

LC/MS Method for the Analysis of Guanine Deaminase

Justin M. Godinho¹, Benjamin P. Libert¹ and Barry E. Boyes^{1,2}

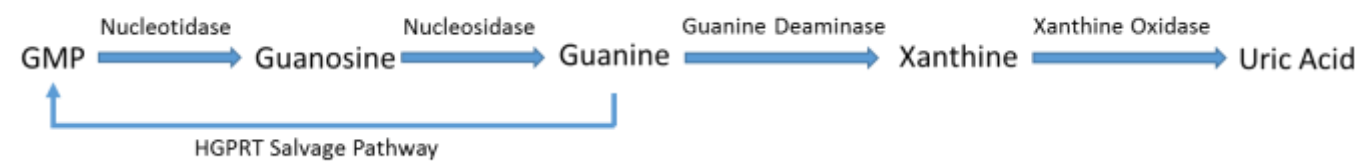
¹Advanced Materials Technology Inc., Wilmington, DE; ²Complex Carbohydrate Research Center, University of Georgia, Athens, GA

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Introduction

Guanine deaminase, also known as "nedasin" or "cypin", catalyzes the purine catabolic commitment step from guanine (G), through xanthine (X), to the elimination product, uric acid. In rabbit and human, the enzyme appears to exist predominantly cytoplasmic as a homodimer, with catalytic domains for the Zn²⁺-dependent hydrolytic deamination of guanine to xanthine plus ammonia. Genomic details for the GDA gene are well mapped, and expression profiling in certain tissues and organisms has been initiated, although the complement of various transcript variants is incomplete.

- Structure: c. 50 kDa subunits with sequences that vary at internal and terminal sites, due to exon selection; at least 4 significant transcripts predicting proteins of various lengths are known.
- Protein Interactions: tubulin, snapin, and the post-synaptic domain protein 95 (PSD-95).
- PSD-95 binding is through the PDZ binding motifs present at the C-terminus of the dimeric structure.
- Sequence variants occur mostly at the protein binding domains, although minor variants lack the deaminase catalytic site.
- In mammalian brain, high enzymatic levels are in telencephalic brain regions; very low levels in white matter and cerebellum; moderate levels in liver and certain other organs; moderate to low levels in plasma/serum, notably altered by liver dysfunction.
- Actual role of guanine deaminase in specialized organ metabolism and synaptic physiology is uncertain, and relatively little is known about the enzyme characteristics outside of rabbit, with significant detail available detail on gene expression patterns, but not at the protein level. Available protein expression surveys have suggested extensive post-translational modifications, that have not yet been detailed with certainty.
- Few useful inhibitors have been described for the enzyme.

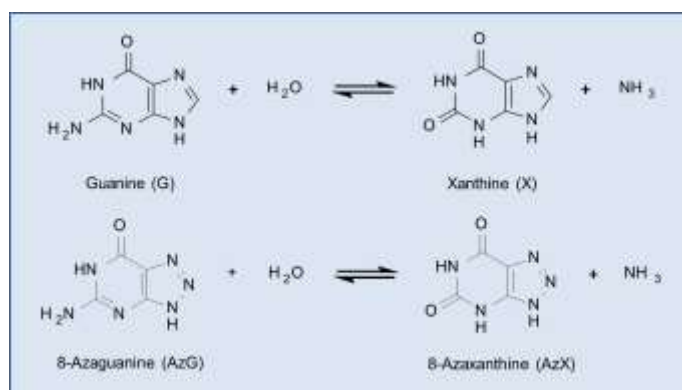


Purpose

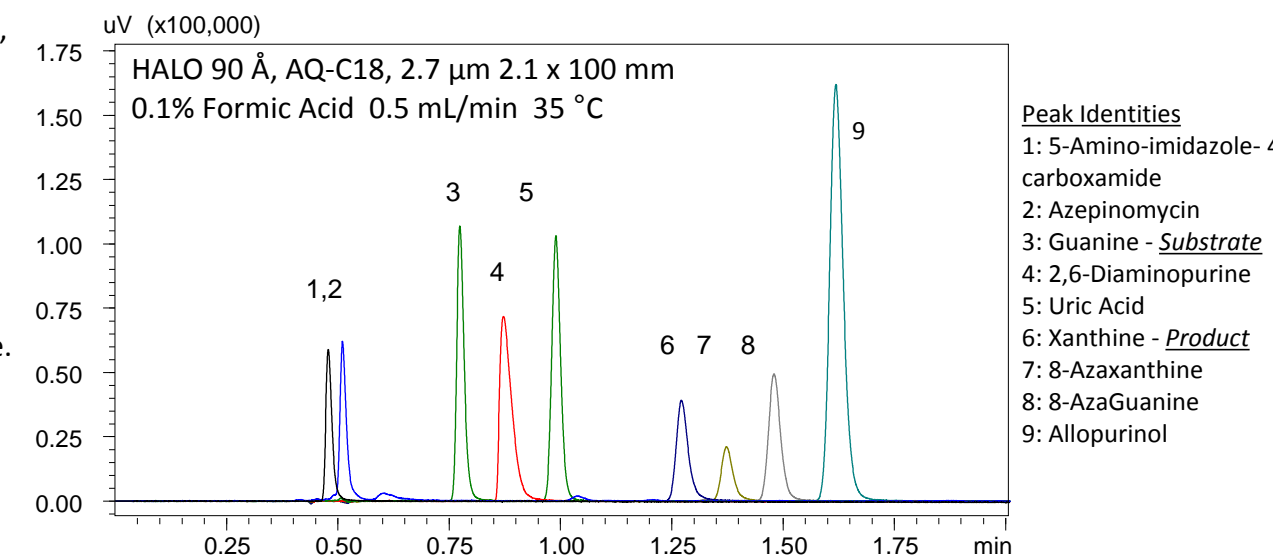
- Previous enzyme assays are problematic, using either the subtle spectral shift from G to X in absorbance spectrophotometry, or coupled enzyme assay with capture of X by Xanthine Oxidase to generate H₂O₂, with a fluorescent dye readout of oxidation products.
- A high throughput spectrophotometric assay uses ammonium liberation, but has uncertain specificity and requires guanine as substrate near the limit of aqueous solution solubility. Although useful for screening purposes, lacks specificity and dynamic range.

To measure tissue enzyme levels, follow purification processes, and assay enzyme kinetics and effects of inhibitors, highly specific LC/UV and LC/MS methods would be useful. Our assay measures direct generation of product xanthine from the deamination of guanine, using rapid separation of these highly polar metabolites by a new reversed-phase material, HALO AQ-C18. To obtain high sensitivity and selective detection, LC-MS/MS was employed in the SRM mode. At high sensitivity, absorbance detection suffers from selectivity concerns. The assay performance was assessed across a range of substrate concentrations, and in the presence of known and potential competitive inhibitors. We have further compared the features of the LC-MS assay using two presently known substrates for guanine deaminase, Guanine and 8-AzaGuanine. We compared kinetic properties of a commercially available recombinant enzyme to one substantially purified from bovine brain.

Substrates for Guanine Deaminase



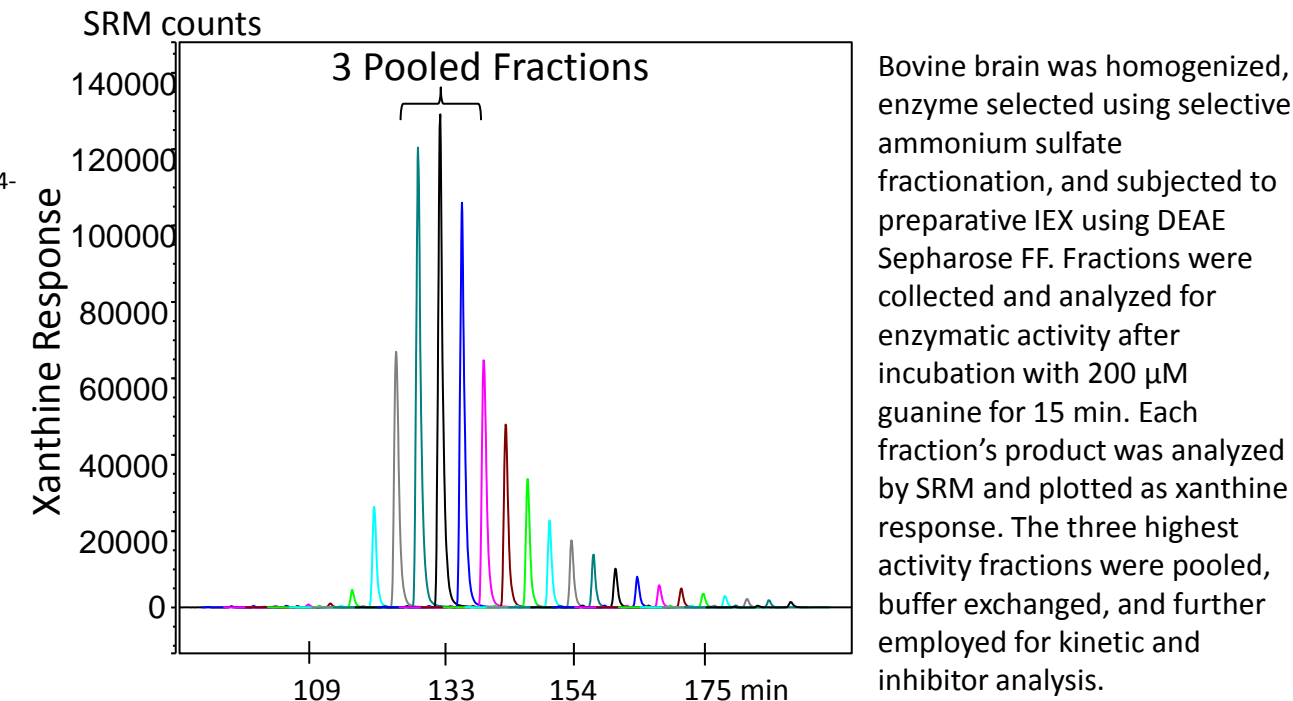
Efficient Separation of Highly Polar Purine Metabolites with HALO® 90 Å, AQ-C18



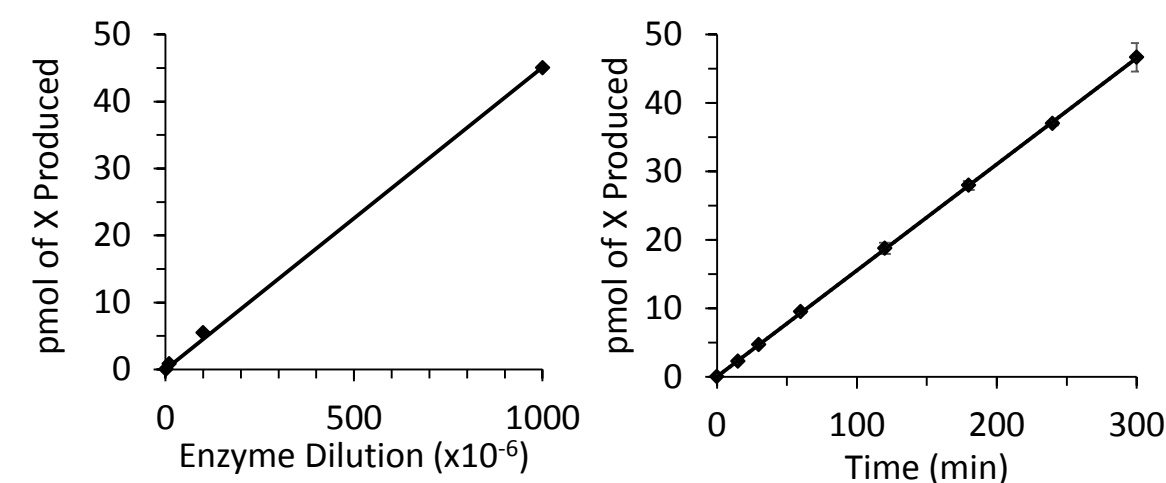
Standard Microplate Enzyme Assay and LC/MS Conditions

Shimadzu Nexera
HALO® 90 Å AQ-C18, 2.7 μm, 2.1 x 75 mm
A=0.1% Formic Acid
B=Acetonitrile
0.5 mL/min, 35 °C, 265 nm, MTP, autosampler 25 °C
Gradient: 0%B 0-1.5min to 70%B @1.7-2.2min to 0%B @2.3-4.2 min
Thermo Orbitrap Velos Pro ETD
MS Run Time (min): 2.20; divert first 0.8 min from MS source
ITMS (-) 0.7 to 1.7 min; ITMS (+) 1.7 to 2.0 min; ITMS (-) 2.0 to 2.2 min
HESI Source Type; Capillary 350 °C; Heater 325 °C; Sheath Gas 40; Aux Gas Flow 10
ITMS Full AGC 50K; ITMS SIM AGC 100K; ITMS MSn AGC 50K
Source Voltage (-kV) 2.70; Ion Trap Full Max Ion Time (ms) 200;
Segment (SEG) Information/Scan Event Details:
ITMS - c low injrf=70.0 (151.00000)->oS(106.0-110.0)
MS/MS: AT CID CE 30.0% Q 0.350 Time 10.000 IsoW 0.8, CV = 0.0V
Assay Conditions
50 mM Bicine pH 7.8, 10 μg/mL BSA, varying Guanine or 8-azaGuanine.
Reaction at room temperature with agitation for >15 minutes, typical volume of 100 μL
Stop reaction by addition of 0.1 volume of 10% Formic Acid.
All purine reagent stocks are maintained in 15 mM NaOH/0.15 mM MgCl₂ until dilution.
Enzyme incubations are conducted in standard 96 well reaction plates, which are directly loaded in the Nexera autosampler, for LC/MS analysis of reaction products.

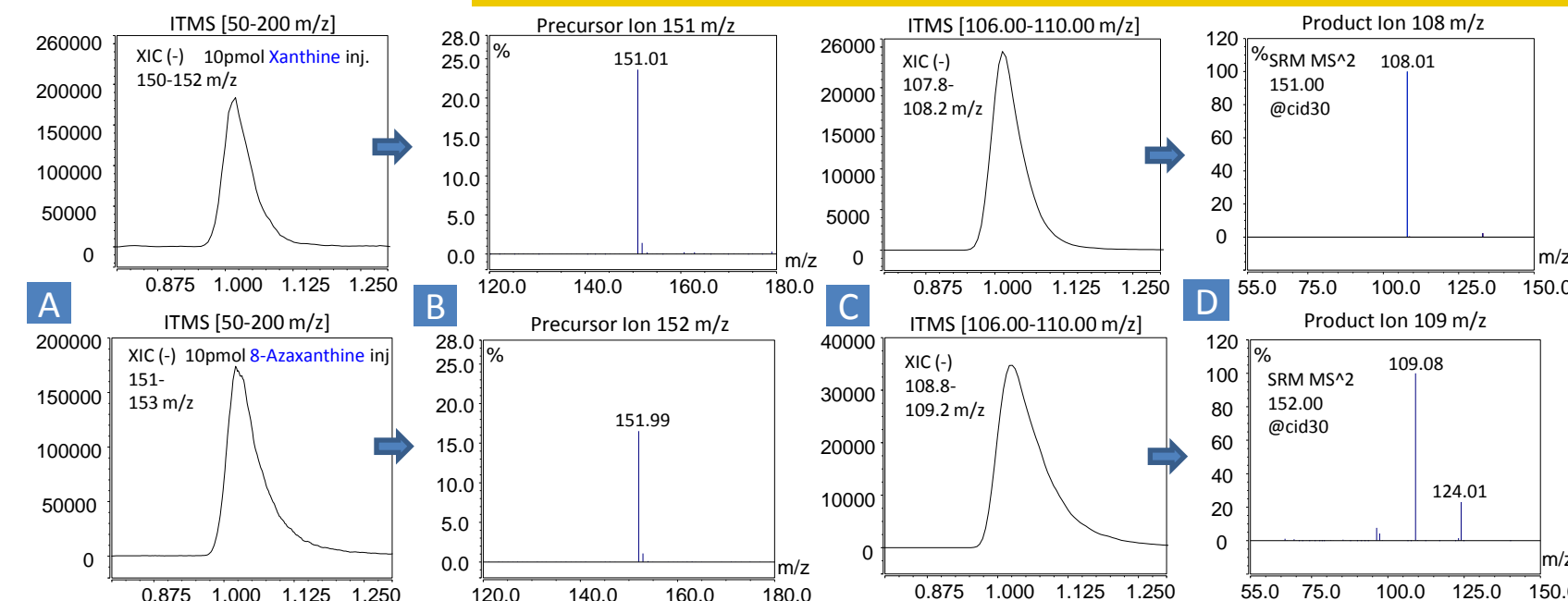
Preparation of GDA from Bovine Brain Homogenate



Validation of Assay Conditions



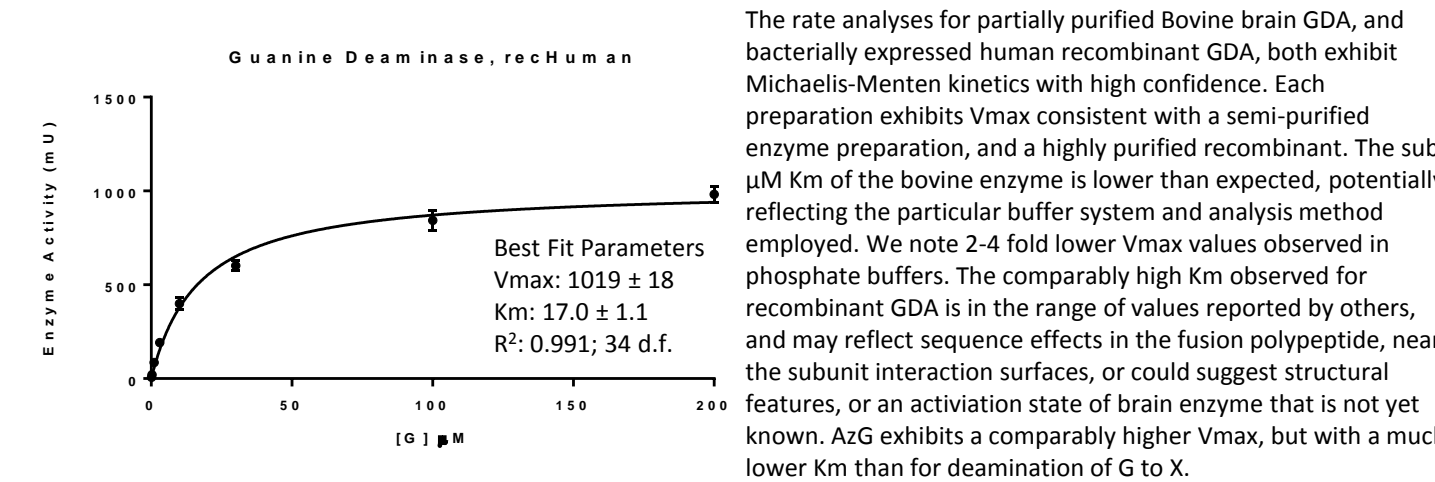
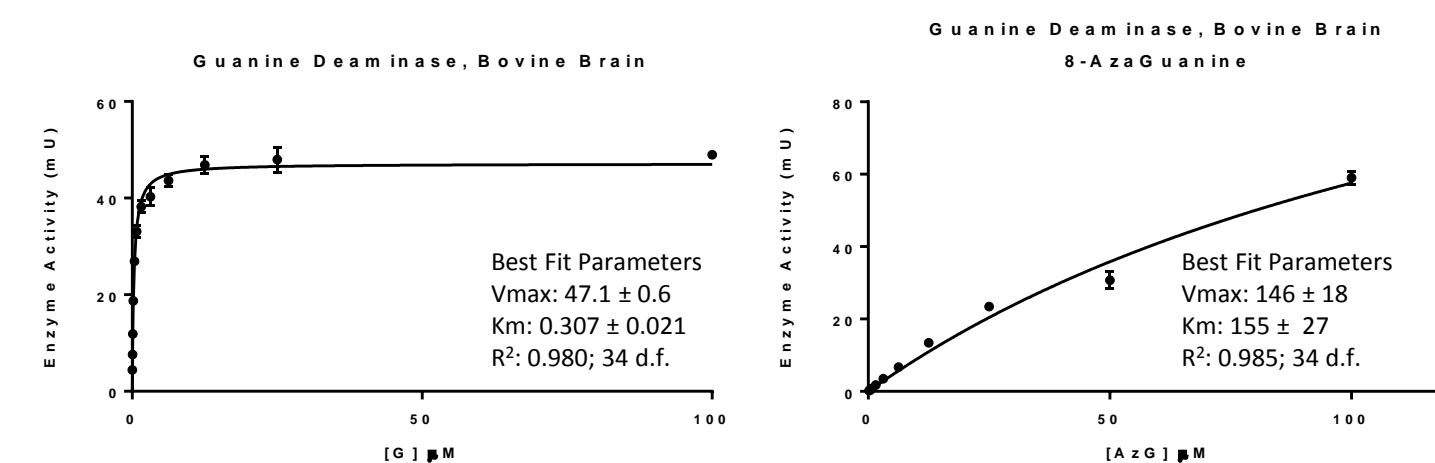
Selective Detection of Enzyme Reaction Products using SRM in the Ion Trap



Panels A-D represent steps taken for selective detection of the enzyme reaction product, xanthine and 8-azaxanthine. Panel A shows a chromatogram of a 10 pmol injection of X or AzX using the extracted ions from 150 to 152 m/z. Panel B shows the spectra with xanthine and azaxanthine precursor ions [M-H]⁻ at 151 m/z (X) or 152 (AzX) chosen for fragmentation. Panel C shows the extracted ion chromatograms centered at dominant fragment ions, and Panel D shows the product ions 108 m/z monitored for X and 109 m/z for AzX.

Xanthine Theoretical Mass [m/z]
152.033 Da monoisotopic, [151.025 m/z (-)]
8-azaxanthine Theoretical Mass [m/z]
153.029 Da monoisotopic, [152.021 m/z (-)]

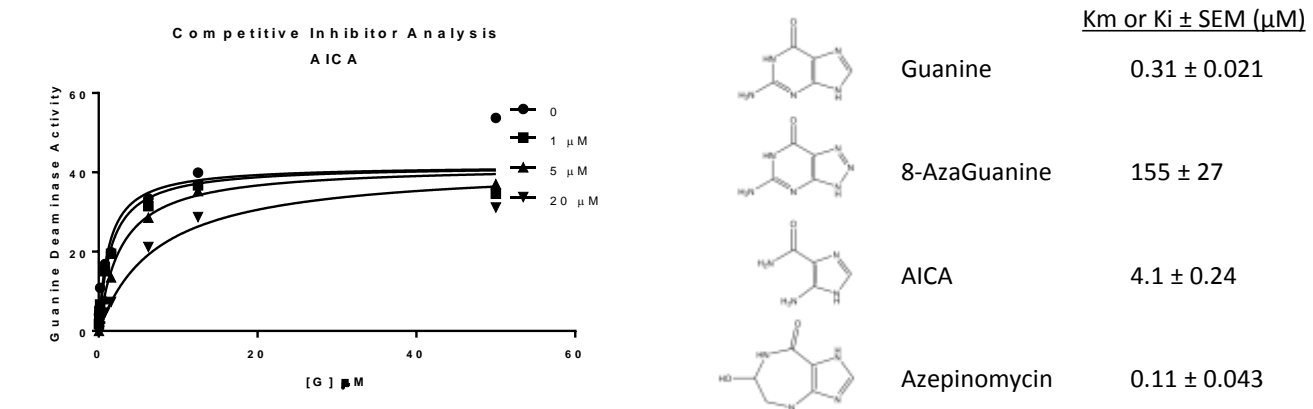
Kinetic Analysis of Recombinant and Bovine Brain Guanine Deaminase



The rate analyses for partially purified bovine brain GDA, and bacterially expressed human recombinant GDA, both exhibit Michaelis-Menten kinetics with high confidence. Each preparation exhibits Vmax consistent with a semi-purified enzyme preparation, and a highly purified recombinant. The sub-μM Km of the bovine enzyme is lower than expected, potentially reflecting the particular buffer system and analysis method employed. We note 2-4 fold lower Vmax values observed in phosphate buffers. The comparably high Km observed for recombinant GDA is in the range of values reported by others, and may reflect sequence effects in the fusion polypeptide, near the subunit interaction surfaces, or could suggest structural features, or an activation state of brain enzyme that is not yet known. AzG exhibits a comparably higher Vmax, but with a much lower Km than for deamination of G to X.

Analysis of Enzyme Inhibitors

Compounds that are potential inhibitors of guanine deaminase have been investigated using the LC/MS assay described, with bovine brain enzyme. As shown for the example of the known competitor inhibitor, 5-aminoimidazole-4-carboximide (AICA), inhibition is concentration dependent, and exhibits typical enzyme kinetic response (apparent Km increase, with no change in Vmax). The effective Ki values for three structurally-related purinergic compounds are shown. Azepinomycin is confirmed to be the most effective inhibitor known to date.



Conclusions

A highly selective and sensitive LC/MS method is demonstrated for the analysis of the enzyme GDA. The HALO 90 Å, AQ-C18, 2.7 μm column was chosen for its ability to resolve the very polar purine metabolites and potential inhibitors of interest. The column's robust stability in 100% aqueous conditions allowed for high throughput assays and the assessment of many thousands of measurements, using a microtiter plate assay format. Studying enzyme kinetics with a highly selective LC/MS/MS method mitigates many of the limitations of previous methods, including lack of sensitivity in complex samples. Selection of the enzyme reaction product through SRM in the ion trap allows for specific detection of the product without interference due to endogenous metabolites and exogenously added potential inhibitors and potentiators.

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