Piper T, Thomas A, Schänzer W, Thevis M

Nitrogen isotope ratios of endogenous urinary AICAR

Center for Preventive Doping Research - Institute of Biochemistry, German Sport University Cologne, Cologne, Germany

Abstract

AICAR (5-Aminoimidazole-4-carboxamide 1β-D-ribofuranoside) is prohibited in sport according to rules established by the World Anti-Doping Agency. Doping control laboratories identify samples suspicious of AICAR abuse by measuring its urinary concentration and comparing the observed level to naturally occurring concentrations. As the inter-individual variance of urinary AICAR concentrations is large, this approach requires a complementary method to unambiguously prove the exogenous origin of AICAR. In parallel to carbon isotope ratios the $^{15}N/^{14}N$ ratio of AICAR should allow for allocation of urinary AICAR regarding its endogenous or exogenous source.
The nitrogen atoms in AICAR (being part of the purine biosynthesis pathway) derive from glutamine, glycine and aspartatic acid. In parallel to amino acids investigated in hair, plasma or bone tissue it is expected that endogenous nitrogen isotope ratios show enriched values of up to +15 ‰ ($\delta^{15}N_{AIR}$).
Sample preparation was done accordingly to the method employed for carbon isotope ratios. As expected, limits of detection were found approx. 5 times higher than for carbon measurements resulting in a minimum required urinary concentration of 1000 ng/mL AICAR for a 3 mL aliquot. Within this preliminary investigation 4 blank urines containing only endogenous AICAR (as ensured by $^{13}C/^{12}C$ measurements), 1 post administration sample collected after oral ingestion of 10 g of AICAR and a reference standard were determined. The standard was also investigated by means of elemental analyzer/isotope ratio mass spectrometry to test for bias introduced by gas chromatographic separation and online conversion of samples prior to isotope ratio measurements. Reference material IAEA 600 was used to calibrate nitrogen tank gas.
Within these first measurements no significant difference was found between the nitrogen isotope ratios of endogenous AICAR, the post-administration value and the standard material. Further AICAR standards and seized black market products should be analyzed to verify a similar homogenous isotopic signature for nitrogen as was found for carbon isotope ratios so far.

Introduction

AICAR (5-Aminoimidazole-4-carboxamide 1β-D-ribofuranoside) is prohibited in sport according to rules established by the World Anti-Doping Agency. Doping control laboratories identify samples suspicious of AICAR abuse by measuring its urinary concentration and comparing the observed level to naturally occurring concentrations. As the inter-individual variance of urinary AICAR concentrations is large, this approach requires a complementary method to unambiguously prove the exogenous origin of AICAR. Analogous to carbon isotope ratios the $^{15}N/^{14}N$ ratio of AICAR should allow for identification of urinary AICAR regarding its endogenous or exogenous source.
The nitrogen atoms in AICAR (being part of the purine biosynthesis pathway) derive from glutamine, glycine and aspartatic acid (Figure 1). With respect to amino acids investigated in hair, plasma or bone tissue it is expected that endogenous nitrogen isotope ratios show enriched values in the range of $\delta^{15}N_{AIR}$ = +7 to +15 ‰ [1-3].

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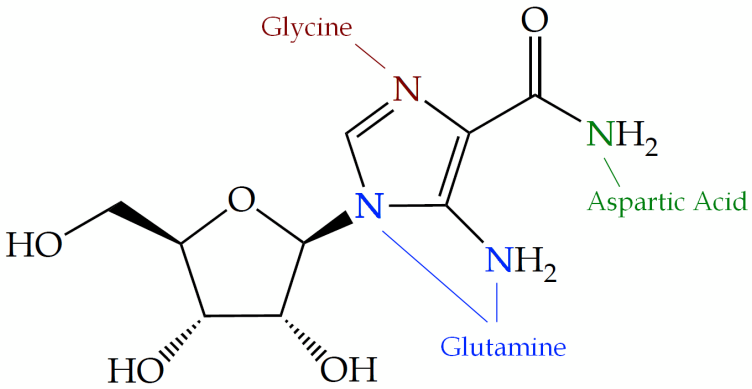

Figure 1: Structural formula of AICAR with the different sources for nitrogen being highlighted.

Experimental

Purification of urinary AICAR was conducted similar to the method employed for carbon isotope ratio anlysis [4]. After twofold HPLC clean up AICAR was derivatized with MSTFA/ethyl acetate (20/80, v/v). As no nitrogen is added during derivatization and silylation of AICAR is not taking place at nitrogen atoms, no isotopic fractionation is expected and no correction of the measured values is necessary.
Nitrogen isotope ratios were measured on a Delta V Plus IRMS (ThermoFisher) coupled to a Trace GC 1310 equipped with a TriPlus RSH Autosampler. Both systems were connected via the GC IsoLink CNH operated at 1040°C and the ConFloIV interface (ThermoFisher). Liquid nitrogen was used to freeze out CO (Figure 2). Injections were performed in splitless mode at 280°C with 2.5 µL of ethyl acetate. The GC column was a J&W Scientific DB-17MS (length 30 m, i.d. 0.25 mm, film thickness 0.25 µm) from Agilent (Waldbronn, Germany). The initial oven temperature was 60°C, maintained at 60°C for 2 min, increased with 40°C/min to 265°C, then with 2°C/min to 285°C, and finally with 40°C/min to 310 °C held for 2 min before cool-down to initial conditions. Carrier gas was He (purity grade 5.0) with a constant flow of 1.5 mL/min. Isodat 3.0 (ThermoFisher) was used for data acquisition and evaluation.

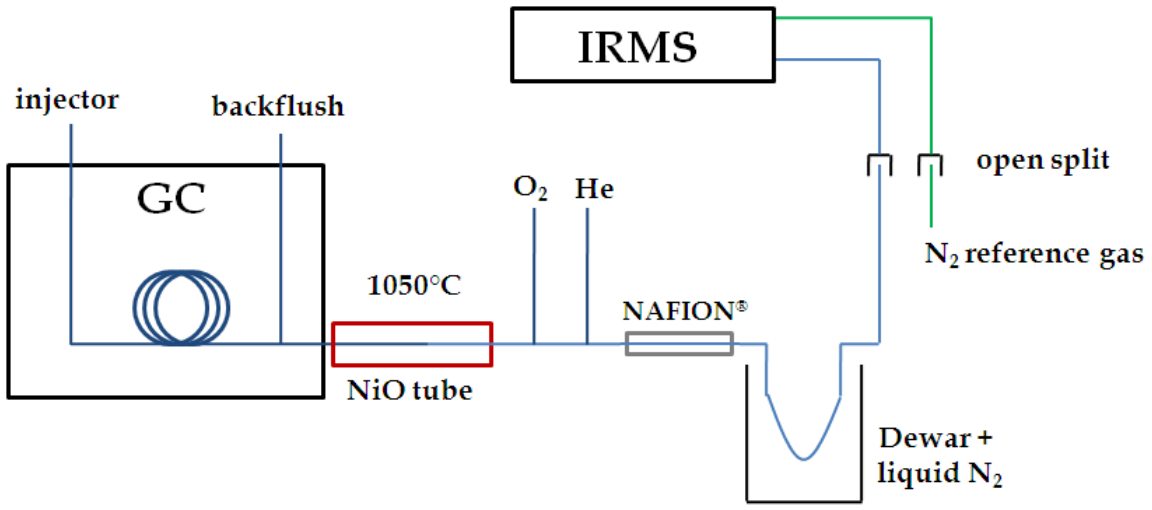

Figure 2: Schematic of GC/C/IRMS system used for $^{15}N/^{14}N$ measurements

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Results and Discussion

In order to evaluate the validity of our $\delta^{15}N$ measurements, an in-house AICAR standard was determined on the GC/C/IRMS system and an elemental analyzer/IRMS. No significant difference was detected between GC/C/IRMS ($\delta^{15}N_{AIR}$ = -4.0 ± 0.52 ‰, n = 12) and elemental analyzer measurements ($\delta^{15}N_{AIR}$ = -3.8 ± 0.18 ‰, n = 4).
As depicted in Figures 3 and 4, the difference between endogenous AICAR at -3.9 ‰ and the AICAR found in urine after administration at -3.6 ‰ is not significant. According to carbon isotope ratio measurements, the urinary AICAR in this post-administration sample perfectly reflects the value of the administered AICAR. Other endogenous values were found between -2 ‰ and -4 ‰ (n = 4).
These values differ surprisingly strong from published values for amino acids [1-3] or human diet [5]. This points towards strong isotopic discriminations during nitrogen metabolism as can also be derived by the large differences in δ^{15}NAIR-values in-between the different amino acids.

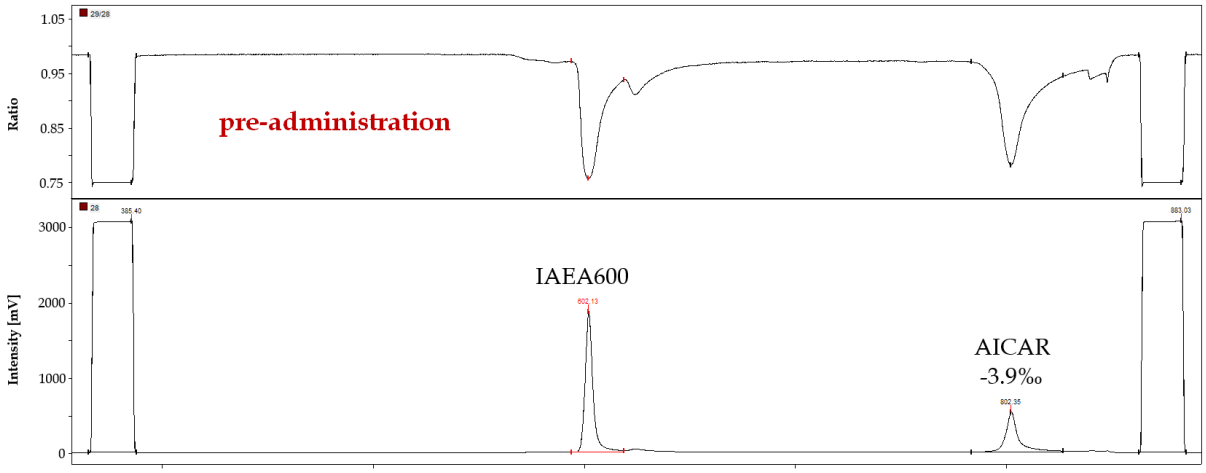

Figure 3: IRMS chromatogram of a blank urine containing approx. 1000 ng/mL AICAR. IAEA600-reference standard (caffeine).

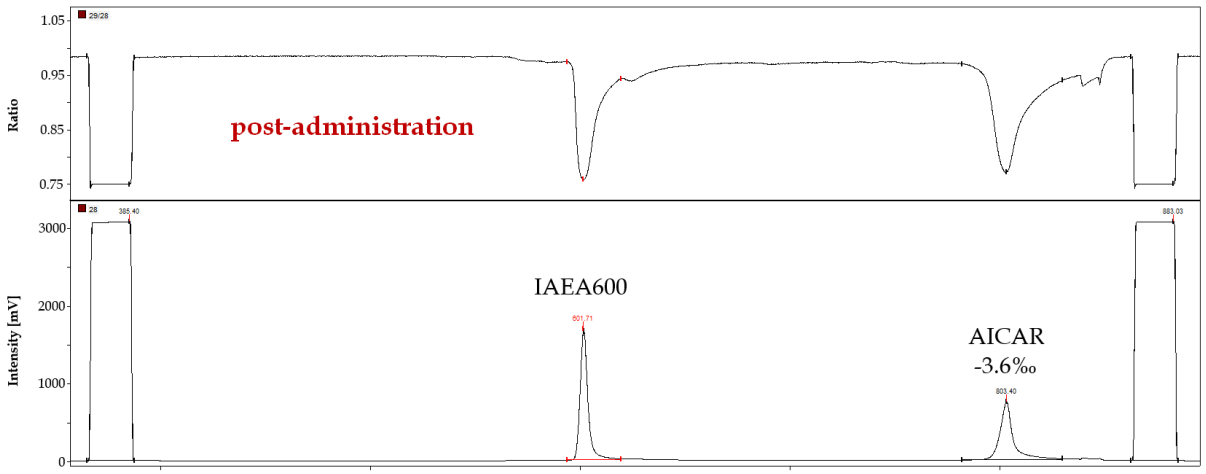

Figure 4: IRMS chromatogram of a post-administration urine containing approx. 6000 ng/mL AICAR. IAEA600-reference standard (caffeine).

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Conclusions

For GC/C/IRMS measurements of nitrogen isotope ratios comparatively large sample amounts are needed. As AICAR can be found in urine with concentrations of more than 1000 ng/mL it is possible to determine its $\delta^{15}N_{AIR}$-values. In contrast to our expectations endogenous AICAR did not show positive $\delta^{15}N_{AIR}$-values but measurements fall between -2 ‰ and -4 ‰. With these values it was not significantly different from the isotopic ratio of the standard substance. Nitrogen isotope ratios seem not to be suitable to distinguish between endogenous and exogenous sources of urinary AICAR.

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