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The Reliability and Practicality of the Arkansas Method Assay of INH Adherence

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Abstract

The Arkansas Method (AM) for Isoniazid (INH) metabolite detection is a relatively inexpensive, simple, objective measure of adherence. The purpose of the study was to explore whether variations in urine sample handling and storage will produce accurate assay outcomes. Participants were a convenience sample of 28 adults and adolescents prescribed INH for Latent Tuberculosis Infection (LTBI). Participants provided one sample to test effects of: mixing processes; durations at room temperature, in a refrigerator, or frozen; and effects of freeze/thaw cycles on AM outcomes. No manipulations had a discernible impact on outcomes with concordant positive rates from 85–100%. Concordance rates of manipulated samples didn't appear to differ from rates of norm samples. Results suggest that urine samples can withstand a variety of manipulations in both handling and storage without affecting the accuracy of AM assay results. These findings have important implications for providers of treatment and researchers, and provide the impetus for both to examine the potential of using the AM of INH metabolite testing as a measure of medication adherence.

Keywords

Arkansas Method; Tuberculosis; LTBI; Adherence; Isoniazid

Background and Rationale

An estimated one-third of the world's population is infected with Latent Tuberculosis (LTBI), with an estimated 14.1 million active Tuberculosis (TB) cases (World Health Organization [WHO], 2007). Tuberculosis causes 1.6 million deaths annually (WHO, 2007). Treatments are available that can reduce the risk of conversion from LTBI to active TB disease by up to 70–90% (US Congress, 1993; International Union Against Tuberculosis Committee on Prophylaxis [IUATCP], 1982; Comstock, Hammes & Pio, 1969; Mount & Ferrebee, 1961). However, many individuals do not complete the medication regimen (Macintyre, Goebel & Brown, 2005; Eidlitz-Markus, Zeharia, Baum, Mimouni, & Amir, 2003; Hollender, Ashkin & Narita, 2003; Hovell, et al., 2003; Palanduz, Gultekin, Erdem & Kayaalp, 2003; Marks et al., 2000; Bock, Metzger, Tapia & Blumberg, 1999; Ailingier & Dear, 1998; IUATCP, 1982). Failure to complete treatment leaves individuals at risk of conversion to active TB disease and increases development of drug resistance (WHO, 2007; Chaulk & Kazandjian, 1998; Sumartojo, 1993; Centers for Disease Control and Prevention [CDC], 1991).

The current CDC treatment recommendation for LTBI in the United States is 9 months of Isoniazid (INH), one pill per day at a maximum dose of 300mg (5mg/kg for adults, 10–20mg/kg for children) (CDC, 2000). INH is also included in first-line treatment regimens for active TB disease in the United States and internationally (CDC, 2003; WHO, 2003). Available methods to improve adherence to INH include: Directly Observed Therapy (DOT) (typically reserved for active TB disease), pill counts, electronic monitoring, and INH metabolite detection in urine samples. Each method has limitations (Hollender et al., 2003; Palanduz, et al., 2003; Perry et al., 2002; Davidson, 1998; Sumartojo, 1993), for example, the cost of DOT and inability to prove ingestion with pill counts or electronic monitoring. Metabolite detection, using the Arkansas Method (AM) (Schraufnagel, Stoner, Whiting, Snukst-Torbeck & Werhane, 1990), is a relatively inexpensive, simple, and objective measure of INH consumption. Research has shown that collection of urine samples for INH

detection, and consequent feedback to patients, may improve adherence (Eidlitz-Markus et al., 2003).

Unannounced collection of urine samples, rather than collection at the time of a scheduled visit, may provide a more accurate assessment of INH compliance by avoiding temporary, reactionary compliance immediately prior to a scheduled visit. Unannounced sample collection for non-institutionalized patients generally occurs in the field, which raises the issue of requisite sample handling and storage conditions. Researchers in the field often do not have immediate access to freezers and may collect several samples before returning to the lab. While testing methods have been developed that may be suited to field testing, such as the Isoscreen point-of-care testing device (GFC Diagnostics Limited, Oxfordshire, UK), costs of these methods may be prohibitive, especially for use in developing countries (Whitfield & Cope, 2004; Cope & Whitfield, 2003; Eidlitz-Markus & Amir, 2003; Palanduz et al., 2003).

Purpose

This study explored various conditions under which urine samples could be handled and stored while still yielding accurate assay outcomes, specifically: sample mixing methods; various durations at room temperature (at 21–23°C), in a refrigerator (at 2–6°C) and in a freezer (at minus 12–16 °C); and freeze/thaw cycles.

Methods

Participants and Design

A convenience sample of 28 adults and adolescents prescribed INH for LTBI was recruited from an ongoing NIH-funded LTBI study and from local community clinics. To be included participants had to be 13 years of age or older, prescribed INH for LTBI, and currently taking the medication. The NIH study participants provided urine samples and completed interviews about medication adherence during the final phase of the LTBI study. Participants recruited from the community clinics were attending routine, monthly follow-up visits. After obtaining applicable informed consents/assents, each participant was asked whether they had taken their INH pill today, yesterday, the day before, and the day before that, as well as the time medication was taken for each day they reported INH consumption. One urine sample was collected from each participant. Participants were provided a small monetary incentive for participation. After varying storage and handling conditions (See Table 1), all samples were tested in duplicate using the AM (Schraufnagel et al., 1990) as described below. All procedures were approved by the San Diego State University Institutional Review Board.

Procedures

Mixing Methods—After collection and transportation to the lab, samples were allowed to sit undisturbed for 12–24 hours in a refrigerator. After the 12–24 hour settling period, two 1ml aliquots were taken from the top of each sample without mixing, two were taken after gentle inversion three times by hand, and two after vigorous mixing (inversion by hand for 15 seconds). These aliquots were then frozen; after approximately one month, they were thawed and tested with the AM.

Temperature Duration—The remaining sample was divided into 48 1ml aliquots after a second round of vigorous mixing. Twenty-four conditions were tested in duplicate from each sample. The first two aliquots (“norm aliquots”) underwent the established procedure of immediate freezing, then were thawed and tested to confirm INH positive status within

approximately one month. Fourteen aliquots ($n = 2$ for 7 conditions) were left at room temperature ($21\text{--}23^{\circ}\text{C}$) for 1, 2, 3, 4, 5, 6 and 7 days before freezing. Twenty-two aliquots ($n = 2$ for 11 conditions) were placed in the refrigerator (at $2\text{--}6^{\circ}\text{C}$) for 1, 2, 3, 4, 5, 6, 7, 10, 14, 21 and 28 days before freezing. In about one month's time, the frozen aliquots were thawed and tested in parallel with the mixing conditions samples. Ten additional aliquots ($n = 2$ for 5 conditions) were frozen immediately (at minus $12\text{--}16^{\circ}\text{C}$) and thawed and tested at 1, 2, 3, 6, and 9 months from collection.

Freeze/Thaw Cycles—After initial testing, positive “norm aliquots” were refrozen to study the effect of freeze/thaw cycles. Samples were frozen, and after at least 24 hours, were thawed, and re-tested, for a total of three cycles.

Urine Assay Protocol—Frozen urine samples were allowed to thaw at room temperature for approximately 20 minutes and then were mixed by inversion and vortexing. Based on AM procedures described by Schraufnagel et al. (1990), 141uL of urine was added to 30mg of Barbituric Acid in a 1.5mL polypropylene graduated microcentrifuge tube. 70uL of Chloramine T (140mg/mL) was added to each tube followed by 70uL of Potassium Cyanide (50mg/mL). Tubes were then vortexed for at least 20 seconds or until sufficient mixing occurred and at five minutes the color change was observed and recorded. The colors of blue, blue-green, and green were considered positive reactions and indicative of samples containing the INH metabolite. The colors of white, peach, yellow or brown were considered negative reactions and indicative of the absence of INH metabolites. Batches of no more than six samples were analyzed at one time, with positive and negative controls (0.1mg/ml INH and 10% methanol, respectively) tested with each batch. All conditions were tested in duplicate as a reliability check. Discrepant results were retested and color change was noted if it occurred outside of the 5-minute window. Assay waste was hazardous and was disposed of according to federal and state regulations.

Results

Of the 28 participants recruited, 26 participants reported taking their pill today, yesterday, or the day before. Two participants reported no INH consumption within this time period and were analyzed separately as negative controls. Six of the 26 participants who had reported taking their pills tested negative for INH metabolites using standard AM procedures and were removed from analyses, leaving a final sample of 20 participants who tested positive for INH metabolites (mean age = 35.7 years, [SD = 19.1; Range 13–74]; 60% female; 85% Latino). The final sample reported having taken INH for an average of 5.15 months (SD = 2.66; Range = 1 to 9) and that INH was last consumed an average of 15.6 hours prior to sample collection (SD = 7.1; range = 2.1 to 25.1 hours).

All positive and negative controls resulted in the expected outcomes. All samples from participants reporting no INH consumption produced negative results for all iterations. All samples were tested in duplicate. A pair of positive outcomes was classified as concordant positive, a pair of negative outcomes was classified as concordant negative, and a pair with one positive and one negative outcome was classified as a discordant pair.

During the first round of testing, there was an overall 95.1% positive concordance rate (510/536 paired samples); 3.5% (19/536) of the pairs were discordant and 1.3% (7/536) were concordant negative. After retests of the discordant and concordant negative results and the inclusion of late-turning samples (i.e., those changing color outside of the 5-minute window but within 20 minutes), there was an overall 99.6% positive concordance rate (534/536 paired samples).

For storage and handling condition comparisons, the most conservative, first pass concordance positive rate was used and those results are presented in Table 1. Norm aliquots had a concordant positive rate of 95%. For the mixing methods, 100% of the unmixed, inverted and vigorously mixed samples tested concordant positive. Of the samples left at room temperature for 1–7 days, in the refrigerator for 1–28 days, and in the freezer for 1–9 months, between 85–100% remained concordant positive. No consistent patterns emerged based on the length of time at room temperature, or in the refrigerator or freezer. Paired samples underwent three freeze/thaw cycles each, resulting in concordant positive rates between 95–100%. Table 2 presents the positive concordant rates inclusive of retests and late-turning samples.

Discussion

To determine acceptable storage and handling conditions for urine samples to be tested with the AM of INH metabolite detection, various deviations from standard procedure were examined. Results suggest that the AM is a robust assay that can detect INH metabolites in urine after a variety of storage and handling conditions. The mixing methods used on the urine samples prior to taking aliquots had no effect on assay outcomes, indicating that individual variations in mixing method are tolerable. Length of time left at room temperature, in the refrigerator, or in the freezer (up to 7 days, 28 days and 9 months, respectively) also had no discernible impact on positive outcomes. Another study similarly found that samples could sit for a 6-hour period at room temperature before testing without altering test results (Hanifa et al., 2007).

Our findings indicate that greater flexibility in the handling and storage of urine samples is acceptable and should not be detrimental to assay results. This allows for greater efficiency, as samples can be stored at room temperature or in the refrigerator or freezer until batches can be divided or tested. Loss of data due to delayed refrigeration or freezing can be minimized and retests can still occur months after initial freezing. While lengthy delays in testing would not benefit patients if used as a feedback mechanism to promote better adherence, delays could be tolerated in clinical or research settings where immediate feedback is not required. Over a nine-month treatment regimen, periodic feedback, even if delayed by days or weeks, might be beneficial to patients.

Finally, the assay was also able to endure three freeze/thaw cycles with no apparent effect on positive outcomes. This finding indicates that samples can be refrozen after initial testing to be reanalyzed at a later date, should it become necessary due to discordant results of matched samples, operator error, or missing data. Refreezing of an original sample for retesting alleviates the need to collect a larger urine sample or to store multiple vials of the same sample, benefiting those with limited storage capacity or budgets.

While a standard protocol is recommended, the deviations from protocol explored in this study are acceptable. The durability of the AM under varying conditions gives confidence in assay results; duplicate negative outcomes suggest truly negative results rather than being due to sample deterioration from poor handling or storage conditions. However, it should be noted that even under ideal, standard conditions, false negative results occur, likely due to operator error or a testing anomaly. For example, accidental exclusion of one of the assay components or poor mixing of the Barbituric Acid could result in a negative outcome.

Other studies have also reported “biologically inconsistent” results (Hanifa et al., 2007). The “norm aliquots” tested in this study, those using established procedures, only had a 95% concordant positive rate when a 100% rate was expected. This discordant rate is similar to what other studies have previously reported (Whitfield & Cope, 2004; Davidson, 1998). It is

interesting to note that all of the discordant and negative concordant results (26/536) were from only 5 (25%) of the final sample participants instead of randomly scattered throughout the sample. There was no significant difference in the length of time between sample collection and INH consumption between this subset and the remaining sample (16.5 vs. 15.1 hours), implying that some other, unknown factor might be affecting these few samples. Samples should be tested in duplicate to reduce the likelihood of false negative reports and optionally retested if discordant or concordant negative results occur.

The above conclusions were based on the most conservative, first pass, concordance rate. However, more liberal estimates of concordance, including late-turning samples, likely represent true agreement and should not be overlooked at the risk of underestimating the reliability of the assay or falsely accusing a patient of failure to adhere to their INH regimen. During assay optimization, the 5-minute wait for color change was found to be ideal. However, other labs using the AM have used windows ranging from 1 to 10 minutes, or have simply noted to observe color change with no time frame specified (Eidlitz-Markus et al., 2003; Palanduz et al., 2003; Davidson, 1998; Sumartojo, 1993). Viewing the liberal estimates of concordance, nearing 100%, further strengthens the opinion that deviations in the handling and storage conditions of urine samples to be tested using the AM, as reported here, are acceptable.

Future studies should examine the effect of heat on samples, to mimic possible conditions of field collection in warm climates, as well as evaluate the potential effects of the concentration of urine samples on the AM. Other studies have reported the INH detection limits to be between 24–72 hours (Hanifa et al., 2007; Whitfield & Cope, 2004; Eidlitz-Markus et al., 2003; Perry et al., 2002; Elizaga & Friedland, 1997), with color changing from blue to blue-green to green as time between INH consumption and urine collection lengthens. No reports were found regarding whether the concentration of the urine sample has a similar effect on assay outcomes. For example, does a dilute urine sample result in a green outcome at one end of the color gradient or simply in a lighter blue color at the other end of the spectrum? Is INH detectable in a dilute sample? Observations within the current study indicated no relationship between the concentration of urine and color gradient, but additional exploration is required.

The color of urine can also be influenced by other medications, including Rifampin, another TB medicine. However, other studies using the AM to measure INH adherence in patients also taking Rifampin have not indicated that urine staining caused by Rifampin interfered with assay outcomes (Whitfield & Cope, 2004; Palanduz et al., 2003). The effect of smoking status on AM assay outcome is unclear, with some reporting influence on the color gradient (Schraufnagel et al., 1990) and others reporting no effect (Hanifa et al., 2007; Elizaga & Friedland, 1997).

The AM requires use of toxic chemicals, including Potassium Cyanide. While use of the Isoscreen point-of-care testing device reduces exposure to such chemicals (as the assay chemicals are enclosed within the testing device), the cost may be unaffordable for some, including low budget clinics, developing and high burden countries. Where cost is prohibitive, safety procedures should be established to protect the health of those conducting the AM assay.

Application

Our findings suggest that the AM may be used to confirm adherence to INH under less than ideal storage and handling conditions of urine samples, without impacting the accuracy of assay results. Concordant negative results are likely to indicate a lack of INH in the urine and use of the AM in clinical settings may prompt practitioner intervention with the non-

adherent patient. Our results have important implications for clinicians providing LTBI treatment and researchers measuring adherence and provide the impetus for both to explore the potential of using the AM of INH metabolite testing as a measure of medication adherence.

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Table 1

Frequency of concordant positive samples for each condition (n=20) * ; 1 st Pass **									
Norm	Mixing	Room Temperature (21-23°C)	Refrigerator (2-6°C)	Freezer (minus 12-16°C)	Freeze-Thaw Cycles				
19/20	No Mix	1 Day	1 Day	1 Month	Cycle 1	20/20			
	Invert	2 Day	2 Day	2 Months	Cycle 2	19/20			
	Vigorous	3 Day	3 Day	3 Months	Cycle 3	19/20			
		4 Day	4 Day	6 Months					
		5 Day	5 Day	9 Months					
		6 Day	6 Day						
		7 Day	7 Day						
			10 Day						
			14 Day						
			21 Day						
			28 Day						

* All variants had n=20 paired samples with exception of refrigerator day 21 (n=19), freezer month 6 (n=6) and freezer month 9 (n=3).

** These results do not include retests or samples with delayed color change.

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Table 2

Frequency of concordant positive samples for each condition (n=20) [*] ; 2 nd Pass ^{**}									
Norm	Mixing	Room Temperature (21–23°C)		Refrigerator (2–6°C)		Freezer (minus 12–16°C)		Freeze-Thaw Cycles	
20/20	No Mix	15/15	1 Day	20/20	1 Day	20/20	1 Month	Cycle 1	20/20
	Invert	15/15	2 Day	20/20	2 Day	20/20	2 Months	Cycle 2	20/20
	Vigorous	15/15	3 Day	20/20	3 Day	20/20	3 Months	Cycle 3	20/20
			4 Day	20/20	4 Day	20/20	6 Months		
			5 Day	20/20	5 Day	20/20	9 Months		
			6 Day	20/20	6 Day	20/20			
			7 Day	20/20	7 Day	20/20			
					10 Day	20/20			
					14 Day	19/20			
					21 Day	19/19			
					28 Day	20/20			

^{*} All variants had n=20 paired samples with exception of refrigerator day 21 (n=19), freezer month 6 (n=6) and freezer month 9 (n=3).

^{**} Results include retests and samples with delayed color change.