
<tr>
<td>8</td>
<td>0.019</td>
<td>15-65</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>9</td>
<td>0.022</td>
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<td>12</td>
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<tr>
<td>10</td>
<td>0.0030</td>
<td>14-100</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>11</td>
<td>0.0020</td>
<td>70-150</td>
<td>50</td>
<td>120</td>
</tr>
<tr>
<td>12</td>
<td>0.0025</td>
<td>50-250</td>
<td>20</td>
<td>90</td>
</tr>
</tbody>
</table>

The slope of the line in the plots \( \frac{v_f}{v_i} \) vs. \( C_i \) was used as a measure of the sensitivity of calix[4]arene determination. The detection limit corresponds to the calix[4]arene concentration which refers to the minimum detectable inhibition level, i.e. 8% decay of the initial rate of enzymatic reaction.

The inhibition constants are consecutively decreasing with the increase of the substituent chain or its branches. The changes in the detection limits with calix[4]arene structure were found much lower than the changes in appropriate inhibition constants.

As was shown earlier\(^{24,25}\), 1,3-substituted calix[4]arenes are able to form inclusion complexes with carbohydrates coordinated in-between planar substituents at the lower rim. The structure of the complexes of compounds 1, 5 and 7 with benzoyl anion and indophenyl acetate was confirmed by IR and \(^1H\)-NMR spectroscopy. This complex with indophenyl acetate can enter the active site of ChE located in the narrow gorge in the distance about 20 Å from the peptide surface. The coordination of calix[4]arene in the gorge (Fig. 5) is additionally promoted by a π-stacking interaction of calix[4]arene substituents and the aromatic residues of the amino acids lining the gorge.

**Fig. 4.** The long-term stability of stabilized ChE preparation in dry conditions at room temperature. 1 - N-phytlylchitozan, 2 - polyglycine, 3 - β-dextran. \( t_{1/2} \) - half-reaction time.

**Fig. 5.** The structure of the complex formed by calix[4]arene and ChE in the proximity of the active site.

The size of the p-tert-butylcalix[4]arene platform is close enough to that of the ChE gorge and the distance between the guest and host species in the complex formed is small enough to establish the close contact necessary for the observation of the inhibitory effect.

It should be mentioned that the concentrations of calix[4]arenes which can be measured in accordance with their inhibitory effect on ChE are comparable with those of some typical anticholinesterase chemicals, e.g. Trichlorfon\(^{25}\). This calls for the necessity of a toxicity assay for the polycyclic aromatic compounds used in industry. On the other hand, enzymatic method for calix[4]arene determination can be recommended for the control of their release from liquid membranes used, for example, for extraction of rare-earth metals or waste water treatment.

**Guest molecules determination**

In accordance with the mechanism of interaction of calix[4]arene and the ChE, the addition of other compounds which can form complexes with host molecules results in the decrease of the inhibitory effect observed for calix[4]arene alone. In these experiments, guest molecules were first added to the buffered calix[4]arene solution and left for 1 hour to avoid the microheterogeneity of the solution. Then 30 µL of the mixture was injected into the cell with stabilized ChE solution and the rate of indophenyl acetate hydrolysis was measured as described earlier.

The influence of guest molecules on the inhibitory effect of calix[4]arenes depends on the guest nature and the reaction conditions. Thus, the addition of sodium benzoate in the concentration range of (2-6)x10⁵ M reduces the inhibitory effect by 30-50% for different calix[4]arenes. Meanwhile, the
sensitivity of the ChE toward calix[4]arenes is not changed with benzoate addition. The effect of benzoate anion becomes lower and less reproducible with the decrease of pH value from pH 8.0 to 6.0. Contrary to that, the addition of glycine in the same concentration range reduces both the inhibitory effect and the slope of calibration curves. The maximum glycine effect is observed at pH 6.0. In both cases, the minimum detectable guest concentration refers to the formation of 1:1 host-guest complex with calix[4]arenes. The relative influence of a guest on different calix[4]arenes corresponds to the changes in their $K_{\text{in}}$ values of ChE inhibition, i.e. to the relative strength of complexation.

The inhibitory effect of amino derivatives on the ChE activity is also sensitive to the presence of heavy metal ions. Probably, this is due to the formation of chelate complexes which are inert toward the ChE. The detection limits of Cu$^{2+}$, Ag$^{+}$, and Cd$^{2+}$ ions were found to be equal to $n = (10^{-8}, 10^{-6})$ M. These concentrations are 10-50 times lower than those inhibiting the ChE in the absence of calix[4]arenes and about 50-100 times lower than the affecting concentrations of calix[4]arenes (see Table 1). It can be proposed, that first a weak complex of the ChE and metal ion is formed and then the cation is binding with calix[4]arene enzyme active site. In this case, the effect of heavy metal cations depends on the concentration of ChE active sites but not on the stoichiometry of host-guest complexation as it was established for amino- and hydroxy acids.

Unfortunately, the narrow range of the $\nu_2/\nu_1$ variation makes it possible only semi-quantitative determination of potential guests. The analytical characteristics of guest detection obtained in optimal reaction conditions for most sensitive calix[4]arenes are summarized in Table 2.

Table 2. Analytical characteristics of guest determination

<table>
<thead>
<tr>
<th>Host</th>
<th>Guest</th>
<th>Concentration range, $\mu$M</th>
<th>Detection limit, $\mu$M</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Benzoate</td>
<td>20-50</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>Oxalate</td>
<td>50-100</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>Glycine</td>
<td>80-200</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>Benzoate</td>
<td>30-80</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>Oxalate</td>
<td>30-80</td>
<td>20</td>
</tr>
<tr>
<td>6</td>
<td>Glycine</td>
<td>20-60</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>Benzoate</td>
<td>30-80</td>
<td>20</td>
</tr>
<tr>
<td>8</td>
<td>Cu$^{2+}$</td>
<td>0.07-0.5</td>
<td>0.04</td>
</tr>
<tr>
<td>9</td>
<td>Ag$^{+}$</td>
<td>0.1-0.5</td>
<td>0.1</td>
</tr>
<tr>
<td>10</td>
<td>Cd$^{2+}$</td>
<td>0.8-10</td>
<td>0.5</td>
</tr>
<tr>
<td>11</td>
<td>Benzoate</td>
<td>70-150</td>
<td>30</td>
</tr>
<tr>
<td>12</td>
<td>Cu$^{2+}$</td>
<td>5-20</td>
<td>1</td>
</tr>
<tr>
<td>13</td>
<td>Benzoate</td>
<td>100-500</td>
<td>50</td>
</tr>
<tr>
<td>14</td>
<td>Cu$^{2+}$</td>
<td>50-200</td>
<td>10</td>
</tr>
<tr>
<td>15</td>
<td>Ag$^{+}$</td>
<td>1-20</td>
<td>0.5</td>
</tr>
</tbody>
</table>

The phenomenon of suppression of inhibitory effect in the system calixarene – ChE – indophenyl acetate offers new perspectives for sensitive detection of host-guest complexation. This makes it possible to extend the number of biologically active compounds to be detected using ChE assay. The sensitivity of biochemical detection of complexation is higher than that of common approaches. The molecular design of calixarene structure makes it possible to enhance the sensitivity of determination due to the achievement of complementary interaction of the reactants. This allows developing specific assay techniques for the rapid and inexpensive detection of particular compounds based on general principles of molecular recognition.

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References