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ARTICLE *in* ANALYTICAL CHEMISTRY · JANUARY 2006

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Inhibitor Screening Using Immobilized Enzyme Reactor Chromatography/Mass Spectrometry

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We describe the coupling of capillary-scale monolithic enzyme reactor columns directly to a tandem mass spectrometer for screening of enzyme inhibitors. A two-channel nanoLC system is used to continuously infuse substrate or substrate/inhibitor mixtures through the column, allowing continuous variation of inhibitor concentration by simply altering the ratio of flow from the two pumps. In the absence of inhibitor, infusion of substrate leads to formation of product, and both substrate and product ions can be simultaneously monitored in a quantitative manner by MS/MS. The presence of inhibitor leads to a decrease in product and an increase in substrate concentration in the column eluent. Knowing the product/substrate ratio and the total analyte concentration (P + S), the concentration of product eluting, and hence the relative enzyme activity, can be determined. Both IC_{50} and K_I values can then be obtained by direct MS detection of the effect of inhibitors on relative activity. Inhibitor screening is demonstrated using reusable, sol−gel derived, monolithic capillary columns containing adenosine deaminase, directly interfaced to ESI-MS/MS. On-column enzyme activity was assessed by monitoring inosine and adenosine elution. It is shown that the method can be used for automated screening of the effects of compound mixtures on ADA activity and to determine the K_I value of the known inhibitor, erythro-9-(2-hydroxy-3-nonyl)adenine, even when the compound is present within a mixture.

Rapid screening of enzyme inhibition is a key method for the identification of drug leads. The most common methods used for high-throughput screening of enzyme inhibitors involve colorimetric or fluorometric assays run in multiwell plate format. However, such assays have inherent drawbacks in that (1) a suitable colorimetric or fluorometric reagent must be available to generate a signal, (2) interferences can arise from compounds that either absorb or fluoresce at wavelengths similar to the reagent, or quench fluorescence, (3) these methods are not amenable to the screening of mixtures, and (4) these methods usually require complex, robotic liquid handling.[1] In cases where spectroscopic assays are not possible, assays are usually done using laborious and time-consuming HPLC-based assays, which are not generally scalable to high-throughput.

An emerging method that can be used to provide more information on modulation of enzyme function with no need for labels is the monitoring of enzyme-catalyzed reactions by mass spectrometry (MS or MS/MS).[2] Several groups have described studies where enzyme reactions were carried out in wells or other vessels containing the free enzyme, followed by off-line MS analysis of substrates, products, or inhibitors to evaluate enzyme activity and ligand binding.[3−7] Other approaches have used immobilized ligands to screen enzymatic activity, with MALDI-MS providing the ability to detect conversion of the ligands.[8] Still other methods have utilized flow-through reactors wherein both the enzyme and substrate/inhibitor flow through a reaction loop followed by infusion of all components into an ESI-MS system to monitor enzyme activity.[9] This latter method, while providing the ability to obtain function-based enzyme inhibition data without labels, requires fresh aliquots of enzyme for each analysis, as the enzyme is infused into the MS system.

An alternative approach that has been reported for screening of enzymes is to prepare immobilized enzyme columns, which are typically used for frontal affinity chromatography with mass spectrometric detection (FAC/MS).[10−12] In this method, a compound mixture is continuously infused though a column containing

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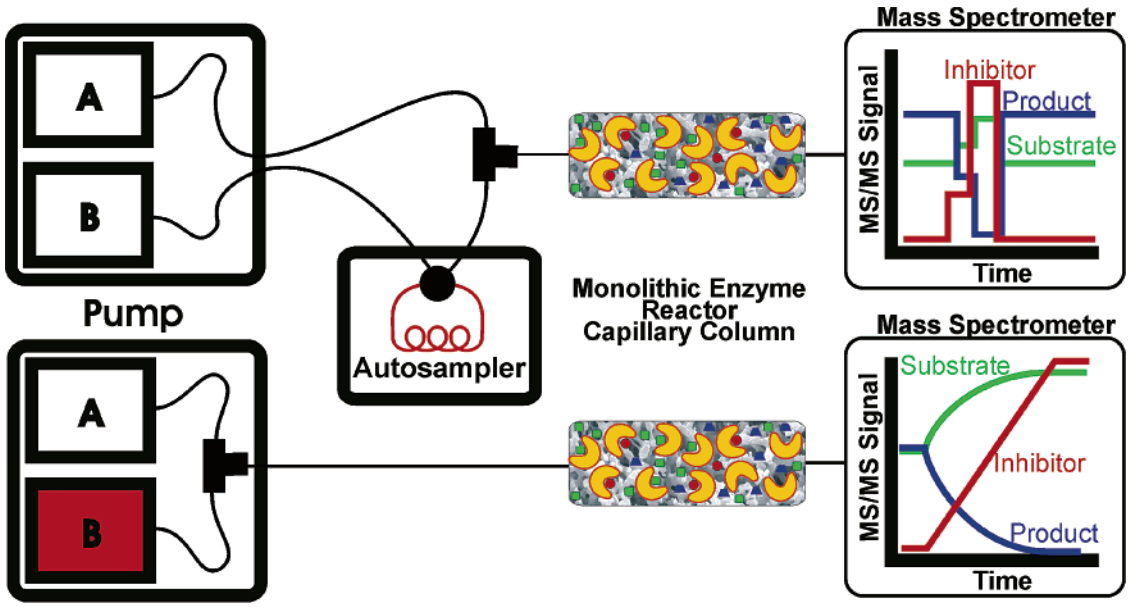

Figure 1. Method for inhibitor screening using enzyme reactor chromatography interfaced to mass spectrometry. All mobile phases and samples contain an identical concentration of substrate, while inhibitors are introduced by pump B, either through an autosampler loop (top) or by loading inhibitors directly into the reservoir for pump B (bottom). The upper configuration allows for automated screening of multiple-compound mixtures while the lower configuration is used for the quantitative analysis of identified inhibitors. Substrate and substrate + inhibitor streams can be infused into the column in any ratio, followed by in-line MS detection. As the concentration of an inhibitor increases, the enzyme within the column is inhibited. Decreased product and increased substrate signals can be used as indicators of enzyme inhibition by unknown test compounds.

a bound protein target. Compounds that show affinity for the protein are retained and thus break through later than nonbinding compounds. By using tandem MS methods, it is possible to determine the identity of compounds that are retained on the column, even when they are present in mixtures. Furthermore, when combined with a second dimension of LC/MS, it can be used for rapid screening of mixtures containing up to 1000 compounds.[12] An advantage of this method is that it allows reuse of the immobilized enzyme, saving on reagent costs. The method is also amenable to a wide range of proteins,[10] aside from enzymes, improving versatility.

While FAC/MS is capable of identifying inhibitors in mixtures, the method suffers from potential drawbacks.[13] First, the retention of a compound is assumed to be due to specific binding to the protein, but this can only be confirmed by assessing nonspecific interactions of each compound in the library with respect to the column matrix. Second, the retention data do not provide information on where the compound binds to the protein, as binding could occur at a nonfunctional site. While such issues can be addressed by using a suitable indicator compound,[10,14] which competes with compounds in the library for binding to the active site, in many cases suitable indicator compounds may not be available.

These issues can potentially be overcome using immobilized enzyme reactors for function-based screening. There are several reports describing the use of immobilized enzyme reactors for examination of enzyme activity and inhibition, although few utilize on-line MS detection. For example, Wainer and co-workers have reported on the combination of an immobilized enzyme reactor (ER) with a reversed-phase LC system using absorbance-based detection as a method for examining the activity of immobilized enzymes.[15] Massolini et al. have developed monolithic columns with covalently bound enzymes to create an immobilized enzyme reactor that was used in conjunction with absorbance detection.[16] Palm and Novotny have used enzyme reactors interfaced with off-line MALDI-MS for evaluation of PNGase F activity.[17] An example of on-line monitoring of an immobilized enzyme reaction by MS was provided by Hindsgaul and co-workers, who used MS to monitor product formation upon introduction of a plug of substrate into an immobilized enzyme column.[11] This method provided a label-free method to assess enzyme activity via MS, but required multiple injections of various levels of substrate and inhibitor to allow construction of a Lineweaver−Burke plot to extract K_I values. Additionally, the Gaussian-shaped profiles of the eluted product suggest that enzyme reaction rates do not achieve steady-state equilibrium at the injected substrate concentration.

In this study, we report on inhibitor screening using a chromatographic mode where immobilized enzyme reactor columns are directly interfaced with tandem mass spectrometry (ER-MS/MS). The basic concept of ER-MS/MS is shown in Figure 1.

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A substrate is continuously infused into an enzyme-doped column, wherein it is partially converted to product molecules. Using multiple reaction monitoring (MRM) mode, ions specific to the substrate and product are monitored independently to obtain a product-to-substrate (P/S) ratio. Since the sum of S + P is a known constant within the system, the P/S ratio can easily and accurately be used to determine the concentration of product eluting from the column. When mixtures of compounds are introduced into the substrate stream, the P/S ratio will remain constant if no inhibitor is present, but will be altered in favor of substrate if an inhibitor is present. By altering the ratio of flow between the substrate and substrate/inhibitor channels, one can alter inhibitor concentration in an automated fashion, to obtain a multicompound screen or a full inhibition curve. When repeated using different substrate concentrations, the inhibition constant can be determined using a single column, saving on reagent costs and assay time. Since substrate and product ions are "separated" by the mass spectrometer, this method eliminates the need for extra dimensions of chromatographic separation and greatly increases throughput. Experiments providing proof of principle for the use of ER-MS/MS in inhibitor screening are presented. We evaluate the method using adenosine deaminase (ADA) as the model enzyme immobilized in a capillary-scale monolithic reactor column, owing to the relevance of this protein in immune disorders[18] and because there are currently no high-throughput screening methods for this enzyme.

EXPERIMENTAL SECTION

Materials. Ammonium acetate and HPLC grade water were purchased from Caledon Laboratory Chemicals (Georgetown, ON, Canada). Erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) was purchased from Calbiochem (San Diego, CA, Catalog No. 324630). Diglycerylsilane (DGS) was prepared by methods described elsewhere[19] using tetramethyl orthosilicate (Sigma-Aldrich) and anhydrous glycerol (Fisher Scientific). ADA (type V, from bovine intestine, EC 3.5.4.4), adenosine, inosine, 2-fluoro-2′-deoxyadenosine, folic acid, pyrimethamine, fluorescein, 10 kDa poly(ethylene glycol), and Bis-Tris buffer salt were obtained from Sigma-Aldrich (Oakville, ON, Canada). Other compounds used for the primary screening experiment are listed in Supporting Information. Fused-silica tubing was purchased from Polymicro Technologies (Phoenix, AZ). Distilled deionized water was obtained from a Milli-Q Synthesis A10 water purification system. All reagents were used as received.

Fabrication of ADA Columns. Columns were fabricated in a fashion similar to that described in our previous report.[13] ADA was exhaustively dialyzed against a solution containing 5 mM Bis-Tris hydrochloride, pH 6.5, with 10% (v/v) glycerol to remove all traces of phosphate buffer and adjust the pH. A 40-μL aliquot of the resultant 40 μM ADA solution was then mixed with 20 μL of a solution containing 0.5 mM Bis-Tris hydrochloride, pH 6.5, with 50% (v/v) glycerol. DGS-based sols were prepared by sonicating DGS with water (1 g + 1 mL) at 0 °C for 15 min to hydrolyze the monomer, followed by filtration through a 0.2-μm filter. A 100-μL aliquot of the resulting sol was rapidly mixed with the 60 μL of ADA solution prepared above, followed by the rapid addition of 40 μL of 40% (w/v) poly(ethylene glycol) in water. The sol solution was then injected into an 80-cm length of 250-μm-i.d., 360-μm-o.d., polyimide-coated fused-silica tubing that was previously cleaned using 1 M NaOH. The liquid sol must be completely mixed, injected, and stationary within the capillary by the time phase separation occurs, ~2 min after mixing. After gelation (~3 min), capillaries were looped such that both ends could be submerged in 50 mM Bis-Tris hydrochloride, pH 6.5, and secured for storage. Columns were aged for a minimum of 5 days to achieve a relatively stable internal structure. After aging, 10-cm column segments were cut from poured columns, as required.

LC/MS Settings. A two-channel, Eksigent nanoLC pump was used for mobile-phase delivery to a MDS Sciex Q-trap mass spectrometer. Note that the Eksigent pump uses direct pneumatic pumping of mobile phase at microliter per minute rates, with no flow splitting. An Eksigent AS-1 48 vial autosampler was fitted with a 250-μL withdrawal syringe and a 150-μL loop of 250-μm-i.d. fused-silica tubing. Thus, only small volumes of inhibitor solutions (~400 μL) are required for screening, although larger volumes can be loaded directly into the Eksigent pump reservoir (1−5 mL depending on configuration) for determination of IC_{50} values. Mobile-phase delivery was controlled by Eksigent nanoLC software v 2.05. Mobile phases were run directly into the MS system, without the introduction of an organic "makeup flow". Mass spectrometer control and data acquisition was done using Analyst v.1.4 software. Precursor−product ion pairs were followed using MRM mode in positive ion mode under the following conditions: curtain gas 30.0; collision gas, medium; ion spray voltage, 5500 V; temperature, 140 °C; ion source gas 1, 40.0; ion source gas 2, 40.0. Specific MS/MS parameters for each ion pair are provided in Supporting Information (Table S1). The total scan time was 2 s per point.

Column Handling. Prior to experiments, a fresh 10-cm column segment (5 μL internal volume) was equilibrated off-line, with mobile phase from channel A, to remove aqueous poly(ethylene glycol) and glycerol. New columns were connected to the pump using 75-μm-i.d. fused-silica tubing, and another 75-μm-i.d. tubing segment was attached to the bottom of the column using Upchurch Microtight unions (Oak Harbor, WA, P.772). Several bed volumes of mobile phase were passed through the column at 0.5 μL/min before slowly increasing the flow rate to 12 μL/min, and finally back to 10 μL/min, for experiments. Columns were attached directly to the Turbospray ion source of an MDS Sciex Q-Trap mass spectrometer with 75-μm-i.d. fused-silica tubing. When mobile phases within the pumps were exchanged, the column was removed from the system and connected top to bottom with a buffer-filled capillary. The column fittings were not adjusted or removed from the column after the initial washing step.

Mixture Screening. Compound mixtures were screened using the autosampler system configuration shown in Figure 1 (top). In this system, the flow from channel A bypasses the autosampler and enters a precolumn mixing tee, while the flow from channel B is directed through the autosampler loop to introduce compound mixtures into the mixing tee prior to passing through the enzyme reactor column. A total of 49 biochemical compounds were selected for screening and were dissolved in DMSO to a

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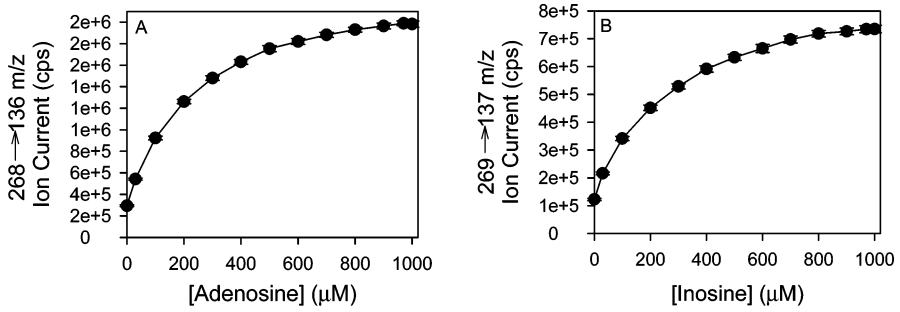

Figure 2. MS/MS signal response to increasing analyte concentration for (A) adenosine and (B) inosine. The nonlinear ionization efficiency of electrospray ionization, within the range of analyte concentrations required for the study of ADA, makes instrument calibration for experiments requiring large concentration changes difficult.

concentration of 10 mM. For the primary screen, compounds were mixed into seven groups of seven compounds each (see Supporting Information for composition of mixtures) and diluted to 10 μM in aqueous 2 mM ammonium acetate buffer containing 200 μM adenosine. Mobile phase, loaded into channels A and B of the Eksigent pumps, contained 2 mM ammonium acetate, 200 μM adenosine, and a DMSO concentration to match the samples (0.7% v/v). The DMSO was included to mimic the solvent composition that would be present in a standard compound library. After suitable column equilibration (see above), sample mixtures were infused from the autosampler loop at 5 and 100% of total flow (based on the ratio of flow rates in channel B relative to channel A) with a total flow rate of 10 μL/min. Under these conditions, the column-entrapped enzyme was exposed to 500 nM, followed by 10 μM concentration of each compound in each mixture. The step profile (see Figure 1, top) was set such that the preequilibration step and postequilibration step (no inhibitor) were each 20 min in duration while exposure to low and high inhibitor concentrations were for 10 min each. In this manner, less than 150 μL of inhibitor solution was used for each two-point screen.

Secondary screening was performed on each of the seven compounds in the active mixture identified by the primary screen. Each compound was individually diluted to 10 μM in 2 mM ammonium acetate plus 200 μM adenosine. The DMSO concentration in the mobile phase was adjusted accordingly (0.1% v/v), and injection was performed as described above, using the same time steps.

Determination of IC_{50} and K_I Values. An alternative pump configuration, shown in Figure 1 (bottom), was used for quantitative determination of inhibition constants. An adenosine solution (62.5, 125, 250, or 500 μM) in 2 mM ammonium acetate buffer was loaded into channel A of the Eksigent nanoLC pump. Channel B was loaded with a solution containing an identical adenosine concentration plus a mixture containing 1 μM concentration of each test compound (fluorescein, pyrimethamine, folic acid, and EHNA (absent in control)).

The MS system was calibrated as described below to provide a means to determine product/substrate ratios from the ratio of intensities for ion pairs related to adenosine (substrate, m/z 268 → 136) and inosine (product, m/z 269 → 137). IC_{50} values were obtained by altering the ratio of flow in the substrate and substrate + inhibitor channels in a stepwise fashion while maintaining a combined flow rate of 10 μL/min. In this manner, the inhibitor concentration could be varied while maintaining a constant substrate concentration. In the programmed infusion profile, each mobile-phase ratio remained constant for 10 min to allow for an equilibrium condition to be achieved within the column. Data collected from a 7-min window, corresponding to the center of each 10-min equilibrium state, were averaged to give each data point. Substrate and product ion pairs were monitored once every 2 s so each data point was determined from 420 individual MRM measurements. The raw data were used to calculate a product/substrate ratio, from which the concentration of product eluting from the column was ultimately determined. This value was normalized by letting the maximum product concentration in the absence of inhibitor correspond to a relative activity of 100%. IC_{50} values were obtained from the point where the relative activity decreased to 50% of its initial value.

The K_I value was determined by extrapolation of IC_{50} values to the point of zero substrate concentration. The method is based on the derivation described by Cheng and Prusoff,[20] as shown in Supporting Information. K_m values were obtained using immobilized enzyme reactor columns by plotting the concentration of product formed upon infusion of a given concentration of substrate and fitting the data to the Michaelis−Menten equation. The data were compared to that obtained using a conventional absorbance-based assay (see Supporting Information).[21]

RESULTS AND DISCUSSION

Calibration of MS Signal Intensity. An issue with the use of MS for quantitative detection of species eluting from the reactor column is the potential for ion suppression. Indeed, the signal response was nonlinear with respect to the concentration of a either adenosine or inosine, as shown in Figure 2. These data were obtained using 2 mM ammonium acetate buffer with no column present. Higher buffer levels led to increased ion suppression (data not shown), and thus, all remaining experiments were done using the 2 mM buffer system. As noted below, the use of the low ionic strength buffer did not lead to any degradation of entrapped enzyme performance, even over multiple runs of the same column. This situation is not likely to hold for all enzymes,[13] and thus, optimization of buffer concentration will likely be required for each new enzyme studied. Alternatively, the reactor columns could potentially be interfaced to MALDI-MS/MS to help

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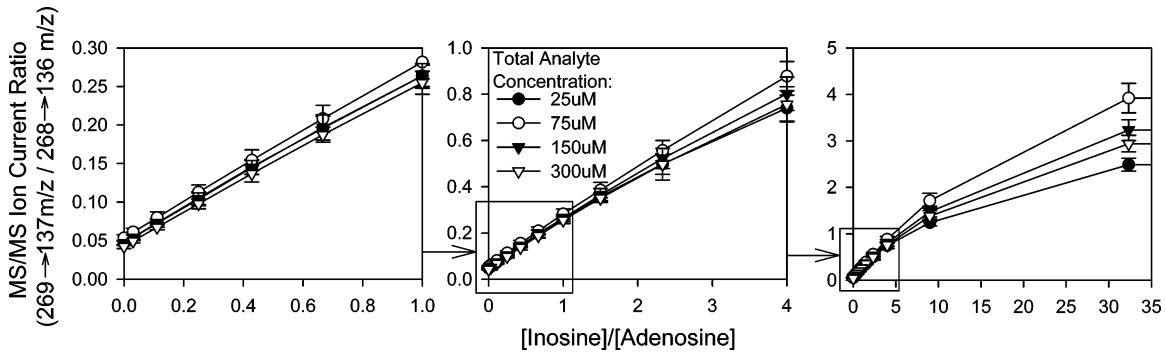

Figure 3. Calibration data for ADA enzyme reactor column. When total analyte concentration is constant, MRM signal ratios are linear with respect to product/substrate ratios over a relatively broad range.

overcome issues with ion suppression and ionic strength limitations.[22]

Given the nonlinear ionization efficiencies seen in Figure 2, we opted to calibrate the system using the ratio of MS signal intensities for product and substrate ions as a function of product/substrate concentration ratio, while infusing a constant total analyte concentration (i.e., constant sum of product + substrate concentrations). Since adenosine is converted to inosine in a 1:1 ratio, the total concentration of these species will be constant regardless of conversion efficiency on column. It should be noted that alternative methods, such as inclusion of an internal standard with ionization efficiency similar to the product ion, could also be used to quantitate the product ion concentration.[7]

For calibration, solutions containing identical concentrations of adenosine and inosine, in 2 mM ammonium acetate, were mixed and infused into the mass spectrometer to provide a constant total concentration of the two analytes. Various total concentrations of analyte (adenosine + inosine) were tested (25, 75, 150, 300 μM). As shown in Figure 3, the ratio of MS signals for inosine and adenosine are linearly related to the ratios of concentration for the two species over a relatively wide range of ratios (up to about 1.5:1 P/S), indicating that "ion stealing" does not occur in the range of analyte concentrations tested (25−300 μM). The *y*-intercept of ~5% is indicative of signal overlap between inosine and the ^{13}C isotope of adenosine, both of which have m/z 269 → 137 as their ion pair for MRM detection. Given that enzyme kinetic studies would generally be done using substrate conversion rates of well under 50%,[23] relative enzyme activity can be determined by simply comparing the ratio of signals of product and substrate. However, higher conversion rates, up to a P/S ratio of >10:1 (>90% conversion), can still be quantitated by working with the nonlinear portion of the calibration curve. Clearly, the linear range and the curvature of the calibration curve are likely to change for different enzymes with different substrate/product pairs, particularly in cases where ion suppression effects are different for the product and substrate. However, we expect that this calibration method should be applicable to a range of other enzyme-catalyzed reactions.

Knowing the concentration ratio, and noting that the concentration of substrate infused must equal the concentration of substrate plus product eluted $[S]_i = [S] + [P]$, it is possible to determine the individual concentrations of substrate and product eluted at any time. Since all assays are performed at the same flow rate (10 μL/min), enzyme activity is thus proportional to the concentration of product ([P]) eluted.

Prior to performing enzyme inhibition studies, the effect of each compound in the test mixture on the substrate and product signals was assessed by directly infusing compounds into the MS system in the presence of product and substrate. In all cases, the inclusion of the test compound at a level of 1 μM, either alone or as a mixture, did not lead to any alteration in the MS signal intensities or in the P/S ratio, indicating that the test compounds did not affect the calibration parameters for the substrate or product.

Inhibitor Screening by Enzyme Reactor Chromatography. Figure 4A demonstrates an automated screen of 49 compounds (seven individual compound mixtures) by enzyme reactor-MS. The infusion profile is shown in the step profile at the bottom of Figure 4A, while the changes in the P/S ratio are shown across the top of the figure, indicating ~40% turnover of substrate in the absence of inhibitor, based on the calibration data in Figure 3. This provides a large range for alterations in P/S ratios upon introduction of inhibitor, making detection of inhibitors relatively easy. In cases where substrate turnover is inefficient, the P/S ratio may be much smaller, reducing assay sensitivity. In such cases, higher enzyme concentrations or lower flow rates (and hence higher substrate/enzyme contact time) could be used to increase the P/S ratio. Alternatively, the MS parameters could be adjusted to enhance the product signal relative to the substrate signal to increase assay sensitivity. Momentary interruptions in column flow between consecutive injections, executed by the Eksigent pumps, are responsible for the vertical lines seen in the data, which conveniently demarcate each injection. The downward drift in the P/S signal ratio is likely due to the presence of DMSO, given that this ratio was much more consistent in subsequent experiments (see below), where DMSO was absent. This suggests that the ER-MS method should be employed with mixtures that do not include DMSO.

Based on the calibration data in Figure 3 and the starting P/S ratio of ~0.25, a decrease in P/S signal ratio to ~0.12 would correspond to substrate turnover dropping from 40 to 20% (50% inhibition). Thus, under the conditions used for the assay, a change in P/S signal ratio to a value of 0.12 is indicative of a

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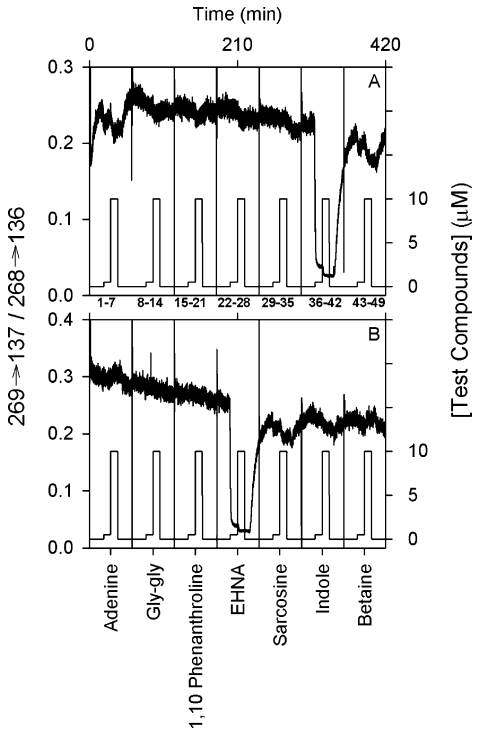

Figure 4. Primary (top) and secondary (bottom) screens of 49 compounds. The primary screen indicates that a compound in the mixture containing compounds 36−42 is inhibiting ADA. Deconvolution of the mixture in the secondary screen reveals that EHNA is the inhibitor of ADA. The square injection profiles, shown by the bottom traces in each panel, are programmed by the Eksigent pump and show when the column is exposed to either 500 nM or 10 μM concentration of the test compound. The P/S signal ratios show the response of the enzyme to the various test mixtures.

compound being present at a concentration equivalent to its IC_{50} value. Using the two-level screen, we can evaluate inhibition at two concentrations (500 nM and 10 μM), allowing for semiquantitative determination of IC_{50} values of mixtures. As shown in Figure 4A, only one of the compound mixtures (compounds 36−42) shows a significant alteration in the P/S ratio to a value below 0.12 when present at 500 nM. This is indicative of a compound with an IC_{50} value well below 500 nM (this is the mixture containing EHNA). On the other hand, none of the other mixtures contains an inhibitor with an IC_{50} value of less than 10 μM. The most potent inhibitor aside from EHNA was 2-fluoro-2′-deoxyadenosine ($K_I = 17$ μM, compound 18), However, this would not be expected to have a significant effect on ADA activity in the presence of 200 μM adenosine, as under these conditions, the IC_{50} value would be ~50 μM. Detection of these weaker inhibitors could be done by using either a lower substrate concentration or a higher inhibitor concentration in the test mixtures.

Several other points should be noted from the data presented in Figure 4A. First, the overall time for the 49-compound screen is 420 min. This is clearly not "high-throughput" but in this case serves to demonstrate the principle of the automated screening protocol. Increased throughput could be easily achieved by increasing the mixture complexity. Indeed, Schreimer and coworkers recently reported on the use of a FAC/MS-based screening method where a mixture of 1000 compounds was tested by FAC followed by deconvolution of the mixture using a LC/MS approach.[12] Analysis time could also be increased by running only a single-point screen, reducing equilibration times, or increasing flow rate, although in our system the Eksigent pumps are limited to a maximum flow rate of 20 μL/min.

A second point is that the system shows excellent recovery after exposure to a potent inhibitor, as demonstrated upon removal of compounds 36−42. In this case, the recovery is slow (30 min) but is essentially complete prior to introduction of the next inhibitor mixture. The slow recovery is likely the result of the slow offrate that is typically associated with high-affinity inhibitors. More rapid recovery would be expected for lower affinity ligands and, indeed, is observed for mixtures that do not contain a potent inhibitor. Importantly, the reversibility of the P/S signal ratio provides clear evidence for the presence of a competitive inhibitor. In cases where irreversible (covalent) inhibitors were present, such recovery would not occur, resulting in loss of column performance. This is a problem with any screening method that utilizes immobilized enzymes (i.e., FAC/MS) and thus is not unique to our approach.

A final point is that the data show that the column remains active over a period of many hours, showing the utility of the sol−gel columns for development of immobilized enzyme reactors. This is particularly important given that some proteins, such as dihydrofolate reductase, do not survive the low ionic strength conditions required for ESI-MS detection.[13]

While Figure 4A shows the presence of an active mixture, it does not provide insight into the identity of the active compound. Figure 4B shows one method for identification of the active compound by testing of the individual compounds present in the active mixture and reveals EHNA to be the active, inhibitory compound (note: this assay was performed with the same column used for the primary screen). This method has the advantage that the determination of the inhibitory compound is based on changes in activity rather than simple binding, minimizing issues such as nonselective binding of test compounds to the column matrix. This method should be suitable in cases where mixtures are not highly complex and where the identity of the compounds in the mixture is known. For more complex mixtures, or in cases where compound identity is not known, alternative deconvolution methods would be required. These might include secondary analysis of mixtures by FAC/MS,[10−12] additional fractionation and retesting by ER-MS, prefractionation of mixtures by reversed-phase LC prior to ER-MS, or use of a bioselective extraction method using an enzyme-doped column to select high-affinity compounds from the active mixture,[24] followed by testing by ER-MS.

Quantitative Binding Analysis by Enzyme Reactor Chromatography. To validate that quantitative inhibition data could be obtained by ER-MS, we evaluated the response of P/S ratios as a function of inhibitor concentration. Figure 5 shows data obtained by injecting many different concentrations of test compounds onto ADA columns. DMSO was not present in these test mixtures to avoid drift in the MS signal with time. Removal of DMSO is also likely to provide more physiologically relevant ligand binding data. In this experiment, adenosine was infused at a constant concentration of 62.5 μM while the concentration of test compounds was increased from 0 to 1 μM over the course of the experiment. As shown in panel A, a mixture of noninhibitors,

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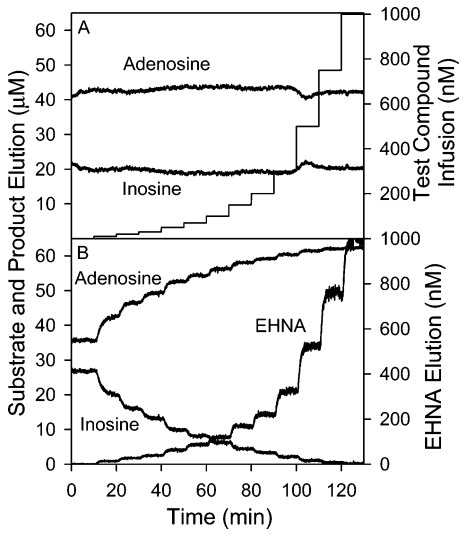

Figure 5. Variation in MS/MS signals for adenosine (substrate) and inosine (product) upon infusion of varying levels of test compounds. Panel A shows infusion of a mixture of fluorescein, folic acid, and pyrimethamine according to the programmed infusion profile. Panel B shows infusion of a mixture of fluorescein, folic acid, pyrimethamine, and EHNA, with the MS signal from EHNA matching the programmed infusion profile. Adenosine was infused at a constant level of 62.5 μM for both mixture screens.

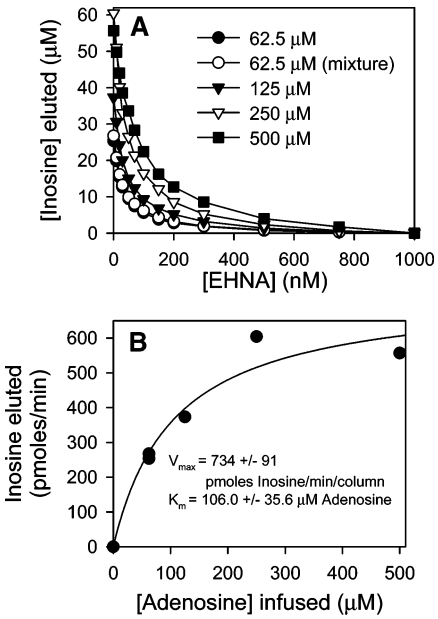

Figure 6. (A) Inhibitor saturation curves obtained by the ER-MS using different concentrations of substrate. The data show the expected decrease in product concentration with increased levels of inhibitor. (B) Assessment of K_m values for ADA entrapped in a macroporous enzyme reactor column. The rate of inosine production from the column as a function of the infused concentration of adenosine was determined by multiplying the product concentration data (at [EHNA] = 0) in panel A by the column flow rate. Data are fit to the Michaelis−Menten equation.

including fluorescein, folic acid, and pyrimethamine, had no significant effect on substrate or product signals, indicating that the activity of ADA was not altered by these compounds. However, when EHNA was added to the mixture (panel B) a concentration-dependent decrease in product and increase in substrate signals, corresponding to a decrease in ADA activity, is apparent. The stepwise inhibition seen in Figure 5B demonstrates the potential for enzyme reactor chromatography/MS to obtain quantitative binding data from high-resolution, primary mixture screens such as the screen shown in Figure 4A. It should be noted that while this assay required ~2 h to complete, most of this time was used to ensure equilibration of the system. Assay time could be reduced significantly either by reducing the number of points or by using shorter equilibration times.

Figure 6A shows inhibitor saturation curves as a function of substrate concentration obtained from the data shown in Figure 5 and subsequent experiments on the same column, at different substrate concentrations. The product concentrations do not scale linearly with adenosine concentration owing to saturation of the enzyme active sites, which reduces the percentage of substrate conversion at high substrate levels. The percent substrate conversion in the absence of inhibitor was as follows: 62.5 μM = 42%; 125 μM = 29%; 250 μM = 24%; 500 μM = 11%. Clearly, it is necessary to assume that the initial reaction rate is proportional to product concentration when using end-point assays to determine kinetic parameters. In general, this only holds up to substrate conversion rates of ~10%,[23] but this depends on the specific concentration of substrate relative to the K_m value. Given the accepted K_m value of 100 μM for the conversion of adenosine by ADA, the substrate concentrations in Figure 6A correspond to 0.625, 1.25, 2.5, and 5.0 K_m. The largest percentage of substrate conversion occurred at 62.5 μM adenosine (0.625 K_m), with 42% conversion. The concentration of substrate drops from 0.625 to ~0.36 K_m over the course of the chromatographic run, with the average substrate concentration during the experiment being ~0.49 K_m. Operating at 0.625 K_m, we expect to have a rate of reaction of 38.5% of V_{max} (recall that at [S] = K_m, the rate is $^1/_2$ V_{max}). When operating at 0.49 K_m, the rate will be 33.1% of V_{max}, corresponding to 85% of the expected rate. Thus, using product concentration directly leads to an underestimation of initial rate by ~15%. Applying the same analysis to the other substrate concentrations, we obtain underestimations of ~7% at 125 μM, 4% at 250 μM, and 1% at 500 μM. It should be noted that, by using the continuous flow method, it is possible to adjust flow rate and column length to suit more or less active enzymes so that conversion rates are in the range where product concentration is proportional to initial rate.

Figure 6B shows the rate of inosine production (picomoles per minute per column) as a function of the concentration of infused adenosine concentration, obtained by multiplying the concentration of inosine eluting from the column at each substrate concentration (from Figure 6A) by the column flow rate. The data show the saturation profile expected for enzyme-catalyzed reactions. Michaelis−Menten analysis of the curve provided a V_{max} of 734 pmol min^{-1} per column and a K_m value of 106 μM of adenosine. The relative errors on these numbers are fairly high (up to 30% RSD) owing to the effects of ion suppression, which made it difficult to obtain points at higher adenosine concentrations, and due to the assumption that product concentration related directly to initial rates, which does not hold at lower substrate concentrations. Indeed, as shown in Figure 6B, the point at 500 μM adenosine lies below that obtained at 250 μM adenosine. Even so, the K_m value obtained is in good agreement with the K_m value obtained for the soluble form of the enzyme using an absorbance-

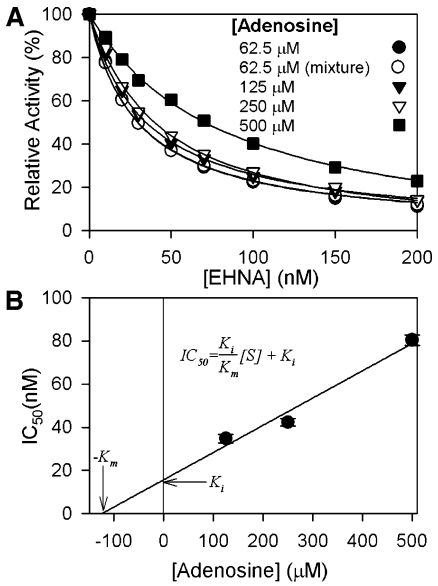

Figure 7. Effect of EHNA on adenosine deaminase activity at various adenosine concentrations. Panel A shows the normalized saturation curves, which were used to calculate IC_{50} [EHNA] values by nonlinear regression. Panel B shows the use of IC_{50} values at various adenosine concentrations to access the EHNA/ADA equilibrium constant, K_I [EHNA], in the absence of competing substrate, as well as the Michaelis constant, K_m, for adenosine/ADA binding.

based assay (89 μM, see Supporting Information),[8] showing that the entrapped enzyme retains binding affinity for the substrate that is similar to that observed in solution. The consistency and resemblance of these data to that obtained by UV absorbance and published data is even more impressive when one realizes that the points shown in Figure 6B were all obtained using the same column. Indeed, these data are only 5 among 60 enzyme activity measurements done using the same immobilized enzyme column.

Figure 7 shows how changes in enzyme activity as a function of inhibitor concentration can be used to determine IC_{50} and K_I values. Panel A is similar to Figure 6A, except that it is normalized to show the 50% inhibition point more clearly. Fitting to a simple ligand binding isotherm provides IC_{50} values (top panel). The nearly identical IC_{50} values obtained for EHNA in a mixture (29.7 nM, open circles) and alone (29.4 nM, closed circles) indicate the usefulness and validity of this technique for quantitative measurements by mixture screening. A novel form of Cheng and Prusoff's equation[20] allows extrapolation of the IC_{50} values to provide the K_I [EHNA] and K_m for the substrate, as shown in Figure 7B. It should be noted that the point at 62.5 μM adenosine was not used in the determination of K_I and K_m values, as this substrate concentration did not allow for the assumption of initial rate being proportional to product concentration, as noted above. The resulting K_I of 15.7 $\pm$ 3.5 nM is in reasonable agreement with the published K_I value of 6.5 nM.[25] The K_m value of 124 $\pm$ 42 μM also matches with values obtained from absorbance assays (89 μM)[21] and enzyme reactor-MS assays (106 μM). The changes in IC_{50} values with substrate concentration provide further evidence for competitive inhibition by EHNA. Hence, the use of the immobilized enzyme reactor column provides kinetic and inhibition data that are of sufficient accuracy to allow the method to be used for both primary screening and quantitative analysis of enzyme inhibition.

CONCLUSIONS

Interfacing of enzyme reactor columns to tandem MS provides a rapid method for function-based assessment of enzyme inhibition and is amenable to direct screening of enzyme inhibitors within mixtures. The use of MS for assessing enzyme activity is highly versatile and avoids the need for labels. The use of an immobilized enzyme column allows reuse of the enzyme for multiple assays, which not only saves on reagent costs but also provides an unprecedented internal control in that the level of enzyme is consistent for all assays. Use of an immobilized enzyme may also provide opportunities to combine a function-based enzyme reactor assay with other assay modes, such as frontal affinity chromatography, to aid in screening of complex mixtures. Further work will focus on decreasing equilibration times, increasing mixture complexity and thus throughput, and extending the method to more complex enzymatic systems. These results will be reported in future papers.

ACKNOWLEDGMENT

The authors thank MDS-Sciex and the Natural Sciences and Engineering Research Council of Canada for financial support of this work. We also thank Dr. Balusubramaniam Easwaramoorthy for collection of absorbance-based enzyme kinetic data. T.R.B. holds a Canada Graduate Scholarship. J.D.B. holds the Canada Research Chair in Bioanalytical Chemistry.

SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review May 3, 2005. Accepted September 27, 2005.

AC050761Q

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