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Progress Towards Simple and Direct Detection of Adenylosuccinate Lyase Deficiency in Human Urine

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Abstract

A rhodamine based boronic acid linearly responds to increasing 5-aminoimidazole-4-carboxamide riboside (AICAr) concentrations in human urine. This method is thus an advance in detecting adenylosuccinate lyase (ADSL) deficiency as AICAr is a model riboside for the ADSL substrates succinyladenosine (S-Ado) and succinylaminoimidazolecarboxamide riboside (SAICAr). ADSL deficiency is a rare but devastating disease of *de novo* purine synthesis in infants. Its diagnosis is also significant as it is one of the autism spectrum disorders.

Multidimensional fluorescence measurements have been widely used in clinical analysis,^[1,2] food science,^[3] and environmental analysis.^[4] Excitation-emission matrices (EEMs) in which the fluorescence response of a sample is monitored at multiple emission wavelengths upon excitation at multiple different wavelengths have a greater information content as compared with more conventional emission spectra collected upon excitation at a single wavelength. This greater information content allows for characterization or determination of substances in complex multi-component mixtures or samples in biological media with a complex background, i.e. methotrexate in urine,^[1] or salicylate in plasma samples.^[2]

Recently, we employed the multidimensional nature of fluorescence in the detection of multiple disease biomarkers using a single indicator via an optimized data collection protocol which scanned EEMs, monitoring the indicator's time- and wavelength dependent response every 4 min for 1 h.^[5] The indicator used was a rhodamine based boronic acid dye (**1**) that we have previously demonstrated displays significant fluorescence enhancement in response to ribose and its derivatives.^[6] Upon further investigation it was discovered that different biomarker analytes displayed distinct time- and wavelength-dependent spectral features. For example, the indicator was shown to simultaneously respond to both fructose and a ribose nucleoside (5-aminoimidazole-4-carboxamide riboside) in mixtures with the response of each occurring in characteristically different spectral regions.^[5] This raises the possibility of detecting an important ribose-containing biomarker in more complex biological media containing many potential interferences.

Herein we investigate the feasibility of using **1** (Fig. 1) for the detection of 5-aminoimidazole-4-carboxamide riboside (AICAr) in human urine. AICAr is a dephosphorylated form of AICAR (AICA-ribotide, also known as ZMP) that is a biomarker of the purine biosynthetic pathway disorder, AICA-ribosiduria.^[7] It is also a commercially available surrogate of succinyladenosine (S-Ado) and succinylaminoimidazolecarboxamide riboside (SAICAr) which accumulate at extremely high mM levels in urine of adenylosuccinate lyase (ADSL) deficient patients.^[8] Structures are shown in Fig. 1. These

serious inborn errors of purine metabolism cause autistic features, mental retardation, and/or neonatal seizures, making this a challenging diagnostic disease.^[9] The Bratton-Marshall reaction has been generally used for the screening, but it can lead to a high rate of false positives.^[8] Some chromatographic techniques such as TLC, GC-MS, or HPLC are currently used for detection,^[9] but simple and improved methods to detect elevated levels of S-Ado and SAICAr at physiologically relevant concentrations (mM in ADSL deficient as compared with undetectable levels in that of healthy groups) directly in urine of potentially ill patients without the need for separations or sample processing are desired.

Rhodamine boronic acid **1** responds to increasing AICAr concentrations with increased fluorescence emission near 590 nm upon excitation in the region of 560–570 nm (Fig. 2). Increasing concentrations of buffered aqueous AICAr solutions were added to DMSO solutions of **1** such that the final solvent composition was 90 % DMSO: 10 % 25 mM pH 7.5 phosphate buffer with a final dye concentration of 40 μ M, and AICAr ranging from 0–200 μ M. It is clear the fluorescence intensity increased linearly as the concentration of AICAr increased. Regression analysis (inset Fig. 2d) of Δ Intensity versus AICAr concentration yielded a straight line ($y = 5.1822x + 66.453$; $R^2 = 0.983$) with a limit of detection of ~ 20 μ M, three orders of magnitude below SAICAr and S-Ado concentrations in the urine of ADSL deficient patients.

Compound **1** also responds to increasing AICAr in a biological matrix, i.e. human urine (Fig. 3). Diluted samples containing 5 % human urine were chosen as a starting point for investigation in this proof of concept because 1) the limit of detection for AICAr in an ideal solvent system containing no interferences is much lower than required, and 2) urine contains many potential interferences and possible quenchers of the fluorescence signal which makes it a difficult matrix to work in. It is clear from Fig. 3a that the indicator responds strongly in the short wavelength region (emission near 540 nm upon excitation near 510 nm) to unidentified interferences present in the urine sample. This region, however, is far removed from the spectral region where the indicator responds to AICAr (emission near 590 nm upon excitations in the region of 560–570 nm). It is interesting to note that response in the short wavelength region is similar to that which was previously reported for fructose.^[5] Comparison of Fig. 3a and 3b demonstrates an increase in long wavelength emission for an AICAr-spiked urine sample. Δ Intensity plots (Fig. 2c and 3c) are very similar in shape for the system containing only buffer and that containing urine and its associated interferences.

When increasing concentrations of buffered aqueous AICAr solutions were combined with healthy human urine and added to DMSO solutions of **1** such that the final solvent composition was 90 % DMSO: 5 % 25 mM pH 7.5 phosphate buffer: 5 % urine with a final dye concentration of 40 μ M and AICAr ranging from 0–200 μ M, the longer wavelength fluorescence signal was observed to increase. Regression analysis (inset Fig. 3d) of Δ Intensity versus AICAr concentration in the long wavelength region yielded a straight line ($y = 0.5557x + 8.8252$; $R^2 = 0.982$). The short wavelength region was not sensitive to the addition of AICAr (Fig. 3e). Concentrations noted in Fig. 3 correspond to AICAr levels above undiluted healthy urine ranging from 0.25–4.0 mM. A similar detection limit of, 20 μ M was observed which corresponds to detecting 0.4 mM AICAr above that of healthy human urine.

These results provide a method to screen for elevated AICAr in a urine sample without the need for separation or sample processing. Work is ongoing to improve the quantification of AICAr with a calibration tool such as PARAFAC.^[2,10] Data processing combined with normal standard addition protocols should allow efficient extraction of the analyte response distinct from interferences and indicator background signals.

In conclusion, we have demonstrated the ability to detect elevated levels of AICAr, a commercially available surrogate of S-Ado and SAICAr, in healthy human urine at levels comparable to those found in ADSL deficient patients. This is a step towards a simple screen for elevated S-Ado and SAICAr.

Experimental

All chemicals were purchased from Sigma-Aldrich and used without further purification. Normal human urine was purchased from Innovative Research. Compound **1** was prepared via microwave-assisted synthesis according to procedures reported earlier.^[5] An InitiatorTM microwave synthesizer (Biotage) was used. Fluorescence spectra were measured on a Varian Cary Eclipse fluorescence spectrophotometer (Agilent Technologies). EEMs were collected every 4 min for 1 h; excitation at 470–620 nm each 10 nm, emission at 520–690 nm each 2.5 nm, both band pass = 5 nm. Dye was dissolved in DMSO and either buffered AICAr solutions or healthy human urine spiked with buffered AICAr was added. One part buffer or aqueous buffer/urine was added to 9 parts dye in DMSO. All samples were vortexed for 15 s before fluorescence scanning. EEM data were processed in *MATLAB Version 7.9.0 (R2009b)*.

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Fig. 1.
Structures of rhodamine boronic acid (**1**), AICAr, SAICAr, and S-Ado.

Fig. 2. EEMs and emission spectra demonstrating the responses of **1** to AICAr at 8 min after mixing in a simple 10 % buffer: 90 % DMSO solvent system. (a) 40 μM **1**; (b) **1** plus 200 μM AICAr; (c) $\Delta\text{Intensity}$, (b) minus (a); (d) emission spectra as a function of AICAr concentrations when excited at 560 nm and linear regression (inset). White lines in (a) and (b) represent the excitation wavelength and emission range for spectra plotted in (d). White circle in (c) represents the data point used for the regression analysis in (d).

Fig. 3.

EEMs and emission spectra demonstrating the responses of **1** to AICAr at 20 min after mixing in 5 % healthy human urine: 5 % buffer: 90 % DMSO. (a) 40 μ M of **1** in presence of 5 % urine; (b) **1** in presence of 5 % urine plus 200 μ M AICAr; (c) Δ Intensity, (b) minus (a); (d) emission spectra as a function of AICAr concentrations when excited at 560 nm and linear regression (inset); (e) emission spectra as a function of AICAr concentrations when excited at 520 nm. White lines in (a) and (b) represent the excitation wavelength and emission range for spectra plotted in (d). White circle in (c) represents the data point used for the regression analysis in (d).