## TECHNICAL NOTE

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# Vitamin $B_2$ Interference with TDx Drugs-of-Abuse Assays*

**REFERENCE:** Kunsman GW, Levine B, Smith ML. Vitamin $B_2$ interference with TDx drugs-of-abuse assays. J Forensic Sci 1998; 43(6):1225–1227.

**ABSTRACT:** Migraine is a headache condition found in significant frequency in the general population. One recent study has shown that riboflavin, Vitamin $B_2$, is an effective prophylactic treatment for this headache condition. One subject in a recent study conducted by the Division of Forensic Toxicology, Armed Forces Institute of Pathology (AFIP) was taking 200 mg of riboflavin twice daily for the prevention of migraine headaches. When that subject's urine was tested using Abbott TDx drugs-of-abuse assays a number of tests resulted in a *MX BKG* error and all samples had *BLK I* values greater than those observed with normal urine specimens. The *MX BKG* error occurs when the *BLK I* value is greater than the upper limit determined by the manufacturer for a particular assay. High *BLK I* values may result if the specimen being analyzed contains a fluorophore that will compete with the fluorescein-labeled antibody used in the assay. This error serves as a notification that an interfering substance may be present and the assay is not performing according to manufacturer-specifications. Upon termination of riboflavin therapy the subject's *BLK I* values began to decrease within 60 h of the last 200 mg dose. A second subject began chronic riboflavin use to confirm this interferent effect. Elevated *BLK I* values resulted within 3 h of a single 200 mg dose and *MX BKG* errors occurred 1 h after a second 400 mg dose. No false negative results were noted with either subject (both subjects used butalbital and the first subject also used hydrocodone and diazepam during the study), suggesting that riboflavin is not an adulterant. Riboflavin use, however, does interfere with the TDx DAU assays and may result in quantitative values being determined which are of questionable validity in the face of an elevated *BLK I* value or may result in only an *MX BKG* error and no quantitative value reported. It is unclear if the interfering fluorophore is simply riboflavin itself or a combination of riboflavin and its metabolic products. Results obtained on urine samples collected from individuals using prophylactic riboflavin for migraine prevention and analyzed by TDx may be of questionable validity. Such samples may require analysis utilizing another immunoassay technique that does not employ a fluorescein-labeled antibody.

**KEYWORDS:** forensic science, forensic toxicology, interferents, riboflavin, drug testing, TDx drugs of abuse assays

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Migraine is one of the most common headache conditions known to man with estimates that 23 to 29% of women and 15 to 20% of men in the general population are affected (1,2). Most migraine patients are initially treated with acute attack-aborting medications. If the headache does not respond to the acute medication then prophylaxis is indicated. In fact, prophylactic drug therapies are frequently necessary to control this condition. Despite their effectiveness, however, prophylactic therapies are typically accompanied by significant side effects and breakthrough headaches (i.e., migraine headaches occur despite treatment). As a result, migraine sufferers have increasingly sought relief through other means. Homeopathic and naturopathic remedies (dietary control, herbal supplements, etc.) as well as a variety of ''alternative'' medical techniques (medical acupuncture, massage therapy, aroma therapy, etc.) have gained popularity and are becoming ''mainstream'' therapies. Many migraine sufferers favor the use of natural products that protect against migraine without the associated sedation and other unpleasant side effects associated with most pharmacologic treatments. One natural product that has recently gained favor is riboflavin (3).

Riboflavin, 7,8-dimethyl-10-(1′-D-ribityl)isoalloxazine (Fig. 1), is one of the B-complex vitamins labeled as $B_2$. It is a yellowish-green fluorescent compound widely distributed throughout nature. It is optically active, light sensitive, amphoteric, decomposes at 280°C, is slightly soluble in neutral polar solvents, decomposes under alkaline conditions, and is relatively stable under acidic conditions (4,5). The recommended daily requirement for riboflavin ranges from 0.06 mg for children up to 1.8 mg for adults; increased amounts are needed during pregnancy and lactation (5). Riboflavin deficiency has been found only in association with deficiencies of other B-complex vitamins, especially niacin and thiamin (5). In a recent study, 49 subjects were administered 400 mg/day of riboflavin. Twenty-three of those subjects were also administered one aspirin per day. Migraine severity decreased by nearly 70% in both the aspirin-plus-riboflavin group and the riboflavin-only group. There is no apparent acute toxicity associated with riboflavin use, but there is no information available concerning long-term use of this vitamin at levels significantly greater than the recommended daily allowance (3).

### Background

During a recent study conducted by the Division of Forensic Toxicology, Armed Forces Institute of Pathology (AFIP), urine was collected from a number of subjects at various times throughout the day over a period of several days. These urine specimens

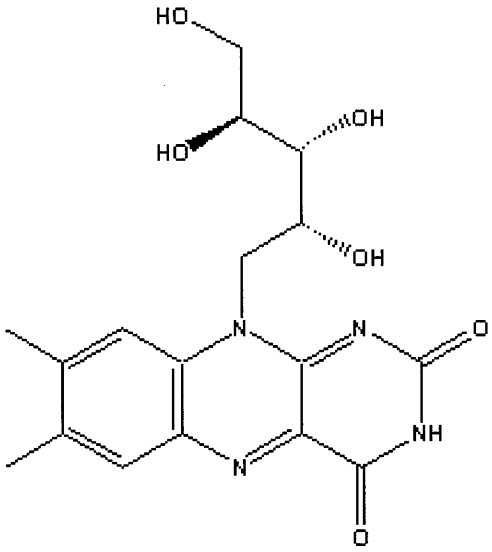


FIG. 1—*Riboflavin structure.*

were subsequently analyzed for drugs of abuse using the Abbott TDx. During this analysis a number of unusual test results and errors occurred associated with the urine of a single subject. This report details the process undertaken to determine the cause for these results.

## Experimental

All urine samples collected as part of the original study were tested for the presence of amphetamine/methamphetamine, barbiturates, benzodiazepines, cocaine metabolite, opiates, phencyclidine, and cannabinoids using Abbott TDx reagents and in accordance with the manufacturer's specifications. The TDx utilizes fluorescence polarization immunoassay (FPIA) technology for the detection of drugs in urine. TDx reagents contain drug-specific antibodies and drug labeled with fluorescein, a fluorescent tracer. The tracer, when excited by plane polarized light, emits fluorescence with a degree of polarization inversely related to its rate of rotation. Unbound tracer becomes randomly oriented and the polarization of fluorescence is low. Tracer binding to its specific antibody results in the tracer rotating at a slower rate and an increase in the polarization of emitted light. Unlabeled drug in the patient specimen competes with the tracer for a limited number of antibody sites. If the patient specimen contains a high concentration of drug the degree of polarization is low. If the patient specimen contains a low concentration of free drug more tracer is bound and the degree of polarization is high. The TDx calculates the concentration of drug present in the specimen based on a calibration curve utilizing the proportional relationship between drug concentration and the degree of polarization (6,7).

## Results

Analysis of the urine specimens from one individual in this study resulted in *MX BKG* errors on some of the assays with all samples having *BLK I* values greater than those observed for the other urine specimens collected and analyzed as part of that study. It was also noted that the *MX BKG* error did not occur in a consistent pattern between different samples (e.g., a *MX BKG* error may have occurred on both the opiate and benzodiazepine assays with one sample and on the barbiturate and cannabinoid assays on the sample collected immediately after that).

This subject was found to be taking 200 mg of riboflavin twice per day for migraine prophylaxis, butalbital as a headache ''rescue'' drug and hydrocodone and diazepam for an unrelated condition. Upon closer inspection of the urine specimens from this subject it was noted that some of the specimens were an unusually bright yellow in color although most did not appear unusual in any way. It was also noted that the intensity of the color increased in some of the samples with increasing storage time.

Results on the benzodiazepine, barbiturate, and opiate assays were positive in a pattern consistent with the subject's use of those drugs. The only instance in which one of these drugs was administered and a positive result was not obtained on the TDx were those runs in which a *MX BKG* error was recorded; the TDx does not report any value in the presence of such an error.

## Discussion

The TDx system calculates the drug concentration in a patient specimen based on the relationship between net polarization and the concentration of drug in the calibrators supplied by the manufacturer. Net polarization is calculated from the measured intensities. The instrument first determines the *BLK I*, which is the background or blank fluorescent intensity of the specimen and the buffer/reagent before the fluorescent tracer is added. After the fluorescent tracer is added to the specimen the *NET I* (net fluorescent intensity) is measured; this intensity measurement is corrected for the *BLK I*. The *NET P* (net polarization) is calculated from the final intensity reading taken after the reaction is complete (8).

The *MX BKG* error occurs when the *BLK I* value is greater than the upper limit determined by the manufacturer for a particular assay. High *BLK I* values may result if the specimen being analyzed contains a fluorophore that will compete with the fluorescein-labeled antibody used in the assay. This error serves as a notification that an interfering substance may be present and the assay is not performing according to manufacturer's specifications (6). The *MX BKG* errors and high *BLK I* values associated with this subject's specimens indicate that an interfering substance may be present in the urine. For those assays in which a high *BLK I* was measured without a *MX BKG* error, the resulting value calculated by the TDx may not be reliable, also as a result of the presence of an interfering substance.

Since riboflavin and some of its metabolites are fluorophores, it was presumed that the riboflavin administration was the source of the interfering substance. To test this presumption the subject agreed to stop taking riboflavin and collect another series of urine specimens. A decrease in *BLK I* values was noted within 60 h after the last 200 mg dose. Further confirmation of the role of riboflavin as an interfering substance in the TDx assay was obtained by recruiting another subject to begin taking riboflavin to determine if the same pattern of results was obtained as noted with the first subject. The volunteer was also a migraine sufferer who was using butalbital as a ''rescue'' medication. The second subject took 200 mg of riboflavin and provided a urine specimen 3 h later; elevated *BLK I* values were noted in all assays. A 400 mg dose was taken 3 h after the first dose and a second urine specimen collected 1 h after that; *MX BKG* errors resulted on 4 of the 7 assays run. This subject also had positive barbiturate results before the *BLK I* values reached a level high enough to trigger the *MX BKG* error. These results clearly point to riboflavin administration as the source of the interfering substance resulting in the elevated blank fluorescent

intensity values in both subjects. It is important to note that both subjects were taking riboflavin in doses well above the recommended daily requirement for this vitamin; therefore, it is unlikely that normal riboflavin consumption in the diet or the use of riboflavin to supplement the diet will result in this interferent effect.

The results of immunoassay systems may be called into question for a number of reasons. One reason is the result of the presence of cross-reacting substances that are not of interest; e.g., codeine is a substance of interest that cross-reacts with some morphine immunoassays, but digoxin-like immunoreactive factors are substances that cross-react with digoxin assays and are not of interest. Another possible problem with immunoassays that utilize a spectrophotometric technique is the presence of substances that affect the measurement of emitted or fluorescent light. Such substances may be interferents (preventing the ultimate measurement of drug present) or adulterants (substances which are consumed or added to specimens in an effort to yield a false negative result). No false negative results were noted with either subject for the barbiturate assay or for the first subject on the benzodiazepine and opiate assays suggesting that riboflavin is not an adulterant. Riboflavin use, however, does interfere with the TDx DAU assays and may result in quantitative values being determined which are of questionable validity in the face of an elevated *BLK I* value or may result in only a *MX BKG* error and no quantitative value reported. To overcome this problem a number of solutions are available: (1) It may be necessary to use another immunoassay that is not based on FPIA; (2) dilute the sample using buffer until the interferent effect is minimized to the point where an error does not occur—this, of course, results in a dilution of the drug of interest and may result in that concentration falling below the cutoff concentration for that assay; or (3) proceed to a more sophisticated technology such as gas chromatography/mass spectrometry.

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