Real-time enzyme kinetics using RapidFire™ Kinetics (RF-MS Kx) with an Agilent 6410 QqQ

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Abstract

Determining enzyme kinetics and reaction rates is an important tool in understanding mechanism of inhibition in enzymatic assays. The need to purify analytes from salts and buffers prior to MS analysis can cause a major bottleneck in running MS-based kinetic assays. Typically, assays need to be quenched at specific time points and the MS analysis is performed off-line. This is challenging if the enzyme kinetics are fast or if analyte stability problems exist. RapidFire™ technology used in high-throughput screening and *in vitro* ADME applications has been modified for real-time kinetic studies by MS. The RapidFire Kinetics system can analyze reactions in 5 second intervals enabling accurate kinetic determinations.

AKT1/PKBα

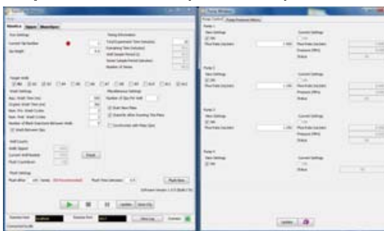
AKT1/PKBα is a serine/threonine kinase that has been studied extensively. AKT1 is a downstream effector of the PI3K pathway and is implicated in oncogenesis and is a potential cancer therapy target. The development of an assay for AKT1 using the RapidFire high-throughput mass spectrometry system has been previously described[1]. The assay was used for the high-throughput screening of a targeted library. The work described here is a continuation of this earlier study and all reagents and methods described are similar.

Assay Conditions

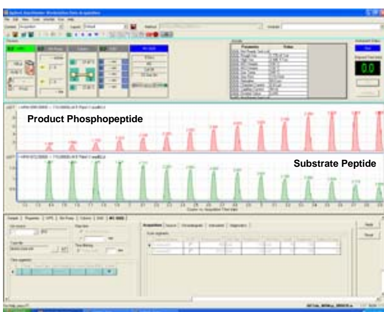
- All assays were monitored using the RapidFire high-throughput mass spectrometry system running the RapidFire Kinetics software package interfaced to an Agilent 6410 triple quadrupole mass spectrometer
  - **Agilent 6410 Conditions**
    - Substrate (M+3H): Q1=599.0 amu, Q3 = 110.0 amu
    - Product (M+3H): Q1 = 572.5 amu, Q3 = 110.0 amu
    - Fragmentor = 135V, Collision Energy = 30V
  - **RapidFire Conditions**
    - Buffer A = Water with 0.09% formic acid, 0.01% TFA; 1.5 mL/min flow rate
    - Buffer B = 80% acetonitrile with 0.09% formic acid, 0.01% TFA; 1.0 mL/min flow rate
    - SPE Column A (reverse-phase C4 chemistry)
- Reactions were initiated on the deck of the RapidFire high-throughput mass spectrometry system with the addition of ATP and substrate to a solution of enzyme
- The substrate peptide and the product phospho-peptide were simultaneously monitored in all experiments

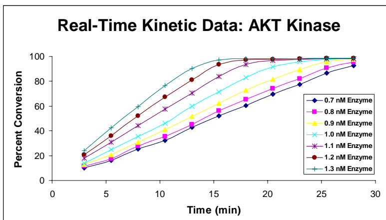
RapidFire System with Agilent 6410

RapidFire Kinetics (RF-MS Kx) Software

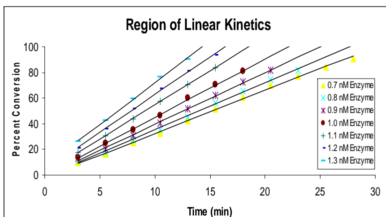


Raw Data Screenshot

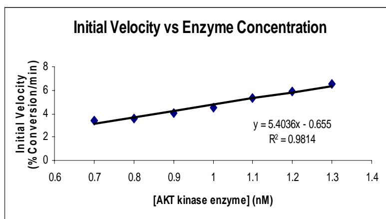


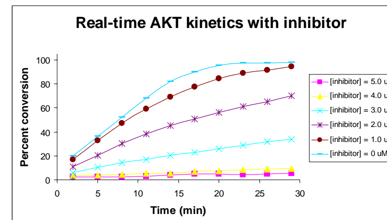


Real-time kinetic measurements of AKT kinase using RapidFire Kinetics. The experiment consists of 7 different enzyme concentrations, run at 10 μM ATP and 5 μM substrate peptide. The reaction was initiated on a plate on the deck of the RapidFire mass spectrometry system and followed for about 30 minutes during which 77 data points were acquired.

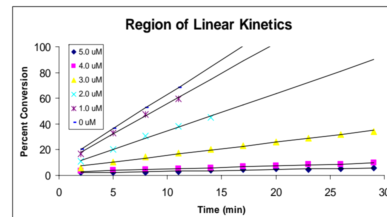


The initial velocity for each of the 7 reactions in the experiment above have been determined from the region of each curve where linear enzyme kinetics are maintained. A plot of initial velocity (calculated as percent conversion per minute) is plotted against the enzyme concentration in the figure below.

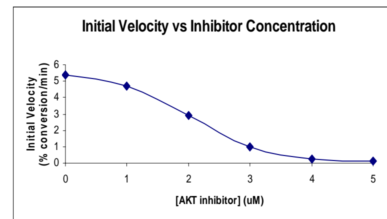


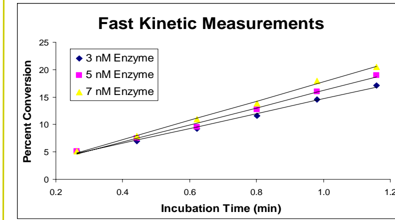


Real-time kinetic measurements of AKT kinase 10 μM ATP and 5 μM substrate in the presence of varying concentrations of an inhibitor. The experiment consisted of 6 different inhibitor concentrations ranging from 0 to 5.0 μM. The effect of relatively small variations in inhibitor concentration (total range = ½ log) is clearly evident in reaction rate.



The initial velocity for each of the 6 reactions in the experiment above have been determined from the region of each curve where linear enzyme kinetics are maintained. A plot of initial velocity (calculated as percent conversion per minute) is plotted against the inhibitor concentration in the figure below.





Accurate determination of the rate of conversion in an assay with rapid turnover due to high enzyme concentration 10 μM ATP and 5 μM substrate. Three separate experiments are shown. A total of 6 MS data points are acquired in under 1 minute enabling the difference in reaction rates as a function of enzyme concentration to be accurately quantified.

Conclusions

- A series of real-time kinetic experiments on AKT1/PKBα kinase using a RapidFire mass spectrometry system running the Kinetics Software interfaced to an Agilent 6410 QqQ MS were run
- Reactions were initiated on the deck of the RapidFire system and the relative amounts of substrate peptide and product phospho-peptide were monitored at rates up to 7 seconds per data point
- Kinetic measurements with soluble enzymes on the RF-MS Kx platform have many advantages over endpoint measurements:
  - Greatly simplified workflow that precludes the need to prepare time course plates by quenching assays at controlled times
  - Real-time determination of kinetic parameters
  - Determination of binding constants and mechanism of inhibition
  - Accurate resolution of assay variations too small to be quantified in endpoint experiments (ie: variations that approach the CV of an endpoint assay)
  - Tracking reactions with fast kinetics that make it unsuitable to prepare time courses

References

1) Quercia AK, LaMarr WA, Myung J, Özbal CC, Landro JA, Lumb, KJ. "High-Throughput Screening by Mass Spectrometry: Comparison with the Scintillation Proximity Assay with a Focused-File Screen of AKT1/PKBα" *Journal of Biomolecular Screening* **2007** Jun;12(4):473-80