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# A Filter Paper Method for Determining Isoniazid Acetylator Phenotype

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Several reliable chemical procedures for determining the isoniazid (INH) acetylator phenotype of persons are available (table 1). Initially, INH was used as the test drug [1, 8] for this purpose. A simpler method of phenotyping persons as rapid and slow acetylators was developed by Evans [10] using sulfamethazine. Variations of this procedure using sulfamethazine [12] as well as other sulfonamides such as sulfapyridine [13, 14] have been described.

The procedures referred to in table 1 are designed to use biological fluids (plasma, serum, and urine) and are best suited for typing persons who are nearby or can come to medical laboratory facilities. They are less adaptable for typing individuals who are remote from these facilities because handling liquid specimens and transporting them to a laboratory for analysis, even in a frozen state, can be difficult. A simple procedure which avoids these difficulties would be useful for collecting pharmacogenetic data on the acetylation polymorphism that are virtually unattainable with procedures now available.

Use of small samples of blood or urine dried on filter paper at the site of collection is a proven, convenient way of collecting genetic data for conditions such as phenylketonuria [15]. Sulfamethazine appeared to be an excellent candidate drug to use in a similar way for acetylator typing because it accurately reflects the INH acetylator phenotype [10], the drug and its acetylated metabolite are quite stable to heat and long-term storage (W. Weber, unpublished data), and assays for these substances in blood and urine are simple, sensitive, and readily adaptable to small dried samples of these biological fluids [16, 17].

This report describes a method of acetylator phenotyping based on modifications of Evans's procedure [10] which requires 0.1 ml of whole blood or urine collected 4½ hr after an oral dose of sulfamethazine (10 mg/kg) and dried on filter paper.

## MATERIALS AND METHODS

### *Materials*

Commercially available sulfamethazine powder (free acid) mixed as a suspension with 20–30 ml of water was used as the test drug.

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TABLE 1

CHEMICAL PROCEDURES FOR DETERMINING INH ACETYLATOR PHENOTYPE

| Test Drug and Administration | Dose (mg/kg Body Weight) | Biological Specimen | Discriminant Used | Reference |
|---|---|---|---|---|
| INH: | | | | |
| Oral | 40* | Plasma | INH concentration 6 hr after ingestion | [1] |
| Oral | 4 | Serum | First-order rate constant for INH elimination | [2] |
| Oral | 10 | Urine | Ratio of acetylated INH to INH in 6–8 hr specimen | [3, 4] |
| Oral, slow release matrix | 30 | Urine | Ratio of acetylated INH to acid-labile INH in 24–25 hr specimen | [5] |
| Intramuscular | 8 | Urine | Ratio of acetylated INH to INH in 6–8 hr specimen | [6, 7] |
| Intravenous | 5 | Serum | Biologic half-life of INH | [8, 9] |
| Sulfamethazine: | | | | |
| Oral | 160 and 40* | Plasma or urine | % acetylated sulfamethazine in 6 hr plasma or 5–6 hr urine | [10, 11] |
| Oral | 44 | Plasma or urine | % acetylated sulfamethazine in 6 hr plasma or urine | [12] |
| Oral | 10 | Whole blood or urine dried on filter paper | % acetylated sulfamethazine in 4½ hr blood or urine | Present report |
| Sulfapyridine, oral | 10 | Serum or urine | % acetylated sulfapyridine in 8 hr serum or 7–8 hr urine | [13] |
| Sulfamethazine, sulfapyridine, or sulfasalazine, oral | 10 | Urine | % acetylated sulfonamide in 5–6 hr specimen | [14] |

NOTE.—Modified from Weber [16].
* $mg/kg^{0.7}$.

Whatman no. 3 filter paper was obtained from Reeve Angel, Clifton, N.J.

N-1-(naphthyl)ethylenediamine dihydrochloride was obtained from Fisher Scientific Supply Co., Springfield, N.J.

*Subjects*

Nineteen healthy volunteer adults whose acetylator phenotype (8 rapid, 11 slow) had been determined previously by a slight modification of Evans's procedure [10] were instructed not to eat after 12 midnight or to take any fluids after 7 A.M. of the day of the test. At 9 A.M. the subjects were observed to ingest 10 mg sulfamethazine/kg body weight with 1–2 oz of water. After 11 A.M. they were permitted to eat and drink as usual. The bladder was emptied of urine at 12 noon and urine was retained until collection of blood and urine specimens. At 1:30 P.M. (4½ hr after sulfamethazine ingestion) samples of blood from a venipuncture and urine were collected and 0.1 ml of each was placed within a small circle penciled on filter paper (Whatman no. 3). The specimens were allowed to dry in air.

*Assay of Sulfamethazine and Acetylated Sulfamethazine*

Each filter paper circle containing the dried blood (or urine) was cut out, cut into between six and eight small pieces, and placed in a test tube (10 mm × 100 mm). One-half

ml of trichloroacetic acid (20% in water) was added to cover the paper in each tube and allowed to stand for 5 min. This was followed by 1.5 ml of water and each tube was allowed to stand for an additional 15–20 min. Addition of the trichloroacetic acid and water in that order elutes the drug from the filter paper and fixes most of the blood proteins to the paper. Each tube was then centrifuged for 10 min at 1,500 *g*. Two 0.5-ml aliquots of the supernatant fraction were transferred to separate tubes for analysis of free (unacetylated) and total (unacetylated and acetylated) sulfamethazine. The analyses of free and total sulfamethazine were carried out by a micromodification of the Bratton-Marshall procedure [18] outlined in table 2. The mixture was centrifuged for 10 min at

TABLE 2

PROCEDURE FOR ANALYSIS OF FREE AND TOTAL SULFAMETHAZINE

| PROCEDURE | SULFAMETHAZINE | |
|---|---|---|
| | Free (ml) | Total (ml) |
| Supernatant fraction | 0.500 | 0.500 |
| 4 N HCl | ... | 0.100 |
| Water | 0.100 | ... |
| Boil 1 hr | — | + |
| Diazotization and coupling: | | |
| Aqueous $NaNO_2$ (0.1%) | 0.050 | 0.050 |
| Mix and leave 3 min | ... | ... |
| Aqueous ammonium sulfamate (0.5%) | 0.050 | 0.050 |
| Mix and leave 3 min | ... | ... |
| N-1-(napthyl)ethylenediamine dihydrochloride (0.05%) | 0.200 | 0.200 |

NOTE.—Micromodification of the Bratton-Marshall procedure [18].

1,500 *g* to remove any particulate matter, and the absorbance of the supernatant fraction at 540 nm was determined in a Beckman DU spectrophotometer using 1.5-ml cuvettes of 1-cm light path against water as a blank. The extent of acetylation was obtained by subtracting the "free" absorbance reading from the "total" absorbance reading and dividing the difference by the total reading. An absorbance of 1 OD unit in this assay corresponds to 0.625 $\mu$mole sulfamethazine/ml blood (or urine).

Control experiments with 0.1 ml of blood or urine assayed directly by this procedure without drying gave results identical with those obtained when the specimen was placed on filter paper, dried, eluted, and then assayed. This indicated that sulfamethazine and its acetylated metabolite could be completely recovered from the paper.

## RESULTS AND DISCUSSION

Evans [10] showed that a satisfactory separation of rapid and slow acetylator phenotypes was obtained by analyzing a 1-hr urine sample collected between 5 and 6 hr after sulfamethazine (11 mg/kg) ingestion. The acetylator phenotypes of our volunteer subjects were determined in our laboratory by this procedure (slightly modified) by giving approximately the same dose of sulfamethazine (10 mg/kg) and collecting blood and urine sequentially up to 6 hr after ingestion [19]. We found that subjects could be classified almost as efficiently from specimens obtained at 4½ hr as at 6 hr after drug ingestion.

For the present study, the same subjects were phenotyped again several months later with specimens of blood and urine collected as described (see Materials and Methods) 4½ hr after sulfamethazine ingestion (10 mg/kg). Specimens on filter paper were coded in a double-blind manner. The results are summarized in tables 3 and 4 and in figure 1.

Analysis of the data in table 3 reveals that individuals in this series can be divided into rapid and slow acetylators by (1) the level of free sulfamethazine in blood; (2) the percentage of acetylated sulfamethazine in blood; (3) the percentage of acetylated sulfamethazine in urine; (4) a plot of percentage of acetylated sulfamethazine in urine versus the percentage of acetylated sulfamethazine in blood (fig. 1); or (5) a plot of the percentage of free sulfamethazine in blood versus the level of free sulfamethazine in blood (not shown). Also, it is evident from inspection that the value of free sulfamethazine in urine is, by itself, a relatively unreliable discriminator of acetylator phenotype except for very slow or very rapid acetylators.

The efficiency of discriminating the two acetylator phenotypes by the filter paper procedure and by Evans's procedure, modified slightly as described, is presented in table 4. Agreement between the two procedures is excellent.

Mild transient headaches have been noted by a few subjects during the Evans

TABLE 3

SULFAMETHAZINE CONCENTRATION AND PERCENTAGE ACETYLATION IN BLOOD AND URINE DETERMINED BY FILTER PAPER METHOD

| | SULFAMETHAZINE (μmole/ml) | | | |
|---|---|---|---|---|
| | Blood | | Urine | |
| SUBJECT | Free | % Acetylated | Free | % Acetylated |
| Rapid acetylators: | | | | |
| LA | 0.010 | 82 | 0.768 | 90 |
| SM | 0.012 | 63 | 0.095 | 92 |
| VZ | 0.019 | 71 | 0.0175 | 85 |
| AF | 0.019 | 67 | 0.243 | 84 |
| KD | 0.022 | 46 | 0.0562 | 89 |
| BS | 0.023 | 54 | 0.329 | 87 |
| JC | 0.024 | 67 | 0.280 | 82 |
| DS | 0.026 | 65 | 0.324 | 91 |
| Slow acetylators: | | | | |
| WW | 0.042 | 33 | 0.465 | 66 |
| PA | 0.042 | 33 | 0.301 | 61 |
| PM | 0.055 | 16 | 1.350 | 59 |
| JS | 0.060 | 27 | 0.681 | 63 |
| JW | 0.060 | 35 | 0.274 | 58 |
| JM | 0.061 | 34 | 1.09 | 67 |
| LG | 0.064 | 27 | 0.528 | 62 |
| HA | 0.064 | 20 | 0.484 | 60 |
| GD | 0.069 | 24 | 1.01 | 50 |
| RA | 0.072 | 20 | 0.494 | 57 |
| NA | 0.083 | 2 | 0.369 | 56 |

TABLE 4

COMPARISON OF FILTER PAPER METHOD AND EVANS'S PROCEDURE* OF ACETYLATOR PHENOTYPING

| PARAMETER TESTED | ACETYLATOR PHENOTYPE | | $t$ | $P$ |
|---|---|---|---|---|
| | Rapid | Slow | | |
| Filter paper method: | | | | |
| Free sulfamethazine, blood† | 0.019 ± 0.0056 | 0.061 ± 0.012 | 9.1 | <.001 |
| % acetylated sulfamethazine, blood | 64.4 ± 10.8 | 24.6 ± 9.9 | 8.3 | <.001 |
| Free sulfamethazine, urine† | 0.26 ± 0.24 | 0.64 ± 0.35 | 2.6 | <.02 |
| % acetylated sulfamethazine, urine | 87.5 ± 3.6 | 59.9 ± 4.8 | 13.7 | <.001 |
| Evans's procedure: | | | | |
| Free sulfamethazine, blood† | 0.013 ± 0.0052 | 0.054 ± 0.0093 | 11.2 | <.001 |
| % acetylated sulfamethazine, blood | 71.5 ± 8.7 | 26.9 ± 11.3 | 9.3 | <.001 |
| Free sulfamethazine, urine† | 0.47 ± 0.50 | 1.23 ± 0.70 | 2.6 | <.02 |
| % acetylated sulfamethazine, urine | 87.9 ± 3.7 | 55.3 ± 9.6 | 9.1 | <.001 |

NOTE.—Values given are mean ± SD.

* Evans's procedure [10] slightly modified as described in text.

† Expressed as μmole sulfamethazine/ml blood or urine.

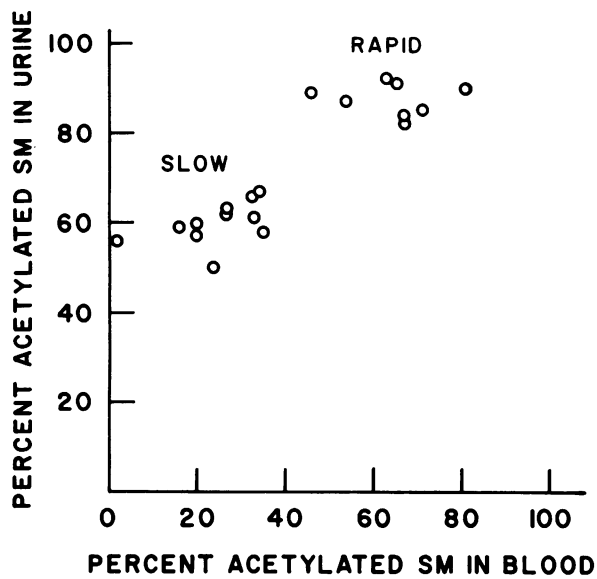


FIG. 1.—Plot of percentage of acetylated sulfamethazine in urine versus the percentage in blood.

phenotyping procedure [10, 20]. A few of our subjects also noted this side effect when they were typed by that procedure. During the test described in this report, in the same subjects, no headaches or other untoward effects occurred. It is possible that the difference in the incidence of this side effect is related to the shorter period of food and fluid restriction used in our test compared to that in the Evans procedure.

SUMMARY

A simplified, safe test is described for drug acetylator phenotyping that uses only 0.1 ml of blood or urine collected 4½ hr after a small oral dose (10 mg/kg) of sulfamethazine and dried on filter paper. Samples are stable and convenient to send to a central laboratory for analysis. The test should be useful for obtaining pharmacogenetic data on the isoniazid acetylation polymorphism, particularly for individuals and populations remote from medical laboratory facilities.

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