SUPPLEMENTAL MATERIAL

Immobilization of ADPH to Silica by the Schiff Base Method. The coupling of 3-*N*-amino-5,5-diphenylhydantoin (ADPH) directly to aldehyde-activated silica through the Schiff base method was one immobilization approach that was initially considered in this report, as adapted from the literature for the immobilization of proteins or other amine-containing agents.[1,2] The basis of this method is illustrated in Figure 1s. In this approach, two samples of 0.6 g Nucleosil Si-1000 diol silica were placed into separate containers. One sample was used to make the immobilized ADPH silica and the other served as a control support taken through all immobilization steps except addition of ADPH. A 20 mL portion of a 90% acetic acid solution and 1 g sodium periodate were added per g silica to each sample and allowed to shake for two hours at room temperature to convert the diol silica into an aldehyde form. These samples were then washed six times with water and three times with pH 6.0, 0.1 M potassium phosphate buffer. Approximately 10 mL of pH 6.0, 0.1 M potassium phosphate buffer per g silica was used to suspend the silica and combined with 16 mg ADPH per g silica (added to only one of the two samples) and 7.5 mg $NaCNBH_3$ per g silica (added to both samples). This slurry was allowed to gently rock at room temperature and in the dark for a period of 10 days.

After 10 days, the silica was washed three times with pH 8.0, 0.1 M potassium phosphate buffer, three times with a 50:50 mixture of ethanol and pH 8.0, 0.1 M potassium phosphate buffer, six times with ethanol, three times with a 50:50 mixture of ethanol and pH 8.0, 0.1 M potassium phosphate buffer, and four times with pH 8.0, 0.1 M potassium phosphate buffer. The total volume was then brought to 15 mL per g silica by adding pH 8.0, 0.1 M potassium phosphate buffer. A 25 mg portion of $NaBH_4$ per g silica was then slowly added over the course of 90 min (note: do this addition with caution; the solution will bubble) with gentle suspension of the silica after each addition. The contents were then washed three times with 0.5 M NaCl in pH

8.0, 0.1 M potassium phosphate buffer and three times with pH 7.4, 0.067 M potassium phosphate buffer. The silica was brought to a final volume of 6 mL with pH 7.4, 67 mM potassium phosphate buffer, resulting in a slurry containing approximately 100 mg silica per mL. These slurries were stored at $4^{o}C$.

The final method used for ADPH immobilization (see Figure 2 of the main manuscript) also began by converting diol silica to an aldehyde-activated form, as described for the Schiff base method. This aldehyde-activated support was then reacted with oxalic dihydrazide at pH 5.0, as described in the literature.[2,3] The dihydrazide-activated silica was combined with 20 mL of pH 5.0, 0.1 M potassium phosphate buffer per gram silica. A 0.6 mL portion of 25% (w/w) glutaraldehyde per g silica was added to the slurry (note: this value represented a five-fold mole excess of glutaraldehyde versus dihydrazide groups). The mixture was shaken for three hours at room temperature. The silica was washed three times with pH 6.0, 0.1 M potassium phosphate buffer and brought to a final volume of 10 mL per g silica. The remainder of the procedure followed the steps described for the Schiff base method, starting with the addition of ADPH to one of the support samples and $NaCNBH_3$ to both support samples. The final supports were stored in pH 7.4 buffer at $4^{o}C$.

Comparison of Immobilization Methods for ADPH. Preliminary studies with the RDIA method were used to compare the response that was obtained on various supports that contained immobilized ADPH. One of these supports had been immobilized by the Schiff base method and used a six-atom spacer arm to attach ADPH to the support, while the second method used a multi-step process that introduced a seventeen-atom spacer arm. The reaction schemes that were used to prepare these two types of supports are provided in Figure 1s and Figure 2, respectively. An application flow rate of 0.1 mL/min was used during these experiments to give

the label maximum time to interact with the immobilized ADPH. In these preliminary studies, a flow rate of 0.1 mL/min was also used to inject the phenytoin samples and allow them adequate time to displace the label from each column. The quantity of label that was injected in each of these experiments was 100 pmol. The sample had a volume of 100 μL and contained either potassium phosphate buffer to serve as a blank or 50 μM phenytoin (i.e., 5 pmol phenytoin) as a positive control. The amount of injected label represented at least a 30- to 60-fold excess versus the estimated total amount of immobilized drug analog in the column. As indicated in the main body of the text, the concentration of phenytoin that was employed in this study was ten-times that seen for free phenytoin in serum at therapeutic levels.[4,5]

Figure 2s shows some typical displacement peaks that were obtained in the RDIA method under these conditions. Injections of phenytoin samples onto both types of ADPH supports gave a displacement peak, as shown in Figures 2s(a)-(b); however, the peak obtained when using a seventeen-atom spacer arm between ADPH and the support was much larger than the signal for the support that used a six-atom spacer arm. It was for this reason that the support with the seventeen-atom spacer arm was used in all further experiments. The fact that these studies were conducted using a relatively long contact time for the sample with the column, thus minimizing kinetic effects, suggested that this difference was due to steric hindrance between the labeled binding agent and the immobilized ADPH when using the shorter spacer arm. This conclusion agrees with previous observations that have been made in affinity chromatography in the use of short versus long spacer arms for the attachment of small ligands for use in the isolation of enzymes, proteins or other large targets.[2]

Similar experiments based on the RDIA method were conducted using a control column in which the support contained the seventeen-atom spacer arm but no immobilized ADPH. A

typical result obtained at an injection flow rate of 0.1 ml/min is shown in Figure 2s(c). It was found in this type of experiment that very little labeled binding agent was retained by this support, leading to no significant displacement peak upon the injection of phenytoin. Using an even higher injection flow rate (e.g., 1.2 ml/min) further reduced this background signal. This low background signal in the absence of any immobilized ADPH indicated that little or no non-specific interactions were occurring between the labeled binding agent and the support or the seventeen-atom spacer arm. Low non-specific binding of human serum albumin and other serum components has also been noted in prior work using similar silica supports that were prepared by the Schiff base method or activated with hydrazide groups.[1,3,4] These results confirmed that the displacement of the labeled binding agent in the RDIA method was from the immobilized ADPH and was not due to the release of this label from other components of the support, such as the spacer arm.

Another set of control experiments were conducted in which the labeled binding agent was injected onto the immobilized ADPH supports followed by the injection of only phosphate buffer. Figure 2s(d) shows the result that was obtained when using the ADPH support with the seventeen-atom spacer arm. It was found that the injection of buffer produced no measurable displacement peak even though the same bound of labeled binding agent had been adsorbed to the immobilized ADPH column as was used in Figure 2s(a) for the injection of a phenytoin sample. It was determined from this result that, under the injection conditions used in this study, the labeled binding agent did not have significant displacement from the ADPH column in the presence of only buffer. Instead, the presence of phenytoin in the sample was required to combine with this labeled binding agent and displace it from the ADPH column.

References

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Figure Legends

Figure 1s. Immobilization of 3-*N*-amino-5,5-diphenylhydantoin (ADPH) onto silica by the Schiff base method. This scheme uses aldehyde-activated silica as the starting material, which can be prepared as described in the text.

Figure 2s. Effect of ADPH support preparation method on displacement peak area in the RDIA method. These results show displacement peaks that were obtained for 100 µL injections of 50 µM phenytoin on (a) an immobilized ADPH column containing a seventeen-atom spacer arm, (b) an immobilized ADPH column containing a six-atom spacer arm, and (c) a control column containing a seventeen-atom spacer arm but no immobilized ADPH. The result in (d) was obtained for a 100 µL injection of pH 7.4, 67 mM potassium phosphate buffer on the immobilized ADPH column containing a seventeen-atom spacer arm. The displacement flow rate was 0.1 mL/min, with a pH elution step being used between the displacement and re-application of the label to the column.

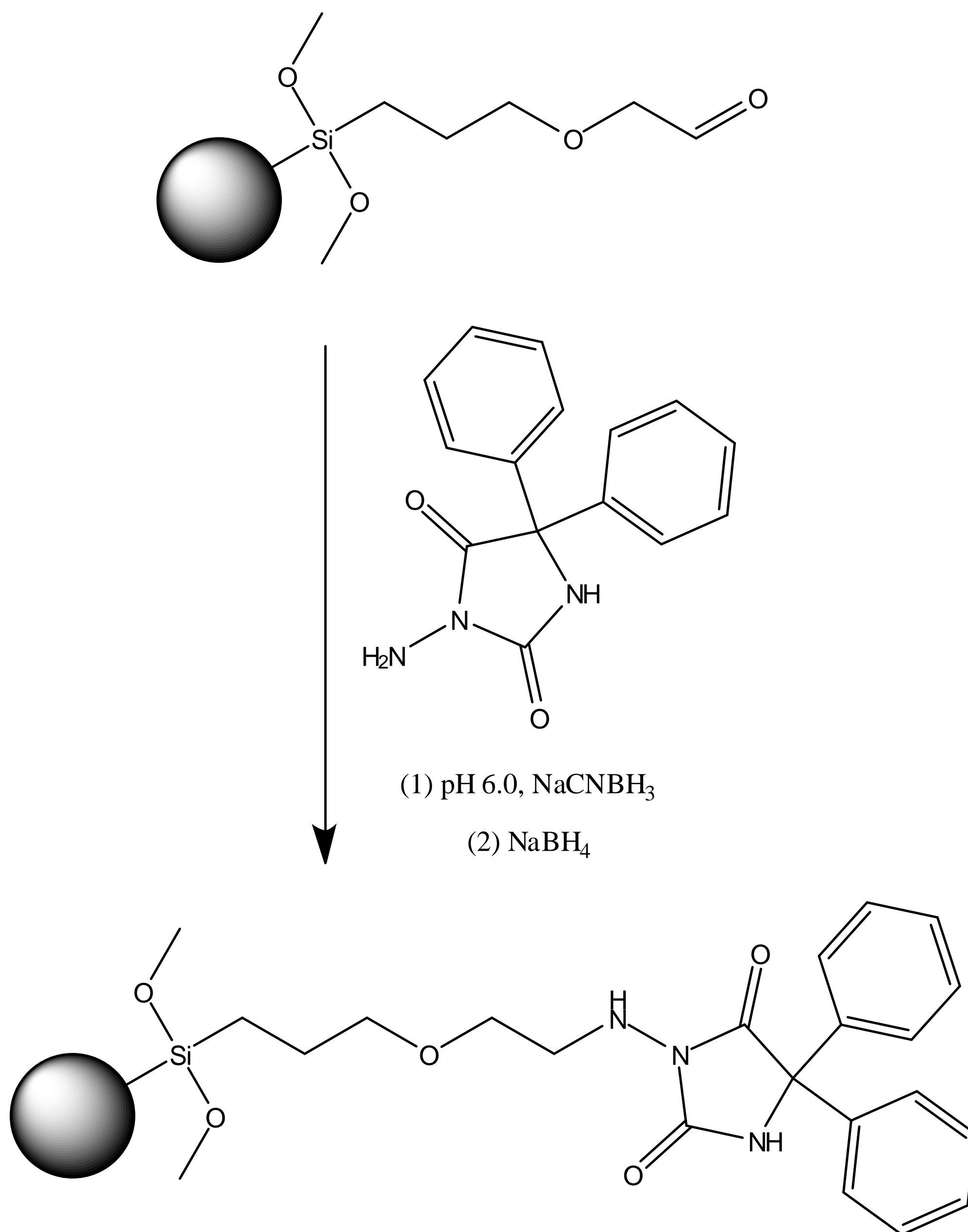

Figure 1s

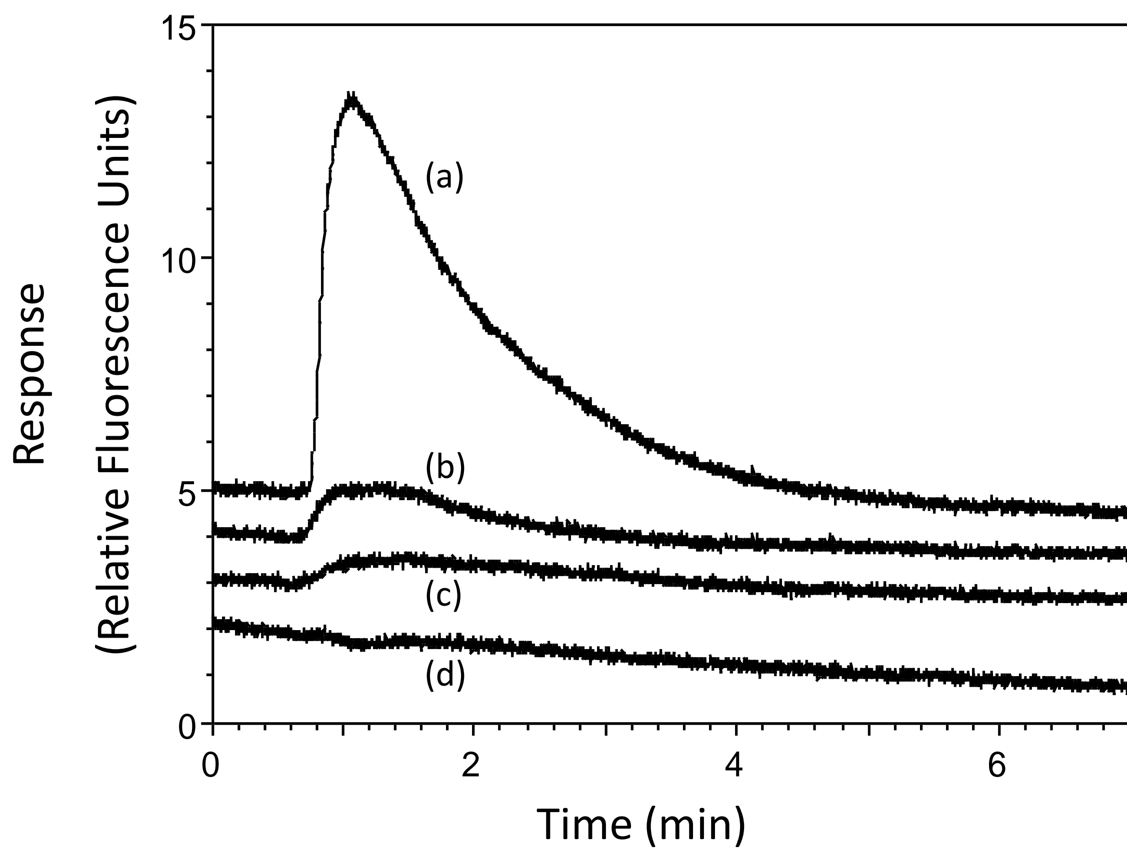

Figure 2s