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Tsuyoshi Minami
Nina A. Esipenko
Ali Akdeniz
Ben Zhang
Lyle Isaacs

See next page for additional authors

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Multianalyte Sensing of Addictive Over-the-Counter (OTC) Drugs


ABSTRACT: A supramolecular sensor array composed of two fluorescent cucurbit[n]uril-type receptors (probe 1 and probe 2) displaying complementary selectivities was tested for its ability to detect and quantify drug-related amines. The fluorimetric titration of the individual probes showed highly variable and cross-reactive analyte-dependent changes in fluorescence. An excellent ability to recognize a variety of analytes was demonstrated in qualitative as well as quantitative assays. Importantly, a successful quantitative analysis of several analytes of interest was achieved in mixtures and in human urine. The throughput and sensitivity surpass those of the current state-of-the-art methods that usually require analyte solid-phase extraction (SPE). These results open up the opportunity for new applications of cucurbit[n]uril-type receptors in sensing and pave the way for the development of simple high-throughput assays for various drugs in the near future.

INTRODUCTION

The increasing cost of healthcare and the rising trend of self-medication drive the increased use of over-the-counter (OTC) medicines. The most popular OTC remedies relieve symptoms of colds and allergies and associated congestion. These OTC remedies typically include analgesics such as acetaminophen or ibuprofen to relieve pain and sedative antihistamines such as diphenhydramine (Benadryl) or doxylamine (NyQuil) to suppress allergic reactions. Decongestants such as pseudoephedrine or phenylephrine and cough suppressants might also be present. The most common brands are NyQuil (Vicks), Robitussin (Pfizer), and Sudafed (Johnson & Johnson), but various store brands are also available. On a less salubrious level, the ingredients of cold medicines and antihistamines are psychotropic drugs frequently abused because of their OTC status. An accidental overdose can lead to death, particularly in children.

The widespread use of antihistamines and decongestants with potential for abuse, such as doxylamine, diphenhydramine, phenylephrine, pseudoephedrine, and dextromethorphan, requires that methods for their detection and quantitative determination are widely available to healthcare professionals, counselors, and law enforcement. Quantitative determination of these drugs generally relies on solid-phase extraction (SPE) followed by gas chromatography/mass spectrometry (GC/MS) or liquid chromatography/mass spectrometry (LC/MS), which are not easily amenable to high-throughput screening (HTS). A further disadvantage of current urine analyses is that a number of urine drug tests display cross-reactivity with antihistamines, and as a result, numerous false-positive urine drug screen (UDS) results are encountered. For these reasons, we decided to investigate methods that could lead to an HTS-amenable assay for addictive components of OTC medicines. In the broader sense, this problem relates to the issues associated with analyzing structurally similar compounds in competitive media and in the presence of potential interferents.

Here, we focus on a direct method that would not require prepurification or pre-extraction of the compounds of interest. We decided to develop an HTS-amenable assay for selected OTC-available drugs such as antihistamines by exploiting the supramolecular properties of these analytes, namely, their ability to undergo protonation while displaying large hydrophobic surfaces to be leveraged in hydrophobic interactions with organic receptors and probes. As receptors, we used cucurbit[n]uril- (CB[n])-10 type receptors containing naphthalene fluorophores that are amenable to fluorescence-based reading (Figure 1).

Although cucurbiturils have previously been used in displacement assays, only recently were they found to be useful in assays based on their intrinsic fluorescence. These probes exhibit unique supramolecular properties enabling simultaneous sensing of multiple analytes in competitive media including human urine.

RESULTS AND DISCUSSION

Both probes display cross-reactive binding of a number of amines and ammonium or pyridinium salts. Specifically, probe 1 with a rigid cucurbit[6]uril macrocycle receptor imparts...
selectivity, and acyclic receptor 2 incorporates cross-reactive binding.\textsuperscript{13,14} The complementarity of selective and cross-reactive features enables recognition and quantification of structurally varied analytes. Both probes contain fluorescent naphthalene units whose fluorescence is partly quenched by Eu\textsuperscript{3+} ions coordinated to C=O moieties. This is due to the energy transfer (antenna effect) from the naphthalene moieties to the Eu\textsuperscript{3+} ions. The Eu\textsuperscript{3+} luminescence is not observed, however, because of the water molecules coordinated to the Eu\textsuperscript{3+} ions.\textsuperscript{15} The spectral properties of the probe–Eu\textsuperscript{3+} complex are then modulated upon formation of host–analyte ensembles. Whether the analytes induce quenching or intensity amplification of the probe fluorescence depends on the interplay between the structure, binding mode, and analyte–receptor affinity.

For example, pyridine (and pyridinium) moieties present in the analyte, such as paraquat,\textsuperscript{16} induce quenching of probe fluorescence. Similarly, depending on the pK\textsubscript{a} values of their conjugate acids, aliphatic amines might be able to quench the naphthalene fluorescence by photoinduced electron transfer (PET).\textsuperscript{17} Finally, nitro groups, also known to induce quenching – add yet another layer of variability in the signal output.

On the other hand, the formation of the complex between a probe and analyte that does not contain quencher moieties results in an increase in probe fluorescence. This is presumably due to the fact that the formation of the complex is associated with increased rigidity of the receptor and limited rotational/vibrational modes that would otherwise cause nonradiative decay. The magnitude of this fluorescence amplification is therefore intimately related to the drug–probe association constants and would serve as a fingerprint for the identification of the analytes.

Taken together, it is clear that OTC-associated medicines exhibit the structural variability to induce highly variable responses from the probes. Such responses are likely to be rich in information and would allow us not only to identify the OTC-related analytes, but more importantly to quantify them in aqueous samples. This is of particular interest because of the well-documented limits of cross-reactive arrays to determine analyte levels in binary and ternary mixtures.\textsuperscript{16}

The binding of the OTC-available drugs to probes 1 and 2 was confirmed using a combination of mass spectrometry (Figure 2) and fluorescence titration experiments (Supporting Information). The two methods independently confirmed binding of the drugs by the probes. Probe 1 was found to be more selective than the highly cross-reactive probe 2 (cf. Table 1).

![Figure 1. Top: Structures of probes 1 and 2. Center: Probes 1 and 2 comprise a cucurbituril-type receptor with naphthalene fluorophores. Both probes are shown as complexes with histamine. Curbit\textsubscript{6}uril-derived probe 1 binds smaller analytes, whereas acyclic probe 2 adapts its size to a wider variety of analytes. Bottom: Analytes associated with Eu\textsuperscript{3+} ions.\textsuperscript{15b} The spectral properties of the probe fluorescein (PET).\textsuperscript{15} Finally, nitro groups, also known to induce quenching react...](image)

![Figure 2. (A) MALDI TOF mass spectrum of the complex of 2 and famotidine. Inset: Calculated isotope pattern for C\textsubscript{70}H\textsubscript{87}N\textsubscript{22}O\textsubscript{24}S\textsubscript{6}\textsuperscript{+}. (B) ESI mass spectrum of the complex of 2 and doxylamine. Inset: Calculated isotope pattern for C\textsubscript{79}H\textsubscript{92}N\textsubscript{18}O\textsubscript{25}S\textsubscript{4}\textsuperscript{+}.](image)

Table 1. Association Constants (K\textsubscript{assoc} M\textsuperscript{−1})\textsuperscript{4} Obtained from Fluorescence Titration and Magnitudes of the Fluorescence Response (I\textsubscript{sat}/I\textsubscript{0}) and the Corresponding Lookup Table (LUT) for Comparison

<table>
<thead>
<tr>
<th>Guest</th>
<th>1</th>
<th>2</th>
<th>LUT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxylamine</td>
<td>3.4 × 10&lt;sup&gt;−5&lt;/sup&gt;</td>
<td>1.1 × 10&lt;sup&gt;−6&lt;/sup&gt;</td>
<td>72</td>
</tr>
<tr>
<td>Phenylephrine</td>
<td>ND&lt;sup&gt;18&lt;/sup&gt;</td>
<td>1.1 × 10&lt;sup&gt;−6&lt;/sup&gt;</td>
<td>92</td>
</tr>
<tr>
<td>Pseudoephedrine</td>
<td>4.5 × 10&lt;sup&gt;−6&lt;/sup&gt;</td>
<td>8.6 × 10&lt;sup&gt;−6&lt;/sup&gt;</td>
<td>81</td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>6.9 × 10&lt;sup&gt;−10&lt;/sup&gt;</td>
<td>1.0 × 10&lt;sup&gt;−10&lt;/sup&gt;</td>
<td>82</td>
</tr>
<tr>
<td>Histamine</td>
<td>5.1 × 10&lt;sup&gt;−7&lt;/sup&gt;</td>
<td>1.9 × 10&lt;sup&gt;−7&lt;/sup&gt;</td>
<td>113</td>
</tr>
<tr>
<td>Phenylephrine</td>
<td>ND&lt;sup&gt;18&lt;/sup&gt;</td>
<td>1.2 × 10&lt;sup&gt;−6&lt;/sup&gt;</td>
<td>92</td>
</tr>
<tr>
<td>Pseudoephedrine</td>
<td>3.5 × 10&lt;sup&gt;−8&lt;/sup&gt;</td>
<td>1.7 × 10&lt;sup&gt;−8&lt;/sup&gt;</td>
<td>92</td>
</tr>
<tr>
<td>Nizatidine</td>
<td>7.4 × 10&lt;sup&gt;−10&lt;/sup&gt;</td>
<td>3.0 × 10&lt;sup&gt;−10&lt;/sup&gt;</td>
<td>189</td>
</tr>
</tbody>
</table>

*Titration were recorded in the presence of Eu\textsuperscript{3+} (300 µM),\textsuperscript{15} and K\textsubscript{assoc} values were calculated based on the change in fluorescence intensity upon addition of each guest (pH 3 in water). The errors of the curve fitting were <18%. K\textsubscript{assoc} could not be calculated.

Table 1 shows the binding constants (K\textsubscript{assoc} M\textsuperscript{−1}) for drugs used in cold remedies, namely, doxylamine (H\textsubscript{1}-receptor antagonist), phenylephrine, pseudoephedrine, and acetaminophen. The binding of other histamine antagonists including famotidine (Pepcid), ranitidine (Zantac), cimetidine (Tagamet), and nizatidine (Axid) used to block the formation of peptic ulcers by OTC-available drugs. Famotidine, ranitidine, and cimetidine are OTC-available drugs. Inset: Calculated isotope pattern for C\textsubscript{70}H\textsubscript{87}N\textsubscript{22}O\textsubscript{24}S\textsubscript{6}\textsuperscript{+}.

![Figure 2. (A) MALDI TOF mass spectrum of the complex of 2 and famotidine. Inset: Calculated isotope pattern for C\textsubscript{70}H\textsubscript{87}N\textsubscript{22}O\textsubscript{24}S\textsubscript{6}\textsuperscript{+}. (B) ESI mass spectrum of the complex of 2 and doxylamine. Inset: Calculated isotope pattern for C\textsubscript{79}H\textsubscript{92}N\textsubscript{18}O\textsubscript{25}S\textsubscript{4}\textsuperscript{+}.](image)
elicit similar signals, the $K_{\text{assoc}}$ values are different, thus enabling a reliable recognition. For example, both doxylamine and acetaminophen induce quenching in both probes 1 and 2. However, acetaminophen is bound by probe 2 with significantly lower affinity ($K_{\text{assoc}} \approx 1100 \text{ M}^{-1}$) than doxylamine ($K_{\text{assoc}} \approx 10^6 \text{ M}^{-1}$). This is important because acetaminophen is present in OTC medicines at higher concentrations than, for example, doxylamine.

Equally important are the two columns on the right (Table 1) that show the fluorescence response to the presence of drugs. The color coding of the response magnitude shows that the responses are specific for each analyte. In combination with pattern recognition methods, \(^{13}\) the analyte responses enable correct identification of the analytes even in situations where the binding affinities are similar.

Figure 3 shows the recognition of cold remedies, their components, and several antihistamines by probes 1 and 2 using pattern recognition (linear discriminant analysis, LDA). \(^{18}\) The ability to correctly classify all analytes was tested by the leave-one-out routine (100% correct classification). Here, OTC cold remedies (NyQuil D, NyQuil Cough, DayQuil) cluster together, and so do their components. Within these groupings, the individual clusters are well-resolved. This is of particular interest because, for example, phenylephrine and pseudoephedrine are structurally very similar, and yet they are resolved. Inspection of the binding constants for phenylephrine and pseudoephedrine in Table 1 suggests that this resolution is most likely due to the fact that probe 1 binds phenylephrine with only marginal affinity.

After we had verified that a small sensor array comprising only two probes is capable of recognition of the studied analytes, we explored the quantitative determination of mixtures of doxylamine (0–22 ppm), pseudoephedrine (0–27 ppm), and phenylephrine (0–27 ppm). This combination of a sedative antihistamine (doxylamine) and the decongestant pseudoephedrine is frequently used in OTC medications—and abused. We were interested in investigating whether these structurally similar amines (particularly pseudoephedrine and phenylephrine) could be quantitatively analyzed in mixtures at concentrations relevant to pharmaceutical use. A simultaneous quantitative analysis of ternary mixtures is difficult, \(^{12b,16}\) and, to the best of our knowledge, has never been accomplished in a simple cross-reactive array in a competitive medium.

First, we prepared doxylamine (DOX)—pseudoephedrine (PSE)—phenylephrine (PE) ternary mixtures in pure water in a way that the concentrations of DOX and PSE were increasing (from 0 to 22 ppm and from 0 to 26 ppm, respectively) as the concentration of PE was decreasing (from 27 to 0 ppm). For each mixture, 20 repetitions were measured, and the standard deviation (<6%) was calculated. First, an LDA was performed to provide insight into the clustering and progression of the changes in the sensor response. LDA (Figure 4) yielded a smooth trend in the data, clear separation of the clusters, and 100% correct classification of the individual ternary mixtures.

To be able to determine the concentration of each of the three components independently, we performed regression analysis for DOX, PSE, and PE. A support vector machine (SVM) algorithm was used. \(^{12b,19}\) Toward that end, we divided the acquired data set into two parts, one for calibration and model development and a second to be used as unknown samples for cross-validation. Thus, for all three analytes independently, two concentrations of nine (22% of the overall data set) were analyzed as unknown concentrations, and the developed model that describes the behavior of the data was used to calculate the corresponding concentrations. Here, the two-probe array yielded a very accurate quantitative regression analysis of the ternary mixtures (Figure 5). Figure 5 also shows the correctly quantified two unknown samples (red circle). Limits of detections (LOD) were determined as 1.0 ppm for DOX, 0.7 ppm for PSE, and 0.8 ppm for PE.

In practice, doxylamine and other potential drugs of abuse are usually determined in urine. Human urine is a complex medium comprising electrolytes, small molecules (urea, aminoacids, hormones, etc.), and up to 1500 different proteins. Because doxylamine shows cross-reactivity with drugs of abuse such as methadone during routine urinalysis, we were interested in direct urinalysis, namely, without solid-phase extraction. Incubation of doxylamine with diluted whole human urine followed by analysis showed a clear dependence on the sensor fluorescence on the concentration of added DOX (Figure 6). Corresponding linear regression also enables the
determination of unknown samples (Figure 6 bottom). This experiment confirmed that doxylamine can be quantified in urine (LOD = 1.0 ppm, which is lower than that reported using SPE and GC/MS).20

Finally, we decided to assay the presence of doxylamine and its two main metabolites (desmethyldoxylamine and didesmethyldoxylamine)21 in urine following the ingestion of NyQuil. Such an experiment is complicated by the highly variable urine matrix, where the concentrations of urine components change during the day as a result of food and beverage intake. In our experiment, a properly hydrated volunteer ingested the manufacturer-recommended amount of NyQuil Cold & Flu (30 mL); urine samples were collected before administration and 1, 3, 5, 6, and 8 h after administration and analyzed by a hospital laboratory for levels of electrolytes, protein, and creatinine. The electrolyte values were found to vary significantly. For example, chloride levels were 10–61 mM; sodium, 12–62 mM; and potassium, 6–17 mM (see Supporting Information for details). Interestingly, regardless of the electrolyte fluctuations, the probe array yielded a clear trend in response (Figure 7).

CONCLUSIONS

In summary, we have demonstrated a simple cross-reactive sensor array based on two cucurbituril probes to recognize drug-related amines with known potential for abuse. The corresponding assay enables rapid analysis of compounds such as doxylamine, pseudoephedrine, and others in a high-throughput fashion. The throughput, sensitivity, and LOD surpass those of the current methods that generally require analyte extraction.5

Specifically, we demonstrated that several histamine antagonists and cold remedies such as the antihistamine sedative doxylamine, pseudoephedrine, and phenylephrine are successfully recognized by probes 1 and 2 and can be analyzed...
by the corresponding array. Furthermore, these drugs can be quantitatively analyzed within binary and ternary mixtures and even in human urine, which is, to the best of our knowledge, a first. Finally, doxylamine, a drug known to show cross-reactivity with important drugs of abuse such as methadone during routine urinalysis, was analyzed in urine. Likewise, urine samples after NyQuil ingestion displaying various concentrations of doxylamine were successfully quantified. Overall, these results showed that probe arrays comprising fluorescent cucurbit[n]uril derivatives and acyclic CB[n]-type receptors are well suited for the development of high-throughput assays for a wide variety of drugs and have potentially high impact in high-throughput clinical care settings.

**ASSOCIATED CONTENT**

*Supporting Information*

Fluorescence spectra, experimental detail of microarray, canonical score plots, and jackknifed classification matrices. This material is available free of charge via the Internet at http://pubs.acs.org.

**AUTHOR INFORMATION**

**Corresponding Authors**
pavel@bgsu.edu; L.Isaacs@umd.edu

**Notes**

The authors declare no competing financial interest.

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Figure 7. Results of direct urinalysis by probes 1 and 2 using urine samples collected 1, 3, 5, 6, and 8 h following the ingestion of NyQuil Cold & Flu (30 mL). The clusters clearly follow a pattern with an apex at 6 h, the time required to reach the maximum doxylamine concentration in urine. Top: Linear discriminant analysis (LDA) above) also shows a clear trend in data analysis, and the two spiked samples (yellow-red arrows) suggest that the up/down response is due to the doxylamine levels.
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