A Simple Fluorometric Assay for Distinguishing Creatine from Creatine Prodrugs

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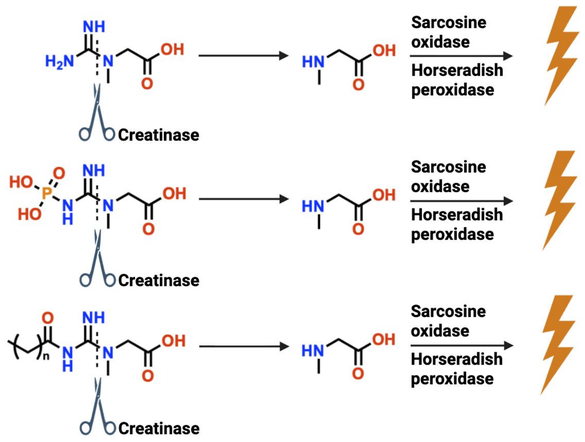
Introduction

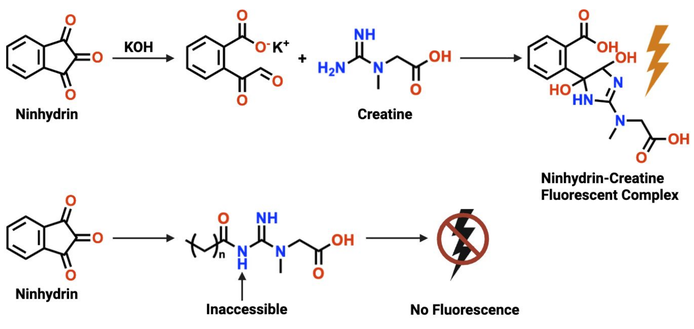
Creatine Transporter Deficiency (CTD) is a rare metabolic disorder, in part characterized by impaired transport of creatine into and throughout the brain. This has motivated the development of **creatine-based prodrug therapeutics** designed to enable transporter-independent cellular uptake of creatine. Accurate and high-throughput analytic methods capable of **distinguishing creatine from structurally related prodrugs** are therefore critical for therapeutic characterization and evaluation.

Commercially available colorimetric and fluorometric creatine detection kits are widely used due to their simplicity and sensitivity; however, these assays **broadly detect creatine-containing molecules**, thereby limiting their utility in prodrug development applications.



Scheme 1. Commercially available creatine detection kit mechanism of action.

Ninhydrin-based creatine detection methods were originally developed in the late 1950s (Conn & Davis, 1959, Conn, 1960, Conn & Anido, 1966) and were later introduced to the CTD research field by Dr. Schulze's group (Tropak et al., 2023). In the present study, we investigated the ability of the ninhydrin assay to **distinguish creatine from creatine prodrugs and other guanidine-containing metabolites**.



Scheme 2. Ninhydrin-based creatine detection predicted mechanism of action.

Methods & Materials

Characterization of Ninhydrin Fluorescence: Kinetics, Dynamic Range, Compound Specificity, and In Vitro Creatine Detection

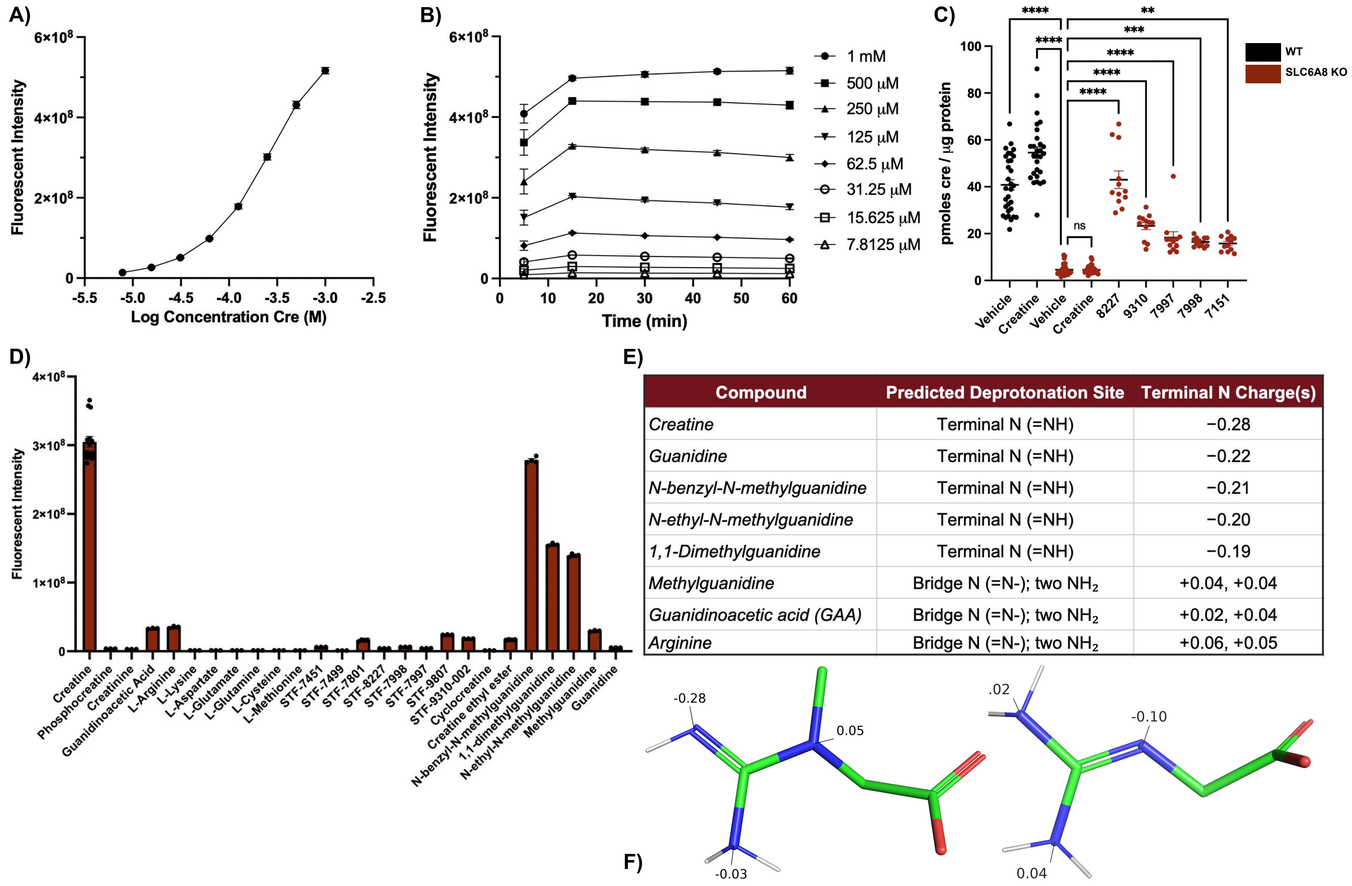
1. **Reagent Prep:** 1% ninhydrin + 10% KOH in 80% EtOH; all samples/standards in 80% EtOH
2. **Sample Prep:** *Characterization:* creatine serial dilution or guanidine compounds at 250 µM in 80% EtOH. *Cell-based:* treat cells (100 µM, 4 hr) → harvest → lyse → deproteinize 1:5 in 80% EtOH at −20°C.
3. **Standard Curve** 9–10 point creatine dilution in 80% EtOH; include blank
4. **Plate Loading & Reaction** 2:1:1 sample:ninhydrin:KOH (e.g. 80 + 40 + 40 µL); seal, spin, shake
5. **Incubation** Dark, RT; kinetics: multi-timepoint, all others: 1 hr fixed endpoint
6. **Read** Ex 310 / Em 510 nm
7. **Analysis** Subtract blank; interpolate from std curve *(Cell-based: normalize to protein BCA)*

Scan me for detailed protocols!

Quantum Mechanical Investigation of Differential Ninhydrin Reactivity:

Tautomeric states and charge distributions of guanidine-containing compounds were determined using **Molecular Operating Environment** (MOE; Chemical Computing Group). Tautomers at basic pH were assigned using the MOE promoter function, which applies the rule-based method of Oellien et al. (2006). Partial charges were calculated via the semiempirical quantum mechanical method **MOPAC**, as implemented in MOE (Stewart, 1993).

Results



Compound	Predicted Deprotonation Site	Terminal N Charge(s)
Creatine	Terminal N (=NH)	−0.28
Guanidine	Terminal N (=NH)	−0.22
N-benzyl-N-methylguanidine	Terminal N (=NH)	−0.21
N-ethyl-N-methylguanidine	Terminal N (=NH)	−0.20
1,1-Dimethylguanidine	Terminal N (=NH)	−0.19
Methylguanidine	Bridge N (=N-); two NH_2	+0.04, +0.04
Guanidinoacetic acid (GAA)	Bridge N (=N-); two NH_2	+0.02, +0.04
Arginine	Bridge N (=N-); two NH_2	+0.06, +0.05

Figure 1. Characterization of Ninhydrin-based Creatine Detection. A) Logarithmic creatine standard curve (8-point, 2-fold serial dilution, 1 mM–7.8125 µM; measured at 1 hr; n=3 ±SEM). **B)** Kinetics of the ninhydrin-creatine fluorescent product across multiple creatine concentrations (n=3 ± SEM). **C)** Intracellular creatine in HAP1 WT and SLC6A8 KO cells after 4 h treatment with vehicle, 100 µM creatine, or creatine prodrug (n=12–32 ± SEM; one-way ANOVA, Dunnett's vs. vehicle-treated KO). **D)** Ninhydrin reactivity of creatine and related guanidine-containing metabolites at 250 µM, measured at 1 hr (n=3–15 ± SEM). **E)** Quantum mechanical simulations of guanidine partial charges under basic (pH 13) ninhydrin reaction conditions. **F)** Representative partial charge maps; left: creatine, right: guanidinoacetate.

Conclusions

The ninhydrin assay is a **simple, sensitive, and selective** fluorometric method that **distinguishes creatine from its prodrugs**, guanidinoacetate, arginine, and other guanidine-containing metabolites, a specificity that commercial creatine kits lack. It is linear across a broad concentration range, works in cell-based systems, and its selectivity is corroborated by **quantum mechanical modeling**.

References

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