A More Sensitive Spectrophotometric Method for Determination of Isoniazid in Serum or Plasma

Leslie Eidus and Anita M. T. Harnanansingh

A sensitive spectrophotometric method is described for determining isoniazid in serum or plasma. It is extracted from biological fluids by a mixture of organic solvents and re-extracted into hydrochloric acid. In this medium, isoniazid reacts with trans-cinnamaldehyde to form a derivative absorbing maximally at 340 nm. The method is five times more sensitive than published procedures in which vanillin or piperonal is used as coupling reagent for the estimation of isoniazid.

Additional Keyphrases extraction with organic solvents • trans-cinnamaldehyde reagent

In the past, bioassay, fluorometric and spectrophotometric methods were used to estimate isoniazid (INH, isonicotinic acid hydrazide) blood concentrations. Bioassay procedures utilizing Myco. tuberculosis strains as test organisms suffer from inherent inaccuracies and require a 14-day incubation, during which INH deteriorates completely in the serum. The fluorometric procedures (1, 2, 3) are sensitive but somewhat cumbersome for a routine laboratory. Among spectrophotometric procedures, two methods (4, 5) are acceptably accurate and are most commonly used. They are based on similar principles, although different organic solvents and coupling reagents are used. The chief drawback of these spectrophotometric methods is that they are not sensitive enough. We therefore attempted to modify a previous procedure of this laboratory (5) to render the method suitable for the estimation of low INH concentrations.

Materials and Methods

Reagents

Organic solvent mixture. Reagent grade n-butanol and chloroform were purified separately by adding activated charcoal, mixing, and filtering the mixture through one sheet of Whatman No. 1 filter paper. Approximately 30 g of anhydrous sodium sulfate was added to 1 liter of each solvent, to dehydrate them. The purified solvents were then passed through three sheets of Whatman No. 1 filter paper. Three volumes of n-butanol were mixed with seven volumes of chloroform.

Hydrochloric acid, 0.1 mol/liter. Prepared by diluting standard 1 mol/liter hydrochloric acid 10-fold.

Trans-cinnamaldehyde reagent (0.04%). A 1 ml/100 ml stock solution of trans-cinnamaldehyde ("chlorine-free," Eastman Kodak, Rochester, N. Y. 14650) is prepared in absolute alcohol. This solution can be stored for 3 weeks at 4°C. Before INH determinations, the stock solution is diluted 25-fold with absolute ethanol to give a final concentration of 0.04 ml of trans-cinnamaldehyde per 100 ml.

Sodium hydroxide, 4 mol/liter.

Equipment

A spectrophotometer with a microcell unit.

Procedure

Three milliliters of plasma or serum is mixed well with 1 drop of 4N sodium hydroxide and 3.2 g of ammonium sulphate in a 250-ml glass-stoppered Erlenmeyer flask. After adding 20 ml of organic solvent, the mixture is shaken for 30 min on a horizontal shaker at 145 excursions per minute. The organic extract is separated and filtered through Whatman No. 1 filter paper. Fifteen milli-
liters of the filtrate is then shaken for 15 min with 1 ml of 0.1N hydrochloric acid, followed by centrifugation for 10 min at 2000 rpm. The aqueous supernatant liquid is withdrawn with a Pasteur pipet, and 0.5 ml of this is mixed with 0.15 ml of alcoholic trans-cinnamaldehyde reagent. The latter must be delivered with a micropipet.

Absorbance of the samples is determined at 340 nm vs. a blank processed in the same way as the samples. The INH concentration of the samples can be calculated directly from the standard curve. The standard solutions are prepared by dissolving INH in serum in concentrations of 0.5 to 8 µg/ml and processing them as described above.

Results

A comparison of the new method with that of Maher et al. (4) and our previous procedure (5), in which alcoholic vanillin and piperonal reagent, respectively, were used, showed that the trans-cinnamaldehyde reagent produced the highest absorbance readings with INH solutions of the same concentration. Figure 1 demonstrates the results obtained with standard isoniazid solutions prepared in 0.1N hydrochloric acid and mixed with vanillin, piperonal, or trans-cinnamaldehyde reagent, respectively, as described in the three methods. The trans-cinnamaldehyde reagent resulted in 1.5 times higher absorbance than the vanillin, and twice as high absorbance as the piperonal reagent. Sensitivity of the new method was further increased by decreasing the quantities of both organic and aqueous extractors. The selection of a more favorable proportion between the extractors yielded a higher concentration of INH in the hydrochloric acid solution.

| Table 1. Recovery of INH in 0.1N HCl Solution after Double Extraction |
|------------------|---|---|---|
| Reference no.    | 1 | 2 | Present |
| Serum (10 µg INH/ml), ml | 3 | 4 | 3  |
| Organic solvent, ml | 40 | 40 | 20 |
| Vol re-extracted, ml | 37 | 35 | 15 |
| Vol 0.1N HCl used, ml | 4 | 4 | 1  |
| Final concentration of INH found, µg/ml | 7 | 8.5 | 22.5 |

Table 1 shows that, if a serum containing 10 µg/ml is extracted according to the method of Maher et al. (4), the final concentration in aqueous hydrochloric acid solution will be 7 µg/ml. The same serum, processed according to our earlier method (5), will yield a concentration of 8.5 µg/ml, while the revised method will give a final concentration of 22.5 µg/ml. As, in addition, the trans-cinnamaldehyde derivative has a higher absorbance than that of vanillin or piperonal, the new procedure became five times more sensitive than either of the two other methods. In the comparison shown in Table 1, no provision was made for the loss of INH during the extraction process, owing to binding of INH to serum. Such a loss was also observed and accounted for in the earlier procedures (4, 5). Based on 80 determinations carried out in human and bovine sera, the average yield of the extractions was calculated for this method as 85% (+3%).

Discussion

This method for estimation of isoniazid in biological fluids is considerably more sensitive than previous spectrophotometric methods based on similar double-extraction procedures (4, 5). The higher sensitivity is achieved by changing the coupling reagent to trans-cinnamaldehyde, and altering the proportion of organic and aqueous reagents used. Under acidic conditions, isoniazid forms a hydrazone with trans-cinnamaldehyde. Depending on the trans-cinnamaldehyde concentration, various absorption maxima were obtained with identical isoniazid solutions. With an increase in concentration of the reagent from 0.03 to 0.8 ml/100 ml, the absorption maxima shifted gradually from 335 nm to 358 nm. Furthermore, only low concentrations of trans-cinnamaldehyde, added in a ratio of 1:3.33 to INH solutions, yield linear standard curves. A 0.04 ml/100 ml reagent produced a lower absorbance than did a 0.1 ml/100 ml reagent; but the compound formed gave a linear absorbance from 0.5 to 12 µg of INH per milliliter. This range can be expected from the usual therapeutic doses of 300 to 400 mg of INH once daily. If higher doses are used, then the samples collected 2 to 3 h after drug administration may have to be
diluted if the absorbance is to fall within the optimal reading range.

The organic solvent mixture extracts about 85% of the INH, varying with the quality of the organic solvents used. Maher et al. (4) recommend including serum standards routinely with estimation of the unknown samples. This practice is preferred as it provides not only a correction factor and a control of the reagents employed, but also a check on the quality of the work.

The sensitivity of the method renders it suitable for phenotyping of INH inactivators by the fall-off technique. For this purpose the patient received 8 mg of INH per kilogram of body weight, intramuscularly, and blood was collected in heparinized Vacutainers before, as well as 2 and 4 h after drug administration. INH deteriorates rapidly in serum stored at room temperature and to some extent even in the refrigerator at 4°C. The deterioration rate depends on the INH content of the sera, being faster with lower concentrations. A loss of 20% to 40% may be expected in serum samples stored for 3 days at room temperature. Blood samples must, therefore, be centrifuged as early as possible and the separated sera or plasma stored in the deep-freeze at −20°C. Deterioration is delayed when the serum is refrigerated at 4°C; recovery is 87% in 3 days and 78% in 9 days. In aqueous solution no deterioration was observed. At −20°C, there was no significant drop in INH concentration (98.02%) during 9 days' storage, as also reported by Peters and Good (6).

References