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Short Communication

Stability of 5-aminosalicylic acid and its metabolites in plasma at −20°C

Formation of N-β-D-glucopyranosyl-5-aminosalicylic acid

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ABSTRACT

The stability of 5-aminosalicylic acid and its metabolites has been investigated when stored frozen. N-β-D-Glucopyranosyl-5-aminosalicylic acid was formed in considerable amounts concomitant with a decrease in 5-aminosalicylic acid in plasma samples spiked with 5-aminosalicylic acid as well as in standard solutions of 5-aminosalicylic acid buffered with potassium phosphate between pH 5.5 and pH 8.0 with 4.0 m*M* glucose added and stored at −20°C. Thus N-β-D-glucopyranosyl-5-aminosalicylic acid migth not, as previously described, be a metabolite of 5-aminosalicylic acid but an artifact formed during storage of plasma samples. The N-glucoside formed could be quantitatively degraded to 5-aminosalicylic acid and glucose by adding 0.2 *M* potassium phosphate buffer pH 3.0 to the sample prior to the analysis. The metabolites of 5-aminosalicylic acid (N-formyl-5-aminosalicylic acid, N-acetyl-5-aminosalicylic acid and N-butyryl-5-aminosalicylic acid) were found to be stable in plasma stored at −20°C for at least eight months.

INTRODUCTION

In clinical trials it is a normal procedure to store plasma samples frozen until they are analysed. Some reaction rates are increased during freezing and during storage in frozen state due to concentration of the reactants [1–3]. As 5-aminosalicylic acid (5-ASA) is a fairly unstable compound amenable to oxidation, an examination of the stability of 5-ASA and its metabolites in frozen plasma and in frozen solutions was performed. Brendel *et al.* [4] have reported that 5-ASA decomposes rapidly in plasma during storage at −20°C, and that addition of antioxidants, on the contrary, accelerates the process. 5-ASA was found to be stable at −80°C for six months. The major metabolite of 5-ASA, N-acetyl-5-ASA (Ac-5-ASA), was reported to be stable at both temperatures.

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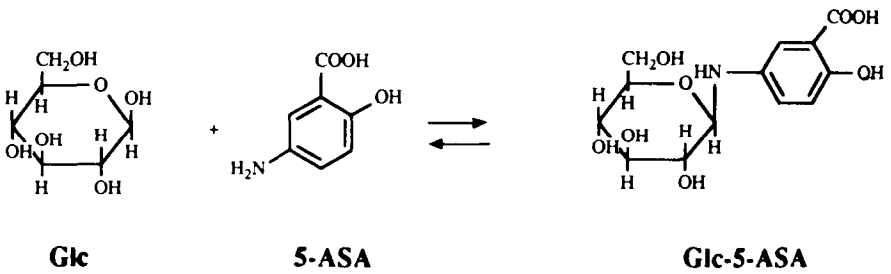

Fig. 1. Equilibrium of 5-ASA, glucose (Glc) and Glc-5-ASA. The equilibrium is shifted to the right during storage at −20°C and to the left in weakly acidic solutions.

This paper reports the formation of N-β-D-glucopyranosyl-5-ASA (Glc-5-ASA) (Fig. 1) in plasma spiked with 5-ASA, as well as in standard solutions of 5-ASA buffered with potassium phosphate between pH 5.5 and pH 8.0 with 4.0 m*M* glucose added and stored at −20°C. The Glc-5-ASA formed could be degraded to form 5-ASA by adding 0.2 *M* potassium phosphate buffer pH 3.0 to the sample prior to analysis. We found that the metabolites of 5-ASA, N-formyl-5-ASA (F-5-ASA) [5], Ac-5-ASA, N-butyryl-5-ASA (Bu-5-ASA) [6] and Glc-5-ASA [7], were stable for eight months at −20°C.

EXPERIMENTAL

Materials

5-ASA was kindly supplied by Ferring (Copenhagen, Denmark) and Ac-5-ASA was kindly supplied by Pharmacia (Uppsala, Sweden). Glc-5-ASA, F-5-ASA and Bu-5-ASA were prepared in the laboratory as previously described [4–6]. Glucose was of pharmacopoeial quality, and all other chemicals were of analytical grade from Merck (Darmstadt, Germany).

Stability in plasma

Three volumes of pooled plasma were spiked with 5-ASA, Glc-5-ASA and a mixture of 5-ASA, F-5-ASA, Ac-5-ASA and Bu-5-ASA, respectively. Each volume was divided into smaller fractions and stored in polyethylene test tubes at −20°C. Blanks were also stored. Concentrations of standard solutions were as follows: 6.3 μ*M* 5-ASA, 5.0 μ*M* Ac-5-ASA, 5.4 μ*M* F-5-ASA, 4.3 μ*M* Bu-5-ASA, 3.3 μ*M* Glc-5-ASA and 2.15 μ*M* 5-ASA. Prior to high-performance liquid chromatography (HPLC) the samples were treated as follows: a 4.0-ml volume of methanol was mixed with 1.0 ml of plasma. After 15 min at 4°C the mixture was centrifuged for 15 min at 3000 *g*. A 1.0-ml aliquot of the supernatant was mixed with 1.0 ml of distilled water or 0.2 *M* potassium phosphate buffer pH 3.0 and analysed.

Stability in buffered solutions

Aliquots of 88 μM 5-ASA and 4.0 mM glucose in 5–20 mM potassium phosphate buffer (pH 5.5–8.0) were stored at −20°C for five weeks. Aliquots of 88 μM 5-ASA in a solution buffered with 20 mM potassium phosphate (pH 5.5–8.0) and of a 5-ASA standard in a 4.0 mM glucose solution were stored at −20°C for five weeks and analysed.

Assay

The HPLC system used consisted of a Knauer (Berlin, Germany) 40 mm × 4.6 mm I.D. stainless-steel column packed with Hypersil (Shandon, UK) 3-μm particles. A saturation column, 150 mm × 4.6 mm I.D., packed with LiChroprep Si 60, 15–25 μm particles, was situated between the pump and the autoinjector. The eluent was methanol–water–0.2 M potassium phosphate buffer pH 6.5 (40:40:20) with 4.0 mM hexadecyltrimethylammonium bromide added. The HPLC system is described in detail elsewhere [8].

Freshly prepared standard solutions of 5-ASA, Ac-5-ASA, F-5-ASA, Bu-5-ASA and Glc-5-ASA were used and were treated as the test samples.

RESULTS AND DISCUSSION

Stability in plasma samples

In plasma samples containing 5-ASA stored at −20°C the concentration of Glc-5-ASA increased concomitantly with a decrease in the concentration of 5-ASA (Fig. 2), indicating a conversion of 5-ASA into Glc-5-ASA. The concentrations of F-5-ASA, Ac-5-ASA and Bu-5-ASA remained constant in plasma during eight months. No decrease in the concentration of Glc-5-ASA was observed in plasma spiked with Glc-5-ASA and stored at −20°C for eight months.

Plasma samples (n = 6) spiked with 5.56 μM 5-ASA were analysed after eight months of storage at −20°C. The concentration of 5-ASA after adding 0.2 M potassium phosphate buffer pH 3.0 to the supernatant of the deproteinized sample was compared with the sum of the concentrations of 5-ASA and Glc-5-ASA when water was added to the supernatant. The concentration of 5-ASA was found to be 5.78 μM (coefficient of variation, C.V. = 1.0%) in the plasma samples treated with potassium phosphate buffer pH 3.0. The concentrations of 5-ASA and Glc-5-ASA were 3.03 μM (C.V. = 3.0%) and 2.61 μM (C.V. = 1.6%), respectively, a total of 5.68 μM (C.V. = 1.4%), in plasma samples with water added to the supernatant. This indicates that Glc-5-ASA was quantitatively degraded to 5-ASA by adding buffer pH 3.0 to the supernatant.

Stability in buffered solutions

The amount of Glc-5-ASA formed in buffered solutions with 4.0 mM glucose added decreased with decreasing pH (Fig. 3). This is due to less formation of Glc-5-ASA with decreasing pH (increasing protonation of the amino group) and

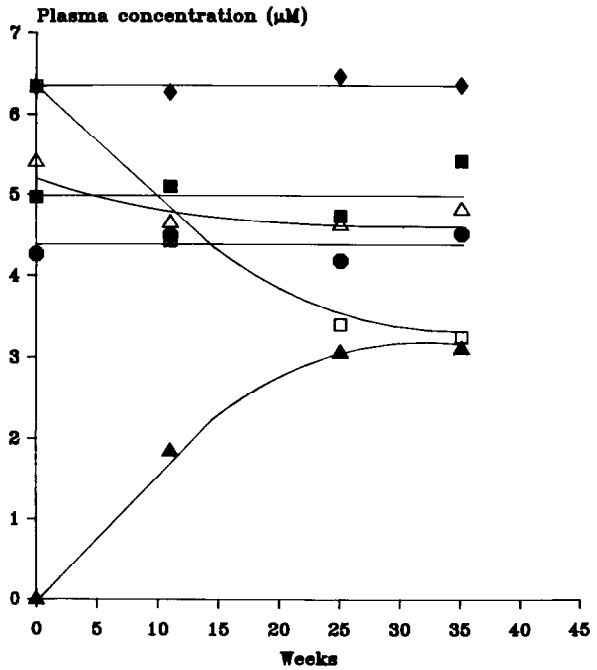

Fig. 2. Stability of 5-ASA (□), Glc-5-ASA (▲), F-5-ASA (△), Ac-5-ASA (■) and Bu-5-ASA (●) in plasma when stored at −20°C for 35 weeks. (◆) Sum of Glc-5-ASA and 5-ASA.

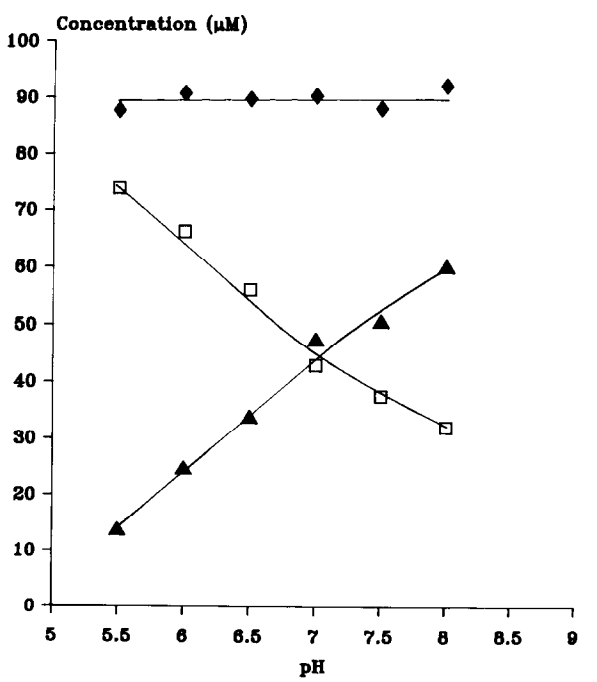

Fig. 3. Formation of Glc-5-ASA (▲) in solutions cointaining 88 μM 5-ASA (□) and 4.0 mM glucose in 0.02 M potassium phosphate buffer pH 5.5–8.0 stored at −20°C for five weeks. (◆) Sum of Glc-5-ASA and 5-ASA.

a concomitantly decreasing stability of Glc-5-ASA. 5-ASA was stable for at least one month in buffered solutions (pH 5.5–8.0) stored at −20°C, when no glucose was added. No Glc-5-ASA could be detected in solutions containing 4.0 m*M* glucose and 88 μM 5-ASA (pH approximately 4) stored at −20°C for one month.

CONCLUSION

The previously reported [7] appearance of Glc-5-ASA as a metabolite of 5-ASA in plasma is most likely an artifact, but the formation of Glc-5-ASA may affect the quantification of 5-ASA in plasma. The Glc-5-ASA formed may be degraded to glucose and 5-ASA in weak acidic solutions and by use of acylation reagents as acetic acid anhydride or propionic acid anhydride. Thus, using analytical methods with sample preparation procedures involving acidification or acylation reagents Glc-5-ASA may be determined as 5-ASA. However, Glc-5-ASA is relatively stable in plasma samples deproteinized with organic solvents as methanol or acetonitrile. Thus, the true concentration of 5-ASA in plasma at the time of collection might be underestimated if Glc-5-ASA is not quantified simultaneously.

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