THE MEASUREMENT OF THIAMINE IN URINE

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The most widely used procedures for the measurement of thiamine in urine are based on the oxidation of the vitamin to thiochrome, which is then extracted into butyl alcohol and measured fluorometrically (1). It has been found possible to omit the butyl alcohol extraction without prejudice to the assay.

By utilizing benzenesulfonyl chloride to obtain blank values, as proposed by Urban and Goldman (2), as well as by incorporating certain other minor changes, a procedure has been developed which appears to combine convenience and simplicity with accuracy.

Procedure

Directions are given for the use of the Coleman fluorometer, model 12. If another fluorometer is used, there may need to be proportionate changes in volumes of reagents.

Exchange columns of the type described by Hennessy (3) are filled with water. Decalso, 60 to 80 mesh, prepared according to Hennessy’s directions, is added to give an 8 to 10 cm. column of adsorbent. After the water has been drained or sucked through, the upper end of the middle segment of the column is plugged with a small rubber stopper on the end of a 6 inch glass rod. Urine, representing not more than a half hour period of collection, is measured into the column above the stopper, and distilled water is added, if necessary, to bring the volume to at least 25 ml. If the urine has been preserved in 0.1 N acetic acid, as usually recommended, approximately 0.05 ml. of 3 M sodium acetate is added per ml. of urine. In the case of unpreserved urine, 1 ml. of a buffer which is 1 M in both acetic acid and sodium acetate is added. The stopper and rod are removed in a manner that will mix the sample and then are rinsed off into the column with a small amount of water. After adsorption, the column is washed with 40 to 50 ml. of boiling 0.01 N acetic acid which may be drawn through by suction. The sample is eluted with 22 to 25 ml. of near boiling 25 per

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cent KC1 in 0.1 N HCl. The eluate is collected in a 25 ml. cylinder or volumetric flask and diluted to volume with the eluting agent. (The exchange column may be used two or three times before recharging with Decalso. Between samples, the columns are washed with 30 to 50 ml. of boiling water and 15 ml. of 3 per cent acetic acid, both of which may be drawn through with suction.)

Three 3 ml. aliquots of the eluate are pipetted into matched fluorometer tubes. To the first tube is added 0.1 ml. of a standard thiamine hydrochloride solution, 5 γ per ml.; i.e., an internal standard equal to 0.5 γ of thiamine hydrochloride.1 To the second tube is added 0.1 ml. of water. While these are being stirred vigorously with filtered air through glass bubble tubes (6), 2 ml. of alkaline ferricyanide (25 volumes of 30 per cent NaOH plus 1 volume of 2 per cent potassium ferricyanide added within 1 hour of use) are plunged in with a syringe pipette.2 After 15 to 30 seconds, 3 ml. of an alcohol-peroxide solution (90 ml. of 95 per cent alcohol plus 0.5 ml. of 3 per cent hydrogen peroxide diluted to 100 ml. with water) are added with another syringe pipette and the bubblers are removed. To the third tube is added, with aeration, 0.1 ml. of 30 per cent sodium hydroxide and immediately 1 drop of benzenesulfonyl chloride. The bubbling must be sufficiently vigorous to emulsify the sulfonyl chloride. After 45 to 90 seconds, this tube is treated as were the first two. This is the blank.

After the bubbles have dissipated, the tubes are read at a sensitivity such that the first tube, with internal standard, reads 80 to 100 scale divisions. By using either the Coleman B-1 or B-1-S filter with or without an additional inserted screen disk, almost any urine sample may be brought to this reading. The other two tubes are read at this same instrument setting.3 If the difference between the readings on the first and second tubes is small, it is desirable to repeat a sample with the addition of 0.1 ml. of a stronger standard. For the sake of accuracy the amount of added thiamine should at least equal the amount initially present. Occasionally, slight turbidity is encountered. If excessive, this may be overcome by adding 0.1 to 0.2 ml. of water to each tube, or, if this fails, the tubes may be centrifuged. All these tubes should be diluted alike, since the ratio of alcohol to water influences the fluorescence. The thiamine in the 3 ml.

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1 This 0.1 ml. volume may be conveniently and accurately added with a constriction pipette (4, 5).
2 Syringe pipettes are available at a reasonable cost from Mr. Herman Ruf, 5023 192nd Street, Flushing, New York.
3 To insure that the sensitivity of the instrument does not change during a set of fluorescent measurements, it is wise to check this for constancy just before each reading. A series of stoppered tubes containing dilute quinine solutions in 0.1 N H2SO4 is convenient for this purpose.
aliquot of eluate is equal to the thiamine added as an internal standard
\(\times \frac{R_2 - R_3}{R_1 - R_2}\), where \(R_1, R_2,\) and \(R_3\) refer to fluorometer readings
for the first, second, and third tubes respectively.

**DISCUSSION**

In agreement with a number of reports (1), urine must not be too con-
centrated if thiamine is to be quantitatively adsorbed on a Decalso column;
hence the specification that not more than a half hour's urine be used.
If a larger sample is desirable, because of a very low excretion, it is per-
missible to use a 1 hour specimen, provided the volume is brought to at
least 50 ml.

The column is washed with a hot solution after adsorption because this
was found to result in an average decrease in the blank of about 35 per
cent in comparison with a cold wash. Hochberg and Melnick (7) and
others (3) have washed with hot water. The wash was made slightly acid
as a precaution against possible destruction of thiamine by heat.

More consistent elution was obtained with hot than with cold KCl.
The recovery of pure thiamine or thiamine added to urine was quantitative
with the hot eluant, but was occasionally low when cold KCl was used.

One of the most vexing questions in regard to urinary thiamine deter-
m inations is how to obtain a satisfactory blank value. Omission of fer-
ricyanide yields a blank which is obviously too large, since not infrequently
the sample treated with ferricyanide will be found less fluorescent than the
unoxidized control sample. Najjar and Ketron (8) recommended using
21 per cent of the fluorescence of this unoxidized sample as the blank
correction, since it was their belief that \(N_1\)-methyl nicotinamide was chiefly
responsible for the fluorescent blank in urine and since under their ana-
lytical conditions this compound gave only 21 per cent as much fluorescence
when treated with both ferricyanide and alkali as when it was treated
with alkali alone. There is now reason to believe that other substances
also contribute to the blank (9), and experiments in this laboratory in-
dicate that the ratio of the fluorescence obtained from \(N_1\)-methyl nicotin-
amide with and without ferricyanide is markedly affected by the concen-
tration of alkali used. Mason and Williams (6) presented evidence that
blanks obtained with bisulfite are more nearly valid than those resulting
from ultraviolet irradiation. In the belief that bisulfite treatment is as
free from objection as any method proposed for obtaining blanks, it was
used for comparison with benzenesulfonyl chloride treatment, which had
been proposed for obtaining a blank in a colorimetric thiochrome procedure
(2). Table I demonstrates the close correspondence between bisulfite and
benzenesulfonyl chloride blanks on the same urine eluates. This agree-
ment increases confidence in both reagents. Since \(N_1\)-methyl nicotinamide
is at least an important contributor to the thiamine blank, the effects of bisulfite and benzenesulfonyl chloride treatment on this compound were tested. As was hoped, pretreatment by either reagent was without effect on the fluorescence produced from the metabolite by alkaline ferricyanide. It may be noted that under the conditions prescribed above the metabolite gives only 7 per cent as much fluorescence with ferricyanide as without it. Because it is simpler to use benzenesulfonyl chloride than bisulfite, this organic blocking agent is utilized in the proposed procedure. The conditions of its use have been modified somewhat from those of Urban and Goldman (2) in order to accelerate the reaction rate with thiamine, which was found to be maximal in 0.1 to 0.2 N alkali. At this alkalinity, with the short treatment given, there is little loss of thiamine unless benzenesulfonyl chloride is present, in which case blocking is complete in 45 seconds.

Ordinarily, after thiochrome is formed from thiamine, it is extracted into butyl alcohol for measurement of the fluorescence. This extraction has two purposes: (1) to leave behind, if possible, some of the non-thiochrome fluorescent materials, and (2) to enhance the fluorescence of thiochrome, which is much weaker in water than in butyl alcohol. Table II indicates that in the case of urine eluates, prepared as described, the blank is nearly as low when measured directly in the aqueous phase as it is when extracted into butyl alcohol. It would, therefore, be unnecessary.

**Table I**

**Blank Values Obtained after Bisulfite and Benzenesulfonyl Chloride Treatment of Urine Eluates**

The results are expressed in micrograms per 24 hours, calculated as apparent thiamine.

<table>
<thead>
<tr>
<th>Subject*</th>
<th>Specimen No. Blank</th>
<th>Subject*</th>
<th>Specimen No. Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Benzene-sulfonyl chloride</td>
<td>HSO₃⁺</td>
</tr>
<tr>
<td>Sp</td>
<td>1</td>
<td>42</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>13</td>
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<td>33</td>
<td>32</td>
</tr>
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<td>27</td>
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</tr>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>6</td>
<td>30</td>
<td>28</td>
</tr>
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</table>

* The subjects were exposed to an experimental procedure which is believed to account for the differences in the blank values on the different occasions.
† The urine eluate was treated with benzenesulfonyl chloride, as described under "Procedure," except that a butyl alcohol extract was used.
‡ Urine eluate heated 15 minutes at 100°; pH 5 with 0.4 per cent NaHSO₃.
to extract the thiochrome if its fluorescence in aqueous media could be increased. At best (0.01 N alkali) thiochrome is only half as fluorescent in aqueous solution as in butyl alcohol. The fluorescence is decreased to 25 per cent of the butyl alcohol value in the strongly alkaline solution necessary for thiochrome formation. By replacement of one-third of the water by ethyl alcohol the fluorescence in such strong alkaline media is increased to 65 per cent of that in butyl alcohol. Further increase in alcohol concentration somewhat augments the fluorescence, but cannot be used in the present instance without precipitating KCl and silicates present in the urine eluates. The resulting 35 per cent decrease in fluorescence with the proposed procedure in comparison with butyl alcohol extraction is compensated by the smaller permissible volume if extraction is avoided. Hydrogen peroxide is a necessary addition to the added alcohol in order to reduce excess ferricyanide which would otherwise cause destruction of thiochrome. The resultant ferrocyanide quenches the thio-

**TABLE II**

*Thiamine Determinations in Urine, with and without Butyl Alcohol Extraction of Thiochrome*

<table>
<thead>
<tr>
<th>Specimen No.</th>
<th>Thiamine</th>
<th>Blank*</th>
<th>Specimen No.</th>
<th>Thiamine</th>
<th>Blank*</th>
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<td>At</td>
<td>B‡</td>
<td>Difference</td>
<td>At</td>
<td>B‡</td>
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<tr>
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<td>126</td>
<td>109</td>
<td>+14</td>
<td>7</td>
<td>5</td>
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<tr>
<td>2</td>
<td>162</td>
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<td>+3</td>
<td>27</td>
<td>11</td>
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<td>294</td>
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<tr>
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<td>14</td>
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<td>+8</td>
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</table>

* Calculated as thiamine.
† Determined by the proposed procedure.
‡ Determined with butyl alcohol extraction.
chromium fluorescence a little and would make it unwise to employ large amounts of ferricyanide in the oxidation. Table II indicates that essentially the same thiamine values are obtained with urine by the proposed direct procedure as by the conventional butyl alcohol extraction.

SUMMARY

A procedure for measuring urinary thiamine by the thiochrome method is described which obviates butyl alcohol extraction without sacrifice in accuracy. Blank values are obtained by a simple treatment with benzene-sulfonyl chloride. Evidence is presented for the validity of blanks obtained in this manner.

BIBLIOGRAPHY

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