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# A METHOD TO PREVENT THE LOSS OF ISONIAZID AND ACETYLISONIAZID IN HUMAN PLASMA

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Plasma concentrations of isoniazid and *N*-acetylisoniazid were measured at varying times after addition of both compounds to plasma (5 μg/ml) and storage at several different temperatures (−70°C, −20°C, 4°C and 20°C). The concentration of both compounds declined log–linearly with time and the half-life of decline was shorter with increasing temperature of storage. Both compounds were stable for at least 5 weeks, however, when stored at −70°C. We conclude that this temperature should be used to prevent loss of both compounds during storage and transport of samples.

## Introduction

Plasma concentrations of isoniazid and acetylisoniazid may be useful in assessing compliance with an efficacy of therapy in tuberculosis. They may also be used to measure the acetylator status of an individual who has developed or who might develop adverse effects of isoniazid or other drugs metabolised by the acetylation pathway in the liver.

Several reports indicate that concentrations of isoniazid and acetylisoniazid are unstable in plasma although the degree of instability is disputed (see Table 1), and some studies show no loss of drug.

All of these studies were performed, however, with relatively non-specific and insensitive spectrophotometric, fluorometric or bioassays and none examined the effects of storage at −70°C. The following study was therefore performed using a sensitive and specific high performance liquid chromatographic method after storage at several temperatures including −70°C for periods of up to 5 weeks.

## Methods

Isoniazid and acetylisoniazid were added to forty samples of blank plasma to achieve concentrations of each compound of 5 μg/ml. These were then stored at room temperature (18–20°C), refrigerated (4°C) and in a −20°C and −70°C freezer. Analysis was performed after exposing two samples to each of these temperatures for 24 h, 4 days, 3 weeks and 5 weeks. Separate samples were spiked with only one of the compounds (5 μg/ml) and stored at room temperature for 24 h to assess possible production of isoniazid from *N*-acetylisoniazid (or *vice versa*) *in vitro*.

Isoniazid and *N*-acetylisoniazid were measured by a modification of the method of Saxena *et al.* (1977). The modification was as follows: the mobile phase used was methanol, 10%, acetonitrile 45%, water 45% rather than methanol 60% water 40%. This resulted in greater column efficiency so that only 1 ml rather than 3 ml plasma samples were needed.

The coefficient of variation of the pairs of samples

**Table 1** Percentage loss of isoniazid after storage in the frozen state

| *Authors* | *Analytical method* | *Time of storage* | *Percentage loss of isoniazid after storage in frozen state* |
|---|---|---|---|
| Poole & Meyer (1961) | Spectrophotometric | 3 weeks | 39% |
| Gangadharam *et al.* (1961) | Biological | 8 weeks | 24% |
| Jenne (1961) | Spectrophotometric | 3 weeks | 39% |
| Peters & Good (1962) | Fluorometric | 8 weeks | 0% |
| | Biological | 4 weeks | 10% |
| Ellard *et al.* (1972) | Fluorometric | 6 months | 0% |
| O'Barr *et al.* (1978) | Fluorometric | 3 weeks | 100% |
| | Biological | 3 weeks | 9% |
| Huffman Dujovne (1976) | Spectrophotometric | 5 weeks | 32% |

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spiked separately and then stored under identical conditions was determined after calculating the standard deviation for the two analyses of the pairs of samples (Barth *et al.*, 1976).

Results were analysed by two way analysis of variance (Francis, 1973) and least-squares linear regression for analysis for time trends (Snedecor & Cochran, 1967).

In all cases, $P < 0.05$ was taken as the minimum level of statistical significance.

## Results

The coefficient of variation of isoniazid and *N*-acetylisoniazid determination in replicate samples was 1.8% and 4.1% ($n = 5$ in each case) respectively. The coefficient of variation of the samples spiked separately and stored under identical conditions was 4.6% for isoniazid and 5.7% for *N*-acetylisoniazid ($n = 40$ in each case). The mean concentrations of these duplicate samples expressed as a percentage of the added concentration (5 $\mu$g/ml) are shown in Table 2.

Two way analysis of variance of the isoniazid results gave the following results. Effect of temperature of storage, $F$ ratio = 167.53, $P < 0.001$; Effect of time of storage, $F$ ratio = 27.63, $P < 0.001$; $F$ ratio for interaction effect = 13.06, $P < 0.001$. Thus we conclude that isoniazid disappears from plasma over time and this time related disappearance is dependent on temperature of storage. For acetylisoniazid, the corresponding values were:- effect of temperature of storage, $F$ ratio = 791.23, $P < 0.001$; effect of time of storage, $F$ ratio = 82.50, $P < 0.001$; $F$ ratio for interaction effect = 20.21, $P < 0.001$. Thus the same conclusions are made for the metabolite.

There was thus a considerable decline in plasma concentration of INH and acetyl INH at 20°C, 4°C and even at −20°C. In all cases except for isoniazid at 4°C this decline fitted more closely a log–linear rather than linear relationship ($P < 0.05$) with half-lives of disappearance of 18.6 days for isoniazid at 20°C and 61.72 days at −20°C. The half-life of *N*-acetylisoniazid disappearance was 0.6 days at 20°C, 1.4 days at 4°C and 37.5 days at −20°C. There was no significant decline in concentration of either compound at −70°C, however, ($P > 0.05$). In the samples spiked with isoniazid only, no production of *N*-acetylisoniazid occurred after 3 weeks at 20°C. Similarly, no isoniazid was seen in samples spiked only with *N*-acetylisoniazid after storage under the same conditions.

The ratio of *N*-acetylisoniazid to isoniazid concentration is sometimes used to determine acetylator status. This ratio (initially unity) in this study was therefore examined at all the times of measurement and the results are shown in Table 2. As would be expected, the ratio at −70°C remained between 1 and 1.05. At all other temperatures, however, the ratio varied more than would be expected from the errors in measuring the two compounds.

Several other approaches were made to stabilise isoniazid and *N*-acetylisoniazid in plasma. These included acidification with acetic acid and alkalinisation with sodium hydroxide. Saturation with salt and addition of acetonitrile, 20% sodium sulphate, sodium fluoride, urea, ethylene glycol, vitamin C or silver nitrate were also tried as well as heating at 55°C, 70°C and 80°C for 5, 15 and 30 min prior to freezing. Samples were also heated at 100°C after acidification to pH 3. None of these manoeuvres was successful in preventing loss of either compound.

## Discussion

The poor agreement concerning isoniazid stability between previous studies may be related in part to the relative non-specificities of the methods used to measure the drugs. The small loss of isoniazid in the two studies in which a biological method was employed may indicate that possible breakdown products may retain antibacterial activity (Gangadharam *et al.*, 1961; Peters & Good, 1962). Similarly, breakdown products of isoniazid may fluoresce accounting for the retention of isoniazid in samples stored frozen for 6 months (Ellard *et al.*, 1972). This observation is markedly different from the apparent complete loss of isoniazid observed by another group (O'Barr, 1978) in samples stored for only 3 weeks under similar conditions and measured by fluorimetry.

The loss of isoniazid at 3 weeks (35%) which we observed agrees closely with Jenne's (1961) results. At 5 weeks, our percentage loss of 37% was also very similar to the 32% loss observed by Huffman &

**Table 2** Drug concentration as a percentage of the original concentration, after storage at different temperatures.

| | *Isoniazid* | | | | *Acetylisoniazid* | | | |
|---|---|---|---|---|---|---|---|---|
| | *1 day* | *4 days* | *3 weeks* | *5 weeks* | *1 day* | *4 days* | *3 weeks* | *5 weeks* |
| +20°C | 76 | 60 | 47 | 21 | 41 | 0 | 0 | 0 |
| + 4°C | 79 | 67 | 52 | 59 | 69 | 14 | 0 | 0 |
| −20°C | 84 | 80 | 65 | 63 | 96 | 90 | 62 | 53 |
| −70°C | 98 | 97 | 100 | 103 | 99 | 102 | 101 | 106 |

Dujovne (1976). Poole & Meyer (1961) observed, like us, that increasing temperature was associated with increased loss of isoniazid, so that loss of isoniazid after 3 weeks was complete at 37°C, 90% at 25°C and 57% at 7°C.

Only two studies of *N*-acetylisoniazid stability in plasma have been recorded. After a week at room temperature, only 7% could be recovered from serum when spectrophotometric or fluorometric methods were used and isoniazid was not formed from *N*-acetylisoniazid (Ellard *et al.*, 1972). At −20°C, its breakdown was equivalent to 3% a week which is much slower than we observed (9% per week) but was certainly faster than the losses of isoniazid that the same group recorded (4% per month). This difference in rate of loss of both compounds is important since it is at variance with the results of another group (Huffman & Dujovne, 1976) who reported equal percentage losses in both compounds (32% in 5 weeks) at −20°C when they measured both drugs by spectrophotometric means. They also reported that the decline was linear rather than the log-linear decline which was observed by two other groups (Gangadharam *et al.*, 1961; Ellard *et al.*, 1972) as well as by us.

There are several clinical implications of our findings. Isoniazid concentrations in plasma are often used to measure compliance. Since the assay is not widely available, delay may occur during which the sample may be at ambient temperatures for a prolonged period. Patient non-compliance may therefore be falsely assumed. Plasma concentrations of the drug are sometimes measured to prevent toxicity. It is recommended for example that isoniazid plasma concentration at 24 h after the dose should be kept below 1 μg/ml particularly in slow acetylators of the drug who have renal disease and who tend to accumulate the parent drug (Bowersox *et al.*, 1973). Losses occurring *in vitro* may similarly cause an underestimate of the true plasma concentration and result in toxicity. The elimination half-life of isoniazid after a single oral dose may be used to determine acetylator status. If the absolute loss of isoniazid was greater at higher drug concentrations, the elimination half-life of the drug might be falsely increased with increasing time of storage. Although we did not specifically test for concentration-dependent drug loss, the log–linear decline in concentration observed by Gangadharam *et al.* (1961), Ellard *et al.* (1972) and by us suggests that this could be the case.

The ratio of *N*-acetylisoniazid to parent compound at a fixed time after a single oral dose of isoniazid is sometimes used to determine acetylator status. According to Huffman & Dujovne (1976) this ratio should remain constant despite drug loss at −20°C. Our findings, however, support the results of Ellard *et al.* (1972) that *N*-acetylisoniazid levels fall faster than those of the parent compound so that this ratio may be perturbed.

Fortunately, it is now possible to prevent loss of both compounds if plasma is stored immediately at −70°C, although freezers which attain these temperatures are not yet widely available. Solid carbon dioxide (temperature −70°C) can be used for storage or transport of samples to centres where the sample may then be assayed. This is of course inconvenient, but in the absence of any other effective preventative measure, the only other way would be to estimate the losses of both compounds based on the conditions and length of time between collection and analysis, and this is likely to be much less accurate.

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