Native Mass Spectrometry using the X500B QTOF System for Fragment-Based Screening

Esme Candish[1], Chris Nortcliffe[2]
[1]SCIEX, MA, USA, [2]SCIEX Warrington, UK,

Native mass spectrometry (MS) is a facet of electrospray ionization MS where proteins are analyzed in non-denaturing solvents. Parameters such as pH, ionic strength and ionization are controlled to maintain the native folded state of the protein in solution and subsequently in the gas phase. This approach has emerged as an important technique to study non-covalent protein interactions including protein-protein interactions as well as protein-ligand interactions.

As native MS monitors the molecular mass of protein complexes, it can provide key insight into the identity of bound ligands, the stoichiometry of protein-ligand binding and even strength of binding. This can be exploited as a starting point in drug discovery research where libraries of small molecule fragments (potential drugs) are screened to identify fragments that bind with the protein target.

Fragment screening campaigns typically employ a wide range of analytical techniques including, and nuclear magnetic resonance (NMR), surface plasmon resonance (SPR), X-ray crystallography and isothermal titration calorimetry (ITC). Native MS provides a complementary insight into the protein-fragment binding; this approach directly monitors the binding and consumes minimal protein and fragment, is label-free and does not require sample immobilization.

Carbonic anhydrase was selected as a model protein for this work for two reasons: First, because of the extensive knowledge of carbonic anhydrase and its inhibitor binding, particularly the sulfonamide inhibitors, and second as carbonic anhydrases are implicated in a range of disorders and diseases, including cancer, glaucoma, obesity, epilepsy, osteoporosis, and altitude sickness.

Presented herein is a proof-of-concept native size exclusion chromatography (SEC)-MS workflow for fragment-based screening. The approach employs the easy to use benchtop X500B QTOF system to identify protein-fragment binding and to probe the binding affinity of sulfonamide inhibitors (Figure 1).

Figure 1. The SCIEX X500B System

Key Feature of X500B System

- High-resolution mass spectrometer for a wide range of biopharmaceutical applications
- Robust, compact benchtop footprint reduces laboratory space requirements
- Easy to use hardware and software accessible for a wide range of users

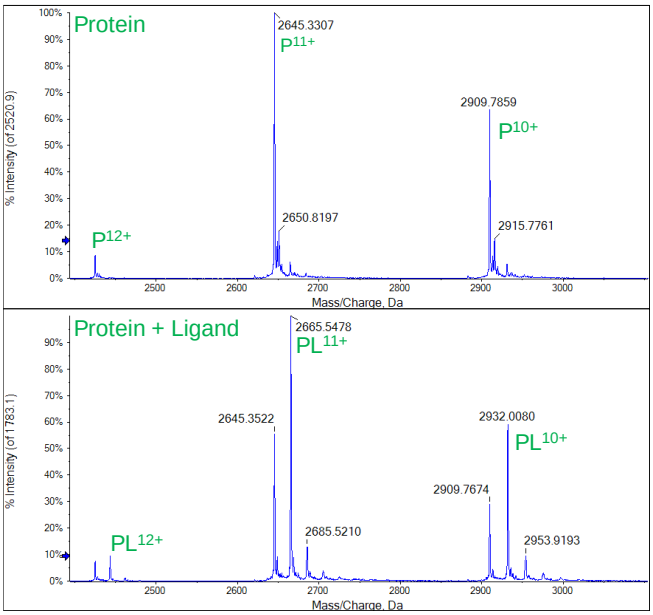

Figure 1. The native spectra of carbonic anhydrase (top). The complex of carbonic anhydrase and acetazolamide (bottom).

Methods

Materials and Sample Preparation: Carbonic anhydrase Isozyme II from bovine erythrocytes, acetazolamide benzenesulfonamide, and ethoxzolamide were purchased from Sigma. The fragments were prepared at a 1M stock solution in dimethyl sulfoxide (DMSO) and working solution, diluted in DMSO, were prepared. A 1 µL aliquot of each working solution was spiked into 99 uL 10 µM of carbonic anhydrase in 50 mM ammonium acetate.

Chromatography: Separation was accomplished using an ExionLC™system fitted with a polyLC polyHYDROXYETHYL A Column 60 Å, 1 mm x 50 mm or 2.1 mm x 50 mm. Mobile Phase A was 100% water while Mobile Phase B was 100 mM ammonium acetate. The isocratic method employed 50% A and 50 % B with a flow rate of 350-600 µL min^{-1}. Flow injection analysis (FIA) was run using a flow rate of 350-µL min^{-1}. A 2 µL injection was employed.

Mass Spec: A SCIEX X500B QTOF-MS system with a Turbo V™ source was employed. SCIEX OS software was employed for data acquisitions, and within the same run experiment, 1 and 2 monitor the protein and the fragment respectively. The MS parameters are listed in Table 1.

Data processing: Data was processed using Explorer and Bio Tool Kit within the SCIEX OS software. Optimal reconstruction was achieved using a start and stop mass of 25000 and 30000 Da respectively. The step mass was 1 Da, and the input spectrum resolution was double the resolution of the raw spectra.

Table 1. MS Parameters

	Experiment 1	Experiment 2
Polarity	*Positive*	
Intact Protein Mode	*On*	
GS1 (psi)	*50*	
GS2 (psi)	*50*	
Curtain Gas	*35*	
CAD Gas	*7*	
Temperature (°C)	*300*	
TOF Start Mass (Da)	*2000*	*100*
TOF Stop Mass (Da)	*4000*	*500*
Accumulation Time (s)	*0.1*	*0.1*
Spray Voltage (V)	*5500*	*5500*
Declustering Potential (V)	*150*	*80*
Collision Energy (V)	*5*	*10*
Time bins to sum	*120*	*6*

Figure 1. shows the mass spectra of carbonic anhydrase in the native state. Compared with the spectra of a denatured protein obtained using a low pH mobile phase there are relatively few peaks in the spectra. The spectrum contains a narrow charge-state distribution with charge states ranging from 10+ to 12+, which indicates that the protein (P) retains a folded, native-like conformation (Figure 1 top). Carbonic anhydrase is a Zn(II)-containing metalloenzyme; the sulfonamide inhibitors non-covalently bind with the protein through chelation to the Zn(II) ion in the binding pocket.[3] Three sulfonamide ligands (L) were investigated in this study; these were acetazolamide (222.25 Da), benzenesulfonamide (157.19 Da) and ethoxzolamide (258.32 Da). As carbonic anhydrase contains just one binding site the ligand is expected to bind at a 1:1 ratio. Figure 1 (bottom) shows typical mass spectra of a positive fragment hit. A positive hit is easily identified by the additional signals at 10+, 11+ and 12+ that corresponds to the protein-ligand complex (PL). In this example, the spectra shown is for carbonic anhydrase and acetazolamide.

The traditional approach of undertaking a fragment screening assay employs nano-infusion for sample delivery prior to native MS. However, the need to implement a robust high throughput solution is apparent for the analysis for fragment-based screening. In this work, we chose to employ SEC prior to native MS. Two key factors must be considered when implementing SEC into the workflow. First, once $P + L \rightleftharpoons PL$ is disturbed time to detection must be minimized to limit k_{off}. Second, desolvation conditions must be soft to maintain the PL complex. For this work, the column employed was a polyHYDROXYETHYL A as analysis could be achieved in less than 10 seconds with a flow rate of 450 µL min^{-1}. This column is also employed as the first dimension SEC separation in an affinity screening mass spectrometry (ASMS) workflow.

Integrating SEC into the workflow affords three key benefits. The first is that buffer exchange is not required prior to analysis as buffer components that may suppress protein signal are resolved from the protein peak due to their smaller size. The removal of tedious sample preparation has the potential to increase assay throughput. The second advantage afforded to the SEC native MS workflow is the ability to utilize higher fragment concentrations (Figure 2). This is of considerable benefit for weaker binding fragments which require a higher concentration for the detection of the complex. The use of high concentrations of fragment can be detrimental to an infusion-based assay as non-specific adduct formation can occur during the ionization leading to false positives (Figure 3 bottom).[1] As SEC separates the ligand from the complex, this phenomenon is mitigated increasing the confidence of a positive hit (Figure 2).

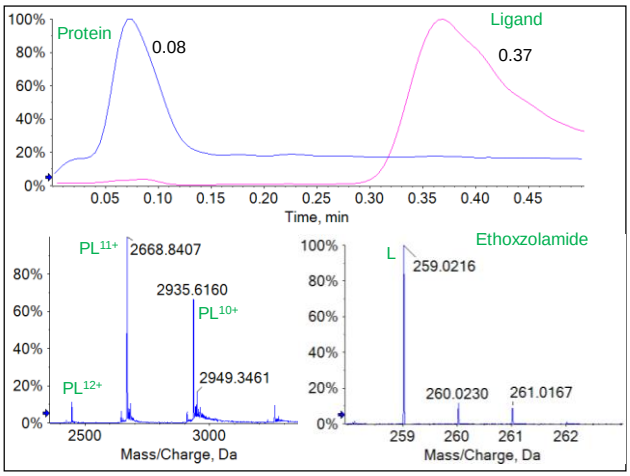

Figure 2. The SEC-MS separation of the 10 µM carbonic anhydrase and ethoxzolamide PL complex (blue) from the unbound ethoxzolamide (pink) with an initial ligand concertation L_0 of 50 µM..

The final benefit of implementing SEC into the workflow is the ability to separate dimethylsulfoxide (DMSO) from the protein. In small molecule drug discovery, the protein is typically stored in DMSO. The presence of DMSO can complicate data interpretation as the presence of a low concentration of DMSO has been shown to shift the charge envelope to a lower charge state (Figure 3 bottom). In addition, DMSO is also a supercharging agent and can concentrate in the gas phase and influence the native structure.[2] In this work, the presence of DMSO does not alter the charge state of the protein when SEC-MS is employed as shown in Figure 3 the dominate charge state with DMSO separated out by SEC-MS remains as +11. In contrast, when a simple FIA analysis is used no separation is achieved, and the presence of DMSO leads to a charge state shift with +8 as the dominate charge state.

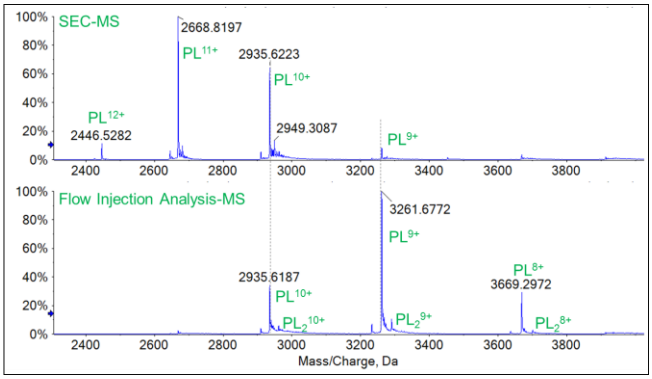

Figure 3. The SEC-MS separation of the carbonic anhydrase and ethoxzolamide complex (top) and flow injection analysis-MS (bottom). P = 10 µM and L_0 = 50 µM..

To ensure the reaction, P + L ⇌ PL had reached a steady state the signal intensity of the P and the PL were monitored over a time course. The fragment was mixed into the carbonic anhydrase and analyzed directly, time point 0 min. The reaction was monitored sequentially at 5 min intervals to until the steady state of PL was reached. Figure 4. demonstrates the time course study of the acetazolamide and carbonic anhydrase complex and highlights that under these reaction conditions the P + L ⇌ PL steady state was achieved by approximately 15 mins. Therefore, all MS analyses were undertaken >15 min post ligand addition.

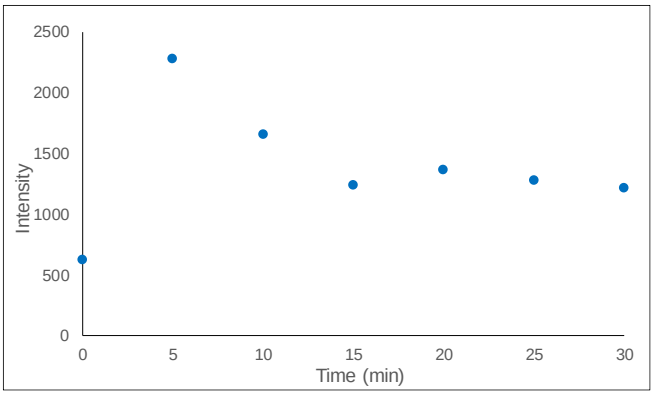

Figure 4. A time course study of the carbonic anhydrase and acetazolamide complex.

The raw native spectra can be quickly reconstructed using the parameters provided in the experimental section. Figure 5. demonstrates the reconstructed spectra of carbonic anhydrase. The mass of the reconstructed protein, 29087.7 Da, compares favorably to the calculated mass of carbonic anhydrase lysozyme II with 5 ppm accuracy.[4] As seen in Figure 5, if the fragment is non-covalently bound to the protein, then the signal of the non-covalent complex of PL is observed in addition to the protein itself. This approach to fragment-based native MS screening provides immediate insight into whether the fragment is a binder or a non-binder. The binding affinity can be calculated by analyzing a ligand titration curve against a fixed concentration of protein. As seen in Figure 4, the binding pocket of carbonic anhydrase is saturated with less than 10 µM of ethoxzolamide. This correlates well with previous studies which have shown the saturation of the binding pocket of carbonic anhydrase at approximately 5. µM.[5]

Additionally, as we are utilizing accurate mass MS, the Δ Da between P and PL provides the molecular weight of the fragment and therefore is highly informative for fragment identification. Figure 5 shows a mass shift of 258.3 Da,

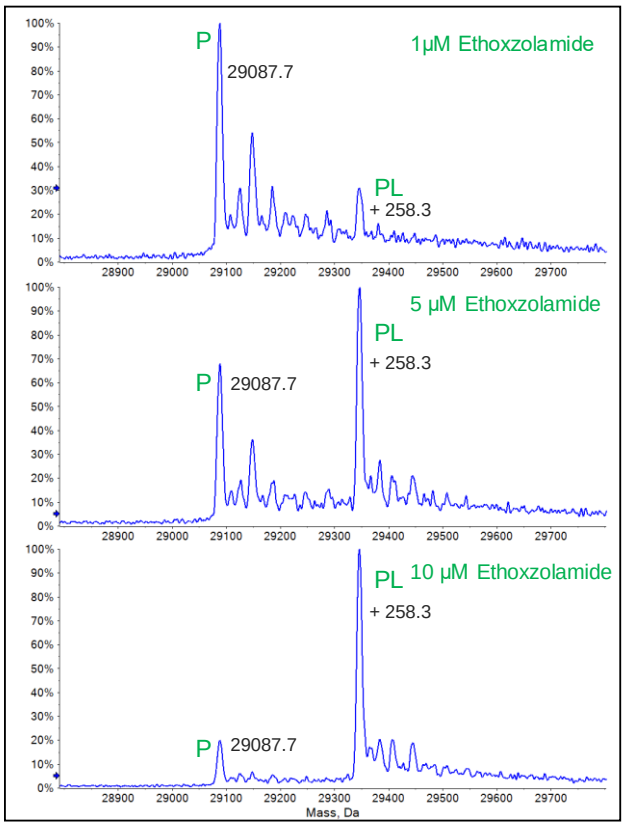

Figure 5. A time course study of the carbonic anhydrase and acetazolamide complex.

However, a simpler approach can be to calculate a relative affinity ranking of the fragment. This can be calculated from the spectral intensity of the protein-ligand complex (%PL) relative to the spectral intensity of the protein.[2] The %PL can be calculated using equation 1.

$$\% PL = [I_{PL}/(I_P + I_{PL})] \times 100 \quad (1)$$

Figure 6. demonstrates the mass shift of ethoxzolamide, acetazolamide, and benzenesulfonamide with mass shifts 258.3, 222.3 and 157.3 Da respectively. The ratio of P to PL can also provide a quantitative insight into the fragment binding affinity. The PL peak for ethoxzolamide and acetazolamide are considerably more intense than the PL peak for the benzenesulfonamide complex suggesting that the ethoxzolamide and acetazolamide have stronger affinities for the carbonic anhydrase.

The relative affinity rankings for ethoxzolamide, acetazolamide and benzenesulfonamide were calculated. The %PL was determined to be to 93%, 84% and 51% for ethoxzolamide acetazolamide and benzenesulfonamide respectively.

Göth and co-workers have determined that a suitable threshold for a positive hit is 20%.[2] Using these criteria fragments analyzed here should be considered positive hit and a suitable fragment for analysis. The %PL determined here are supported by the dissociation constants (K_d) of, 0.7, 7 and 440 nM for ethoxzolamide, acetazolamide and benzenesulfonamide respectively as described by Connell *et al.*.[6]

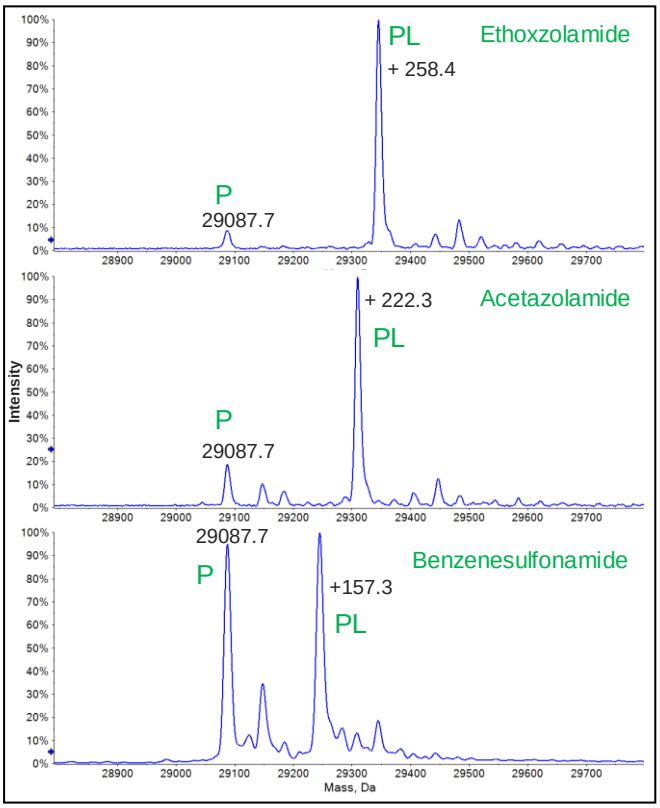

Figure 6. The reconstructed spectra of carbonic anhydrase complexed with ethoxzolamide (top), acetazolamide (middle) and benzenesulfonamide (bottom). P = 10 µM and L_0 = 50 µM.

The robustness of the MS, in-particular the source design, is critical for native analysis due to the continual introduction of a high concentration of salt in the mobile phase. The robustness of the system is demonstrated in Figure 7. This figure shows 50 injections of carbonic anhydrase during an overnight sequence. Each of the 50 raw spectra was reconstructed, and the mass accuracy of the protein was determined. As Figure 7 reveals, the ppm error was within 5 ppm for each of the 50 injections confirming the robustness and suitability of the X500B QTOF system for native MS.

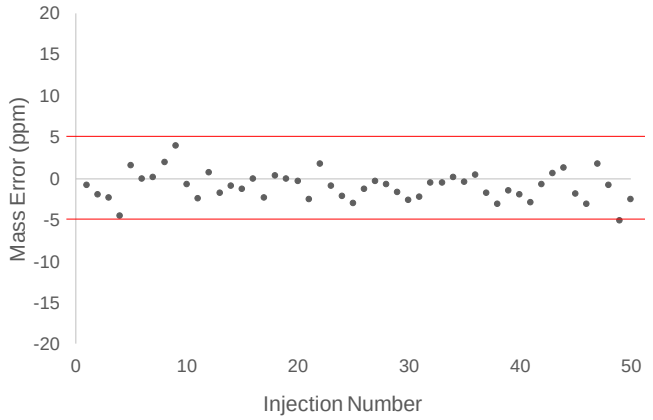

Figure 7. The robustness of the X500B system highlighted by the <5 ppm error over 50 injections.

Conclusions

- Intact native SEC-MS analysis was demonstrated to be suitable for a proof-of-concept fragment-based screening assay. Integrating SEC into the native fragment screening workflow can improve confidence in the results as the complex is separated from excess fragment and buffer components which may be detrimental to native ESI-MS.
- The protein and protein-ligand complex were easily differentiated, and the fragment could be identified with the use of the accurate mass MS
- A relative affinity ranking (%PL) was calculated and could be employed to screen fragment binders from non-binders
- The system robustness of the X500B QTOF system was demonstrated to be suitable for native MS which requires the continual introduction of high salt concentrations

References

1. Kitova, E. N., *et al.* Reliable Determinations of Protein-Ligand Interactions by Direct ESI-MS Measurements. Are We There Yet?. *J. Am. Soc. Mass Spectrom*. 2012, 23, 431–441.
2. Göth, M,. *et al*. Critical Evaluation of Native Electrospray Ionization Mass Spectrometry for Fragment-Based Screening. *Chem. Med. Chem.* 2017., 12, 1201-1211
3. Gao. J., *et al*. Probing the Energetics of Dissociation of Carbonic Anhydrase-Ligand Complexes in the Gas Phase. *Biophysical Journal*. 1999., 76, 3253-3260.
4. Senko, M, W. *et al*. High-Resolution Tandem Mass Spectrometry of Carbonic Anhydrase. *Anal. Chem*. 1994., 66, 415-417.
5. Jecklin, M, C. *et al.* Label-free determination of protein-ligand binding constants using mass spectrometry and validation using surface plasmon resonance and isothermal titration calorimetry. *J. Mol. Recognit.* 2009. 22. 319-329
6. Connell, T, N. *et al*. Solution Based Indirect Affinity Selection Mass Spectrometry-A General Tool For High-Throughput Screening of Pharmaceutical Compound Libraries. *Anal. Chem*. 2014., 86, 7413-7420.

Document number: RUO-MKT-02-9132-A

Headquarters
500 Old Connecticut Path | Framingham, MA 01701 USA
Phone 508-383-7700
sciex.com

International Sales
For our office locations please call the division headquarters or refer to our website at
sciex.com/offices