Condensation of Isoniazid and Acetaldehyde

To the Editor:

Isoniazid (isonicotinic acid hydrazide, INH), a commonly used tuberculostatic drug (1), has various adverse effects, including potentially fatal hepatic injury (2). Because such hepatotoxicity is more prevalent in Oriental patients and in non-Oriental patients of the rapid acetylator phenotype, it has been suggested that metabolites produced after acetylation are involved in the pathogenesis (3). However, other studies (4) have not confirmed this observation. Alcohol ingestion may possibly play a role. The consumption of ethanol increases the risk of hepatic damage induced by INH, although it is not known whether this arises solely by pre-existing alcoholic liver disease or in conjunction with some more direct effect of ethanol (5). In studies with the acute administration of ethanol and INH in dogs, ethanol pretreatment did not enhance the toxicity of INH (6). A study with chronic administration was not reported.

Acetaldehyde, a metabolite of ethanol, reacts in vitro and in vivo with various endogenous and exogenous compounds (7). The chemical structure of INH prompted us to examine the reaction of INH and acetaldehyde. We describe here the synthesis and characterization of the hydrazone adduct of INH and acetaldehyde, and its forma- tion and degradation at physiological pH levels and temperature.

The HPLC system consisted of a pump (M45G; Waters Associates) equipped with a universal injector with a 0.1-mL injection loop (CV-6-UHPa-N60; Valco), a C18 reversed-phase column (25 cm, 10-μm particle size; Waters Associates), a fixed-wavelength (254 nm) ultraviolet detector (LC 15; Perkin-Elmer) and a strip-chart recorder (Model 24; Perkin-Elmer). The mobile phase was acetonitile/water (6/94 by vol), the flow rate was 2 mL/min. Before analysis, samples were diluted with an appropriate volume of mobile phase. The retention times and the number of theoretical plates of INH and the adduct were 2.6 and 5.4 min and 675 and 1340, respectively. Peak heights were used for quantification.

1H and broad-band decoupled 13C NMR spectra were taken at 80.13 and 20.15 MHz, respectively, with a Bruker WP-80SY spectrometer. Solutions were 0.1 mol/L (1H) or 0.9-1.0 mol/L (13C) in D2O. 13C assignments were made on the basis of reported (8) values for chemical shifts of pyridine derivatives and signal intensities.

Reference NMR spectra of isoniazid were taken as follows: 1H NMR (D2O) δ 7.68 (2H, m, H3,5), 8.67 (2H, m, H2,6). 13C (1H) NMR (D2O) δ 124.09 (C3,5), 142.98 (C4), 152.22 (C2,6), 169.29 (C = O).

To synthesize the adduct, we dissolved INH (8 g, 58 mmol, mp 172 °C) in 60 mL of water and added 8 g (180 mmol) of acetaldehyde. The solution was stirred for 1 h at room temperature and the water and unreacted acetaldehyde was removed in a rotary evaporator. The crude product was recrystallized from ethanol to give white needles, mp 183-185 °C (lit. 178-179 °C) (9), with the following NMR spectra: 1H NMR (D2O) δ 2.13 (3H, d, J = 5.4 Hz, CH3), 7.73 (2H, m, H3,5) 7.8 (1H, m, CH =) 8.70 (2H, m, H2,6). 13C (1H) NMR δ 20.48 (CH3), 124.33 (C3,5), 142.76 (C4), 152.18 (C2,6), 157.44 (CH =), 166.31 (C = O).

To study the reaction of INH and acetaldehyde under physiological conditions, we dissolved each reactant in buffer to give a concentration of 100 μmol/L. The buffer consisted of 0.1 mol/L potassium phosphate dibasic added to 0.1 mol/L potassium phosphate monobasic to give a pH of 7.35. We mixed 10 mL of each solution in a tube with Teflon-lined screw cap and kept this at 37 °C in a water bath. The reaction reached equilibrium after about 80 min. The yield was 24% of the theoretical (expected) amount.

To study the decomposition of the adduct, we kept a 5 μmol/L solution of the adduct in buffer (pH 7.35) at 37 °C, and analyzed aliquots removed at various intervals during 70 min. The concentration of the adduct was only half the original concentration by 45 min. To study possible reduction of the adduct, we added ascorbic acid to a final concentration of 56 μmol/L. The 4-h decomposition profiles of the adduct with and without ascorbic acid were superimposable; evidently, ascorbic acid has no effect.

There are at least two conceivable routes by which INH may react with acetaldehyde. One route would yield a bicyclic compound in a manner similar to that in which dopamine and acetaldehyde yield salsolinol (7); there is no evidence for this route. The other route involves formation of the hydrazone. The reversibility of the reaction is consistent with the reactions of other hydrazones.

The 1H and 13C NMR spectra of the adduct are fully consistent with its formulation as the acetaldehyde hydrazone, and inconsistent with a cyclic structure lacking symmetry in the pyridine ring. Comparison of the 1H and 13C NMR spectra of the adduct with those of INH shows that the signals arising from the INH moiety remain essentially unchanged in the adduct; the additional signals from the CH2CH = moiety appear as expected. In the 1H spectrum of the adduct, the CH = signal partly overlaps the multiplet arising from H3,5.

The yield of the hydrazone under physiological conditions is greater than the yield of salsolinol from acetaldehyde and dopamine (7). Salsolinol has been detected in the urine of patients who have consumed ethanol (10), so it might be possible to determine the hydrazine in biological fluids of patients who have ingested both INH and ethanol, but this seems unlikely because our data show that the compound is unstable under physiological conditions. There is also the possibility of enzymic in vivo catabolism. Thus, the time necessary for the sampling of biological fluids and the time required for analysis would substantially decrease the possibility of detecting the compound. The results of the experiment with ascorbic acid suggest that the potential stabilization of the INH-acetaldehyde adduct by in vivo reduction, as has been found with protein-acetaldehyde adducts (11), is unlikely to be efficient, as compared with the decomposition to INH.

We conclude that the reaction between acetaldehyde and INH under physiological conditions yields the hydrazone, which is unstable under these conditions. The presence of the hydrazone may be a factor in increasing the risk of hepatic damage when ethanol is taken in conjunction with INH. In addition, the formation of the hydrazine may also be a factor in decreasing the efficacy of INH by reducing the circulating concentrations of INH.

References


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Improved Dye-Binding Procedure for Determining Microgram Quantities of Protein in Cerebrospinal Fluid—Once Again

To the Editor:

In 1976, Bradford (1) published her paper on quantification of microgram quantities of protein, based on the principle of protein dye binding, with use of coomassie Brilliant Blue (CBB). Since then, several authors have applied the method to determination of protein in cerebrospinal fluid and urine. The assay is easy to perform, requires no special equipment, and has advantages over other procedures in terms of sensitivity, rapidity, and economy. Unfortunately, the color produced varies widely with different proteins. This poses standardization problems, when working with physiological solutions.

Several efforts have been made to overcome this drawback. Read and Northcote (2) evaluated the effect of increased dye concentration and reduced phosphoric acid concentration in the reagent solution. Macart and Gerbaut (3) added sodium dodecyl sulfate (SDS) to improve the assay. Bio-Rad Laboratories has introduced a kit method (4) that is almost identical to that described by Bradford, except that methanol is used.

I modified the reagent composition of the kit as follows: CBB was replaced by a proportionally smaller concentration of Serva Blue G, which is a higher-purity dye with approximately double the color-producing capacity, and methanol was used instead of ethanol. Finally, I included SDS, this being the major modification. Thus I used the following reagent composition, per liter: Serva Blue G (cat. no. 35050; Serva Feinbiochemica GMBH, D-6900 Heidelberg 1, F.R.G.), 50 mg; methanol, 1.2 mol; phosphoric acid, 1.6 mol; and SDS (BDH Chemicals Ltd., Poole, Dorset BH 12 4NN, U.K.), 20 mg. The reagent is stable for two weeks at room temperature.

The test is performed by adding 1000 μL of reagent to 20 μL of sample or standard in disposable polystyrene cuvettes. Color production, complete within 1 min, is stable for at least 1 h. The samples and standards are read at 595 nm vs a reagent blank.

Compared with albumin, the specific color yield (absorption at 595 nm per milligram of protein per millilitre) of γ-globulins is 87% as great, that of transferrin 103%. Read and Northcote (2) found the color yield of γ-globulins to be 42% or 65% that of albumin, depending on the reagent composition. With the described method, color production is linearly related to protein concentration up to 2.0 g/L, whereas the Bio-Rad kit method is linear only up to 1.0 g/L (4).

I tested the assay with γ-globulin and transferrin because these proteins generally pose the greatest differences in color yield. Moreover, next to albumin, γ-globulin is quantitatively the most important group of proteins in cerebrospinal fluid. Although the color yield is close enough to 100% to allow for standardization against human albumin, for greater accuracy I use human multiprotein LN standard (cat. no. OSAU 03; Behringwerke AG, Marburg, F.R.G.).

The enhanced linearity and ease of performance allows the possibility of determination of protein in cerebrospinal fluid and in urine in one set-up.

Also, samples that are too concentrated may be diluted and re-run against the same standards within the 1-h stability period. The assay can easily be automated.

References


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Affinity Chromatography of Urinary Brush-Border Enzymes on Concanavalin–Sepharose

To the Editor:

It has been suggested that the separation of urinary enzymes into their multiple forms can improve the clinical significance of enzyme activity determinations in urine and our understanding of release of renal enzymes into urine (1). Ultracentrifugation, ion-exchange chromatography, electrophoresis, gel filtration, and affinity chromatography have been recommended as separation methods (2–4). Comparing these methods for the separation of urinary γ-glutamyltransferase (EC 2.3.2.2), Rambabu et al. (2) found that, from an analytical viewpoint, affinity chromatography was best suited. As far as we know, no experiments with affinity chromatography have been made with the two other typical brush-border enzymes in urine, aminopeptidase (microsomal) (EC 3.4.11.2) and alkaline phosphatase (EC 3.1.3.1).

To determine whether the multiple forms of urinary brush-border enzymes obtained by ultracentrifugation (particulate and soluble variants) correspond to those obtained by affinity chromatography (bound and unbound variants), we investigated the affinities of the particulate and soluble variants of these three enzymes on concanavalin A (Con A)–Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden). We