Relationships among Urinary Aminoimidazolecarboxamide in Urine and Folate, and Vitamin B₁₂ Concentrations in Serum

J. W. Harrison, B. A. Slade, and W. Shaw

Urinary aminoimidazolecarboxamide (AIC), serum folate, and serum vitamin B₁₂ values were determined in 84 apparently healthy individuals. An automated system for determination of AIC in urine is described. Despite claims to the contrary, we found no evidence of a strong relationship between elevated (e.g., >1.3 μg/mg of creatinine) AIC excretion as reflected in a casual sample of urine and folate or vitamin B₁₂ deficiency. Urinary AIC values ranged from 0.10 to 5.20 μg/mg of creatinine. The mean for the population examined was 1.36 ± 1.02 μg/mg of creatinine.

Additional Keyphrases: megaloblastic anemia, diagnosis • mass screening of population • automated analysis for AIC normal values • frequency distributions • purine nucleotide biosynthesis • index to 24-h urine collection • AutoAnalyzer

The isolation of AIC¹ in sulfonamide-inhibited bacterial cultures (1–3) led to an understanding of the mechanism of action of the sulfonamide drugs and the elucidation of the pathway for purine biosynthesis. AIC ribotide is derived in several biosynthetic steps from glycine and 5-phosphoribosyl-1-pyrophosphate. N¹⁰-formyl FH₄, a folic acid coenzyme, is required for conversion of AIC ribotide to FAIC ribotide. AIC is formed when AIC ribotide is converted to AIC and ribose-5-phosphate (Figure 1).

The increased concentration of AIC in sulfonamide-inhibited bacterial systems is thought to result from the prevention of folic acid synthesis by the bacteria. Presumably decreased concentrations of folic acid coenzymes led to an increased concentration of AIC ribotide and AIC.

Subsequently an elevated urinary excretion of AIC was reported to be associated with folic acid and (or) vitamin B₁₂ deficiency in man and experimental animals (4–8).

Oace et al. (8) determined urinary AIC by a colorimetric method involving the Bratton and Marshall reagents for diazotizable amines. However, this method proved unsuitable for us because gas bubbles evolve during color development, seriously interfering with spectrophotometric analysis, which results in less than satisfactory standard curves. We therefore decided to automate this procedure to eliminate the interference by the gas bubbles. Because deficiency of either folic acid or vitamin B₁₂ can result in megaloblastic anemia (9), we thought that a reliable method for AIC determination might be useful as a screening test for megaloblastic anemia in population surveys. We also thought that an automated method would synchronize the addition of reagents (a problem with the manual method) and increase the precision of the method and the number of samples that could be assayed.

Materials and Methods

Serum folic acid was measured by Lactobacillus casei bioassay (10), serum vitamin B₁₂ by L. leichmannii bioassay (11). Serum was stored at −20 °C until assayed. Each serum folic acid and vitamin B₁₂ determination was performed in duplicate.

Figure 1: Biosynthetic pathway for purine ribotides

---

¹ Nonstandard abbreviations used: AIC, 4-amino-5-imidazolecarboxamide; N¹⁰-formyl FH₄, N¹⁰-formyl tetrahydrofolic acid; FAIC, 5-formamidoimidazole-4-carboxamide; and FH₄, tetrahydrofolate.

Received May 18, 1973; accepted June 14, 1973.
Serum and urine were collected from apparently healthy white and Negro volunteers of both sexes, with ages ranging from 4 to 75 years. At the time of collection, urine samples were acidified to pH 4.5 by dropwise addition of glacial acetic acid and frozen at −10 °C until used. Creatinine in urine was analyzed by an automated Jaffé procedure (12).

AIC standards: A 1 g/liter solution of AIC (ICN Nutritional Biochemicals, Cleveland, Ohio 44128)\(^2\) in distilled water was stored at 4 °C in a dark bottle. An intermediate working standard was prepared by diluting 1.0 ml of the stock standard to a volume of 100 ml with distilled water. Working standards were prepared in the range 0.5 to 6.0 µg/ml in distilled water.

Brij-water solution: With distilled water, 0.5 ml of “Brij-35” (30% solution; Technicon Corp., Tarrytown, N. Y. 10591) was diluted to a volume of 1.0 liter.

Other reagents: All of the following reagents were prepared in distilled water: sodium nitrite 1 g/liter; ammonium sulfamate 5 g/liter; and N-(1-naphthyl)-ethylenediamine dihydrochloride, the color reagent, 1.5 g/liter.

Equipment for automation: The colorimeter, sampler, pump, recorder, tubing, flow cell, and mixing coils were obtained from the Technicon Corp. (Figure 2). A 520-nm filter was used in the colorimeter.

Sample preparation: Urine samples were centrifuged to remove particulate matter. A 10-ml urine sample was adjusted to pH 2 with concentrated H\(_2\)SO\(_4\) and then purified by ion-exchange chromatography. A 7-mm (i.d.) column was filled with a slurry of AG2-X8 resin (100–200 mesh, chloride form; Bio-Rad Laboratories, Richmond, Calif. 94804) to a height of 70 mm. Of the acidified urine, 5 ml was introduced and the resin then washed with 1.0 ml of H\(_2\)SO\(_4\) (5 mmol/liter), for a total eluate volume of 6.0 ml. Recoveries of AIC and AIC added to a urine sample before column treatment ranged from 96 to 100% (Table 1).

Pretreatment of urine with acetic anhydride acetylates interfering aromatic amines that might otherwise react with the color reagent (1). To acetylate these compounds, one drop of acetic anhydride was mixed into 2 ml of the column eluates before the eluates were loaded on to the sampler. Acetylation was 97–100% complete when the automated system described below was used. Acetylation was stopped by introducing H\(_2\)SO\(_4\) (0.18 mol/liter) into the sample stream. The reaction mixture passes through a 15-min time-delay coil before entering the colorimeter to allow for maximum development of color. The reaction was read by using a 520-nm filter in a recording colorimeter. Samples were run at the rate of 40/h with a 2:1 ratio of wash to sample. Blanks that corrected for the endogenous color of urine eluates were prepared by substituting the Brij-35 water solution for color reagent and sampling the corresponding column eluate which contained one drop of acetic anhydride per 2 ml of eluate.

Results

A typical standard curve (Figure 3) is linear over the concentration range used. The system we used showed good washout characteristics (Figure 4). With 40 samples/h at 2:1 ratio of wash to sample, the peak heights for discrete samples were about 92% of the steady-state values, and replicate samples were reproducible at all concentrations studied (Figure 5).

Replicate determinations (n = 10) on column eluates from human urine agreed closely (Figure 6). The coefficient of variation of these replicate determinations was 3.3%. This automated assay is sufficiently sensitive for measuring the concentration of AIC in normal human urine.

The frequency distribution of serum vitamin B\(_{12}\) concentrations (n = 83) is given in Figure 7; the range was 150 to 1290 pg/ml, and the mean was 531 (±244 SD) pg/ml. The median value was 490 pg/ml and values for the lower and upper deciles were 254 and 900, respectively.

The frequency distribution of serum folate values (n = 84) is given in Figure 7. Serum folate values ranged from 2.3 to 20.0 ng/ml (mean, 7.2; SD, 3.7; median, 6.0 ng/ml; lower and upper deciles, 3.5 and 11.9, respectively).

The frequency distribution of urine AIC values (n = 79) is also given in Figure 7. Urinary AIC values ranged from 0.10 to 5.20 µg/mg creatinine (mean,
1.36; SD, 1.02; median, 1.00 μg/mg of creatinine; lower and upper deciles, 0.50 and 2.90, respectively).

Middleton et al. (4) reported the range of urinary AIC excretion for non-anemic hospital patients as 0.4–1.3 μg/mg of creatinine. The range they found for healthy males was 0.2–1.3 μg/mg of creatinine. They reported that patients with both folic acid and vitamin B₁₂ deficiency had a urinary AIC value of 4.7 μg/mg of creatinine, the highest value they reported. However, we found an individual with an AIC value of 5.2 μg/mg of creatinine. This individual had a serum folate concentration of 10.3 ng/ml and a serum vitamin B₁₂ concentration of 540 pg/ml; both of these values are within the normal range. Two individuals had deficient (<3.0 ng/ml) serum folate concentrations; one also had a serum B₁₂ value of 150 pg/ml, the lowest value we found. This individual had an increased (>1.3 μg/mg of creatinine) AIC value. The person with folate deficiency alone did not. Relationships among serum folate, serum vitamin B₁₂, and urinary AIC are summarized in Figure 8. This Figure indicates that there is little or no relationship between an abnormally high urinary AIC excretion and deficiency of either folate or vitamin B₁₂ in serum. These data indicate that the AIC concentration in casual urine sample would be unsatisfactory for use in estimating the incidence of megaloblastic anemia caused by deficiency of folic acid and (or) vitamin B₁₂ in large population surveys.

Discussion

Lubhy and Cooperman (5) reported that urinary AIC excretion was not enhanced in folate deficiency but was abnormally large in vitamin B₁₂ deficiency.
However, Herbert et al. (6) and Middleton et al. (4) reported an increased AIC excretion in either folate or vitamin B₁₂ deficiency. Middleton et al. questioned the way that Lubby and Cooperman interpreted the latters' data and claimed that the data of Lubby and Cooperman also indicated an increased AIC excretion in folate deficiency.

One very important point that has apparently been neglected by the investigators in this area is the fact that folic acid coenzymes are required in two of the enzymatic steps in the biosynthesis of purine nucleotides (13). Although a folate coenzyme is required for conversion of AIC ribotide to FAIC ribotide, a folate coenzyme, N⁵,N¹⁰-methenyl tetrahydrofolate, is also required in one of the earlier enzymatic steps in purine biosynthesis. This earlier reaction is the enzymatic conversion of glycaminide ribotide to α-N-formylglycinamide ribotide (Figure 1). A decrease in folic acid would thus not only decrease the conversion of AIC ribotide to FAIC ribotide but would also presumably decrease the intracellular concentration of AIC ribotide. Thus, a decrease in the supply of AIC ribotide, at the same time that its conversion to FAIC ribotide was inhibited, might result in no net change in the steady-state intracellular levels of AIC ribotide regardless of the intracellular concentration of folate coenzymes.

The AIC test might be of some use as a screening test for megaloblastic anemia if 24-h urine specimens could be obtained. However, we have no evidence to support this speculation since we have not yet analyzed 24-h urine specimens.

McGreer et al. (7) found that the 24-h excretion of AIC is remarkably constant from day to day, showing a coefficient of variation of 16% as compared to 19% for creatinine. They also found the correlation between AIC excretion per day and body weight was highly significant and that there was a highly significant correlation between urinary excretions of AIC and creatinine by normal persons. Because excretion of AIC is relatively constant, the amount of AIC in 24-h urine could be used as an index to the completeness of a urine collection.

References