ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 1973, p. 130-133

Vol. 3, No. 1
Printed in U.S.A.

NOTES

Screening of Isoniazid Inactivators

LESLIE EIDUS AND MARY M. HODGKIN

National Reference Centre for Tuberculosis, Laboratory Centre for Disease Control, Department of National Health and Welfare, Ottawa, Ontario, Canada

Received for publication 5 September 1972

A method is described for phenotyping of isoniazid inactivators. After a test dose of isoniazid, free isoniazid and its acetyl derivative are estimated in urine by the same colorimetric reaction.

For phenotyping isoniazid (INH) inactivators, a urine test was introduced by this laboratory in 1971 (3). In response to the increasing demand for a more rapid screening procedure, however, a further simplification of the urine test became necessary. The method described here for screening of isoniazid inactivators is easy to perform and well adaptable to inadequately equipped laboratories.

The patient receives an oral dose of 10 mg of INH per kg. After 6 hr, he empties his bladder completely, and this urine is discarded; 2 hr later, a urine specimen is collected to determine its INH and acetylisoniazid concentrations.

A 4-ml amount of the urine specimen is acidified with 2 ml of 0.5 N hydrochloric acid and kept for 15 min at room temperature. From this solution, two samples of 1.5 ml each are transferred into separate test tubes. To one sample, one drop of acetic anhydride (reagent grade) is added, shaken for 1 min, and neutralized by one drop of 7 N sodium hydroxide. To the other sample, two drops of distilled water are added in lieu of the above solutions to ensure equal volumes. Thereafter, 0.5 ml of 0.5 N sodium hydroxide is added to both samples.

Acetylisoniazid determination (11). To 2 ml of the neutralized urine samples, the following reagents are added successively: (i) 1 ml of 0.5 M potassium phosphate buffer, *p*H 6 (prepared by mixing 87.7 ml of 0.5 M KH_2PO_4 and 12.3 ml of 0.5 M K_2HPO_4), (ii) 1 ml of a 20% aqueous solution of potassium cyanide (prepared daily), (iii) 4 ml of a 12.5% aqueous solution of Chloramine T, Eastman-Kodak (prepared daily), and, after 1.5 min, (iv) 5 ml of acetone (reagent grade).

In concentrated urine samples, a slight precipitate may occur in the presence of acetone. This can be cleared by centrifugation or filtration.

One drop of acetic anhydride promptly acetylates INH in aqueous solutions. In the samples untreated with acetic anhydride, the color reaction is produced only with acetylisoniazid originally present in the urine, whereas in the treated ones the color intensity is due to acetylisoniazid as above and to free INH artificially acetylated. The results of the test can be read by means of a colorimeter, a simple comparator, or with the naked eye.

Reading by colorimeter. Acetylisoniazid yields a red color in this procedure with maximal absorbance at a wavelength of 550 nm. The color intensity may be estimated with a colorimeter. The results of the optical density readings can be converted to micrograms per milliliter by means of a standard curve, prepared with aqueous solutions of 10, 20, 40, 60, 80, and 100 μg of acetylisoniazid/ml. Amounts of 2 ml of these solutions are used for performing the acetylisoniazid test.

Acetylisoniazid concentrations are determined in samples not treated with acetic anhydride. The difference between optical density readings of acetic anhydride-treated and untreated samples indicates the free INH content of the specimen. As free INH is estimated after its conversion to acetylisoniazid, the value read from the standard curve must be multiplied by 0.761 to obtain the actual INH concentration in urine.

The ratio between acetylisoniazid and INH is termed the inactivation index. For calculation of the inactivation index, the acetylisoniazid concentration expressed in micrograms per milliliter is divided by the free INH concentration in micrograms per milliliter. Individuals exhib-

iting an inactivation index of 3 or less are regarded as slow inactivators, whereas fast inactivators produce indexes greater than 5.

Visual readings. The specimen of a slow inactivator contains acetylisoniazid and a high concentration of free INH acetylated in vitro. Therefore, this sample exhibits a considerably higher color intensity than the other one in which only acetylisoniazid originally present is estimated. Although these differences are striking in slow acetylators, they are insignificant in urine specimens collected from fast inactivators (Fig. 1).

Dilution of the urine samples. For colorimetric and particularly for visual evaluation of the results, it may be necessary to dilute the specimens prior to phenotyping to secure the optimal reading range. In a preliminary test, undiluted and 1:4 and 1:8 diluted urine samples are assayed for their acetylisoniazid content. This test is undertaken on a white tile with semicircular depressions (1), with the use of the same potassium cyanide and Chloramine T reagents as in the method described herein. Into separate depressions, four drops of urine and its dilutions are placed, followed by two drops of potassium cyanide and seven drops of Chloramine T solution. Dilutions giving a distinct pink color reaction are selected for visual reading, whereas a more intense color is used for photometric evaluation.

The two samples of the phenotyping procedure must be diluted in accordance with the preliminary test. It should, however, be borne in mind that acid-treated samples are already diluted to 1:2. Further dilution is undertaken prior to acetylisoniazid determination (11). A 2-ml amount of the final dilution is then employed for estimation of acetylisoniazid concentrations.

This new method gave in 44 volunteers the same results for INH phenotyping as the previous procedure (3), in which a spectrophotometer with ultraviolet range was used for estimation of INH (2). Of 44 volunteers, 19 were classified by both methods as slow and 25 as fast inactivators. These groupings were also confirmed by the fall-off technique performed with plasma specimens of the patients.

The individual inactivation indexes determined by the two methods in identical urine samples are shown in Table 1. The indexes of the 19 slow inactivators were within a narrow range, yielding an average index of 1.41 for the present procedure and 1.51 for the previous one. The variations between the individual indexes were greater in fast acetylators, exhibiting an average index of 21.61 for the present and 14.00 for the previous test. Past experience has shown that inactivation indexes of this group extend from 5.50 to 75.00 or above (3, 6). This may explain the greater individual variations observed here. A comparison of the index values produced in the fast acetylators by the two methods revealed striking differences in a few cases. In two specimens, the present test yielded indexes close to five times higher than the spectrophotometric method. These differences can be attributed to the fact that INH concentrations in diluted urine specimens of fast inactivators are occasionally below the accuracy limit of the acetylisoniazid method, whereas the more sensitive spectrophotometric procedure is capable of estimating them. Whether a rapid inactivator exhibits 13.67 or considerably higher indexes (62.87) is negligible for classification of the patients into fast and slow acetylators. It is rather the low index values (5.15, 6.06, and 6.38) which present the critical concentrations of these methods. We may note from Table 1 that the low critical index values estimated by the two methods are in good agreement. Discrepancies in the compared procedures occurred only in well-defined fast inactivators with high indexes (derived from low INH concentrations of the urine) and are therefore of no practical interest.

Acidification of the specimens prior to performance of the acetylisoniazid test is essential and cannot be omitted. A small portion of INH is excreted in the form of hydrazone derivatives or may react with ketones in the urine during

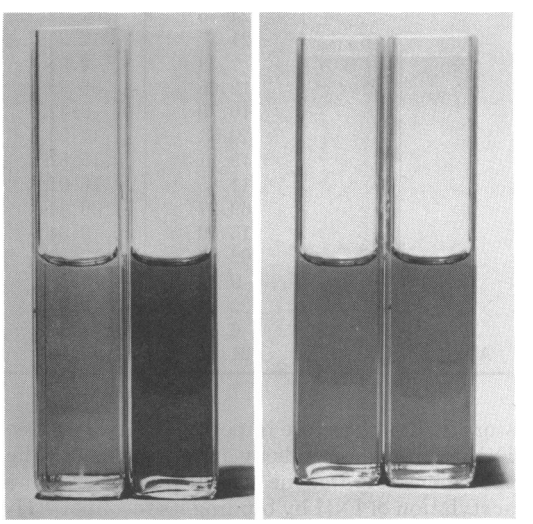

FIG. 1. *Color intensities observed in specimens of slow (left) and fast (right) inactivators.*

TABLE 1. *Comparison of the inactivation indexes of the present method with previous spectrophotometric procedures in 19 slow and 25 fast acetylators*

Groups	Inactivation index	
	Present	Previous
Slow inactivators	1.41	1.54
	1.36	1.36
	1.56	1.48
	0.89	1.05
	1.38	1.28
	0.92	1.03
	1.59	1.36
	1.08	1.49
	0.77	1.03
	1.92	2.06
	1.46	1.27
	1.31	1.35
	1.34	1.45
	1.40	1.44
	1.77	1.91
	1.46	1.44
	1.96	2.15
	2.09	2.21
	1.20	1.88
Averages	1.41	1.51
Fast inactivators	6.06	6.19
	19.38	8.28
	21.96	12.07
	5.15	5.27
	10.97	6.30
	16.12	7.63
	7.76	7.96
	8.82	6.08
	93.59	19.93
	6.96	7.62
	29.18	11.99
	24.45	30.72
	21.57	17.35
	7.34	9.98
	12.65	56.99
	10.70	9.42
	24.53	11.99
	15.11	15.87
	11.35	16.02
	69.27	36.44
	15.60	9.34
	62.87	13.67
	9.94	7.52
	22.64	9.55
	6.38	5.80
Averages	21.61	14.00

storage. It is therefore important to liberate free INH from these bonds by acid treatment of the specimens. Hydrazone formation prevents the acetylation of INH by binding its terminal NH_2 group. On the other hand, the hydrazones may also produce some faint color reactions in the acetylisoniazid procedure. Therefore, in nonacidified specimens the acetylisoniazid values are somewhat elevated, and free INH concentrations are reduced as a result of incomplete acetylation of this compound, yielding higher inactivation indexes as compared to their acid-treated samples. A comparison of indexes with and without acidification of the specimens is demonstrated in Table 2. Nineteen slow inactivators gave an average index of 1.41 with acid treatment, whereas without acidification the average increased to 3.83. In three cases, indexes were as high as 7.82, 7.94, and 8.10. With these indexes, the slow INH inactivators would erroneously be classified as fast acetylators.

Urine tests introduced in the past for phenotyping of INH inactivators (5, 7, 10) did not produce satisfactory results as observed in this laboratory (3, 4). Recently, Russell published a semiquantitative screening test for phenotyping INH inactivators (8). This method is likewise based on the proportion of acetylisoniazid versus the INH concentration in urine specimens. Critical evaluation of this procedure revealed a few drawbacks which have been discussed elsewhere (9). In addition, the screening test of Russell omits the acid treatment of the urine specimens. As shown in our experiment, liberation of INH from its bindings is essential for proper grouping of the patients.

TABLE 2. *Indexes of 19 slow inactivators determined in identical urine samples with and without acid treatment*

Inactivation index	
Acidified	Nonacidified
1.41	2.43
1.36	3.40
1.56	3.10
0.89	2.05
1.38	3.36
0.92	1.83
1.59	3.28
1.08	3.06
0.77	2.90
1.92	3.72
1.46	3.64
1.31	2.15
1.34	3.04
1.40	3.02
1.77	4.57
1.46	3.42
1.96	7.94
2.09	7.82
1.20	8.10
1.41[a]	3.83[a]

[a] Average.

The screening test described here involves acid treatment of the samples. The method employs identical color reaction for INH and its acetyl derivative, which enables a direct comparison of the color intensities produced. It is simple and reliable for rapid phenotyping of INH inactivators.

We gratefully acknowledge the assistance of Paul Varughese in performing the tests.

LITERATURE CITED

1. Eidus, L., and E. J. Hamilton. 1964. A new method for the determination of N-acetylisoniazid in urine of ambulatory patients. Amer. Rev. Resp. Dis. **89**:587-588.
2. Eidus, L., and A. M. T. Harnanansingh. 1971. A more sensitive spectrophotometric method for determination of isoniazid in serum or plasma. Clin. Chem. **17**:492-494.
3. Eidus, L., A. M. T. Harnanansingh, and A. G. Jessamine. 1971. Urine test for phenotyping isoniazid inactivation. Amer. Rev. Resp. Dis. **104**:587-591.
4. Eidus, L., G. M. Ling, and A. M. T. Harnanansingh. 1971. Isoniazid excretion in fast and slow inactivators and its practical aspect for phenotyping. Arzneimittel-Forschung **21**:1696-1699.
5. Iwainsky, H., W. Gerloff, and W. Schmiedel. 1961. Die Differenzierung zwischen INH-Inactivierern and normal abbauenden Patienten mit Hilfe eines einfachen Testes. Beitr. Klin. Tuberk. **124**:384-389.
6. Jeanes, C. W. L., O. Schaefer, and L. Eidus. 1972. Inactivation of isoniazid by Canadian eskimos and indians. Can. Med. Ass. J. **106**:331-335.
7. Maier, N., and V. Moisescu. 1964. Eine schnelle Methode for Bestimmung der Konzentration des durch die Nieren ausgeschiedenen INH. Beitr. Klin. Tuberk. **128**:213-214.
8. Russel, D. W. 1970. Simple method for determining isoniazid acetylator phenotype. Brit. Med. J. **3**:324-325.
9. Russel, D. W., and L. Eidus. Correspondence between D. W. Russel and L. Eidus. 1972. Simplified isoniazid acetylator phenotyping. Can. Med. Ass. J. **106**:1155-1156.
10. Teichmann, W., and R. Kohler. 1964. Beitrag für schnelle Differenzierung des individuellen INH-Stoffwechsels. Prax. Pneumol. **18**:535-541.
11. Venkataraman, P., L. Eidus and S. P. Tripathy. 1968. Method for estimation of acetylisoniazid in urine. Tubercle **49**:210-216.