A method is reported for determining urinary xanthurenic acid (XA), a metabolite of tryptophan. XA is extracted from urine with isobutanol (2-methyl-1-propanol), transferred to ammonium hydroxide, and separated from other urinary metabolites on thin-layer cellulose plates with use of 0.2 acetate buffer, mol/liter, pH 5.4. The XA is measured spectrophotometrically after diazotization with 2-chloro-4-nitroaniline. The method, which is specific for XA, can be used to measure amounts as low as 0.4 µg/ml of urine. It has a range from 0 to 24 µg/ml, and results within this range are reproducible within approximately ±5%. Data obtained by this procedure are comparable to results reported for other methods.

Additional Keyphrases
- thin-layer chromatography
- spectrophotometry
- diazotization with 2-chloro-4-nitroaniline
- tryptophan metabolism
- vitamin B₆ deficiency

The measurement of urinary xanthurenic acid has been used clinically to study vitamin B₆ deficiency (1), febrile disorders (2), and the effects of steroid hormones and contraceptive agents on tryptophan metabolism (3).

Current methods for measuring tryptophan metabolites in human urine are slow, cumbersome, and often unreliable. Among the techniques used have been ion-exchange resin chromatography (4), thin-layer and paper chromatography (5–9), solvent extraction and separation, and various methods of spectrophotometry and fluorometry (10–14).

This report presents a sensitive, specific, and accurate method for the determination of a tryptophan metabolite, xanthurenic acid (XA),¹ in urine. XA is extracted from urine with isobutanol by a modification of Cohen’s procedure (10), isolated by thin-layer chromatography (6), and finally the concentration is determined spectrophotometrically after diazotization with CNA, a reagent described by Bartels and Boehmer (16) for the determination of bilirubin in plasma.

Material and Methods

Reagents

Unless otherwise stated, reagents of the highest quality available were obtained commercially. In addition to the usual chemicals and solvents, these included L-tryptophan (Sigma grade, Sigma Chemical Co., P.O. Box 14508, St. Louis, Mo. 63178), β-glucuronidase (“Ketodase,” 5000 units/ml, Warner-Chilcott Labs., 201 Tabor Rd., Morris Plains, N. J. 07950), isobutanol (2-methyl-1-propanol, ACS grade, bp 108°C, Fisher Scientific Co., Pittsburgh, Pa. 15219), xanthurenic acid (4,8-dihydroxyquinalidic acid, Sigma grade), dl-kynurenine (Sigma grade), anthranilic acid (o-amino-benzoic acid, Sigma grade), hydroxykynurenine (3-hydroxy-dl-kynurenine, Sigma grade), 3-hydroxyanthranilic acid (Sigma grade), sulfanilic acid (Fisher, ACS grade), chloronitroaniline (2-chloro-4-nitroaniline, practical grade, Eastman Kodak Co., Rochester, N. Y. 14650), and kynurenic acid (4-hydroxyquinoline-2-carboxylic acid, Sigma grade). Thin-layer plates, 20 × 20 cm, were prepared from a 20 g/100 ml slurry of cellulose (“Selectacel no. 65” without additive, Schleicher & Schull, Inc., Keene, N. H. 03431) spread at a thickness of 0.75 mm with an applicator (Brinkmann Instruments, Westbury, N. Y. 11590). After

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¹ Abbreviations used: XA, xanthurenic acid; CNA, 2-chloro-4-nitroaniline; DAS, diazotized sulfanilic acid.

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was concentrated duplicate man carried which adding immediate 90°C the 12-ml centrifuge tube, tubes. These contained areas (Rf about 0.35) were identified by their fluorescence under shortwave (260 nm) ultraviolet light. They were scraped into 12-ml centrifuge tubes, and 2 ml of a mixture of equal parts of water and methanol was added to each tube. The tubes were shaken for 1 min each at the beginning and end of the 1-h elution period, then centrifuged at 700 g for 10 min.

To verify that all the xA had been removed from tracted twice with 1-ml portions of isobutanol. xA in the pooled isobutanol layers was then back-extracted with 1 ml of 1 mol/liter NH₄OH. The colorless isobutanol layer was discarded and the xA-containing ammonia portion was dried under nitrogen at 80°C.

Purification. The dried samples were dissolved in 100% methanol (two 100-μl portions), and transferred quantitatively with disposable micropipets to thin-layer plates, being applied to a line 1.5 cm from the edge of the plate, which was divided into four equal segments. Three samples and a 3-μg aqueous reference standard were applied to each plate. The chromatograms were developed at 4°C with sodium acetate buffer (0.2 mol/liter) that had been adjusted to pH 5.4 with NaOH (1 mol/liter). Under these conditions chromatography was usually completed within 1 h, when the solvent front was approximately 1 cm from the top of the plate. The plates were dried for 15 min in an oven at 80°C.

The xA-containing areas (Rf about 0.35) were identified by their fluorescence under shortwave (260 nm) ultraviolet light. They were scraped into 12-ml centrifuge tubes, and 2 ml of a mixture of equal parts of water and methanol was added to each tube. The tubes were shaken for 1 min each at the beginning and end of the 1-h elution period, then centrifuged at 700 g for 10 min.

To verify that all the xA had been removed from

Procedure

Extraction. After thawing, measurement, and filtration through Whatman no. 1 filter paper, duplicate 6-ml urine aliquots were transferred to 12-ml centrifuge tubes. The pH of each specimen was adjusted to about 1 by adding 2 or 3 drops of concentrated (12 mol/liter) HCl. An amount of NaCl known to saturate the urine (about 0.75 g) was added to each sample. Urinary xA was ex-

Fig. 1. Standard curve for xA-CNA chromophore

To 1 ml of standard solutions (0.75, 1.5, 3, 6, 12, and 24 μg of xA per milliliter) was added 0.5 ml of CNA reagent and 1 ml of KOH (10 mol/liter) at room temperature.

the plates dried at room temperature and at 80-90°C for one-half hour, they were stored in a desiccator.

The CNA spraying reagent, which was used to verify removal of xA from the plates, was prepared immediately before use from one part of a solution of 1 g of sulfanilic acid in 100 ml of HCl (10:100, by vol) one part of a solution of 5 g of NaNO₂ in 100 ml of water, and two parts of a solution of 10 g of Na₂CO₃ in 100 ml of water (5). These components were stored at 4°C.

The CNA reagent consisted of equal volumes of a saturated solution of chloronitroanil in 0.1 mol/liter HCl and a solution of 0.25 g of NaNO₂ in 100 ml of water. These solutions were stored at room temperature and mixed just before use.

Urinary reference solutions were prepared by adding known amounts of xA to pooled urine in which endogenous xA was not detectable. Aliquots of these solutions, which were stored frozen, were carried through the entire procedure in duplicate with each set of unknown urine specimens.

All spectrophotometric measurements were made on a Model DB-G spectrophotometer (Beckman Instruments, Fullerton, Calif. 92634).

Fig. 2. Effect of urinary contaminants on xA-CNA absorption spectra

Three types of materials were extracted, purified, and measured as described in the text: A, xA aqueous standard, 15 μg/ml of distilled water; B, urine specimen collected over an 8-h interval after ingestion of 2 g of L-tryptophan; C, 8-h urine specimen (no tryptophan ingestion). These curves confirm the relative purity of the isolated xA fraction, the linearity of the baseline absorbance resulting from nonspecific contaminants, and the validity of mathematical correction for such contaminants.

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the plates, we sprayed the scraped plates with the DSA reagent. Any \( \text{XA} \) remaining on the plate is colored red by this reagent.

**Measurement.** A 1-ml eluate was transferred to a 5-ml test tube. A blue complex, with absorption maximum at 640 nm and inflection points at approximately 590 and 690, was formed by adding 0.5 ml of the CNA reagent to the eluate, followed by 1 ml of KOH (10 mol/liter). Color intensity vs. concentration was linear over the range of 0 to 24 \( \mu \text{g/ml} \) (Figure 1).

Because traces of bilirubin or other urinary contaminants might have accompanied xanthurenic acid through the procedure, the resulting spectra occasionally showed trace contamination (Figure 2). Therefore, the final concentration was calculated by triangulation, as described by Allen (16).

Reference solutions of known concentrations, carried simultaneously through the entire procedure, were used to prepare standard concentration-absorbance curves, and in this way recovery losses were corrected for internally. Standard curves are shown in Figure 3. The upper curve was constructed by connecting points representing absorbance at 640 nm only; the lower by applying Allen's correction to the same data. Since these curves obey Beer's law, final levels of \( \text{XA} \) per ml were determined either directly from the curve or by appropriate calculations in absorbance units per microgram.

The entire procedure for extraction, purification, and measurement of urinary \( \text{XA} \) is summarized in Table 1. Each of the steps was analyzed before being incorporated into the final procedure, and is discussed below.

**Experimental Study**

**Hydrolysis of conjugates of \( \text{XA} \).** Rothstein and Greenberg (17) have shown that urinary \( \text{XA} \) is conjugated as the glucuronide in the rat, and as the sulfate in the rabbit. To determine whether human urinary \( \text{XA} \) is similarly conjugated, we subjected aliquots of the 8-h urine output of a human subject who had previously ingested 3 g of L-tryptophan to \( \beta \)-glucuronidase hydrolysis. The enzymatic hydrolysis was performed by adding 1 ml of undiluted "Ketodase" and 1 ml each of sodium acetate and acetic acid (each 0.2 mol/liter) to 6 ml of urine and incubating the solution at 47°C for various times.

Concentrated HCl was used for acid hydrolysis both at room temperature and by refluxing at 100°C.

\( \text{XA} \) was extracted from the resulting aqueous solutions, purified, and measured. Results of the hydrolysis experiments (Table 2) indicate that, at these concentrations, the amount of \( \text{XA} \) detected increased little or none after hydrolysis with acid or glucuronidase. Thus, if \( \text{XA} \) is present in human urine in the conjugated form, it is either hydrolyzed

![Fig. 3. Effect of baseline correction on XA-CNA standard curves](image)

Urinary standards containing 0.1, 1.2, 4, and 8 \( \mu \text{g} \) of \( \text{XA} \) per milliliter were processed according to the method in the text and the final eluates were reacted with the CNA reagent. ---, \( A_{690} \); \( \Delta \cdots \Delta \cdots \), values obtained after use of the Allen correction for background contamination:

\[
A_{\text{corr}} = A_{690} - \frac{(A_{690} + A_{590})}{2}
\]

**Table 1. Quantitative Determination of \( \text{XA} \) in Urine: Procedure**

**Extraction**
1. Filter urine through Whatman no. 1 filter paper.
2. Pipet 6 ml into a conical centrifuge tube.
3. Adjust to pH 1 with 2 to 3 drops 12 mol/liter HCl.
4. Add approximately 0.75 g solid NaCl.
5. Extract twice with 1 ml isobutanol.
6. Extract combined isobutanol portions with 1 ml 1 mol/liter NH\(_4\)OH.
7. Evaporate NH\(_4\)OH solution to dryness under nitrogen at 80°C.

**Purification**
8. Dissolve dried extract in two 100-\( \mu \text{l} \) portions of 100% methanol.
9. Apply quantitatively to thin-layer cellulose plates.
10. Develop for approximately 1 hour at 4°C in 0.2 mol/liter acetate buffer, pH 5.4.
11. Dry plates at 80°C for 15 minutes.
12. Identify fluorescent \( \text{XA} \)-containing areas with short-wave ultraviolet lamp, scrape into centrifuge tubes.
13. Add 2 ml 50% methanol. Elute for 1 hour with 2 one-minute shakings. Centrifuge.

**Measurement**
14. To 1 ml of eluate, add 0.5 ml chloronitroaniline reagent and 1 ml 10 mol/liter KOH.
15. Measure absorbance at 640 nm, 590 nm, and 590 nm. Calculate corrections.
16. Determine \( \text{XA} \) in \( \mu \text{g/ml} \) from standard curve or by calculation.
Table 2. Hydrolysis of Urinary Xanthurenic Acid

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Time, h</th>
<th>XA found, μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>4.8</td>
</tr>
<tr>
<td>pH 1</td>
<td>6</td>
<td>4.8</td>
</tr>
<tr>
<td>pH 1</td>
<td>15</td>
<td>3.2</td>
</tr>
<tr>
<td>pH 1</td>
<td>24</td>
<td>4.1</td>
</tr>
<tr>
<td>pH 1, 100°C, reflux</td>
<td>0.25</td>
<td>4.8</td>
</tr>
<tr>
<td>β-glucuronidase</td>
<td>0</td>
<td>5.2</td>
</tr>
<tr>
<td>β-glucuronidase</td>
<td>6</td>
<td>4.8</td>
</tr>
<tr>
<td>β-glucuronidase</td>
<td>15</td>
<td>5.2</td>
</tr>
<tr>
<td>β-glucuronidase</td>
<td>24</td>
<td>4.3</td>
</tr>
</tbody>
</table>

Table 3. Recovery of XA from Aqueous Solutions with Use of Various Solvents

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toluene</td>
<td>8</td>
</tr>
<tr>
<td>Methylene chloride</td>
<td>18</td>
</tr>
<tr>
<td>Chloroform</td>
<td>22</td>
</tr>
<tr>
<td>Ether</td>
<td>36</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>70</td>
</tr>
<tr>
<td>Ether-alcohol</td>
<td>81</td>
</tr>
<tr>
<td>Isobutanol</td>
<td>100</td>
</tr>
</tbody>
</table>

During the acidification that precedes butanol extraction or it is resistant to both modes of hydrolysis.

Extraction. The extraction solvent was chosen on the basis of recovery experiments in which various solvents were used (Table 3). From an aqueous XA solution containing 6 μg/ml, the pH of 5-ml aliquots was adjusted to approximately 1 with concentrated HCl, and the XA was extracted as in steps 1–7, Table 1. Dried samples were dissolved in 2 ml of methanol:water (1:1) and measured as in steps 14–16, Table 1. The recoveries (Table 3) demonstrate that isobutanol extracts essentially all of the xanthurenic acid from aqueous standards, whereas recoveries are markedly lower if other, less polar immiscible solvents are used. When other known urinary metabolites of tryptophan were substituted for XA, it was found that kynurenine, hydroxyxanthanilic acid and anthranilic acid are also extracted by isobutanol, whereas kynurenine and hydroxykynurenine are not.

Purification. Attempting to avoid a chromatographic step, we reacted the XA in the dried extract (step 8, Table 1) directly with the CNA reagent, but nonspecific chromogens obscured the final color reaction. Therefore, both silica gel and cellulose thin-layer chromatographic systems were tested for their ability to separate XA from other tryptophan metabolites as well as from other urinary substances. On silica gel, two solvent systems, ethyl alcohol–34% ammonia (7:3, by vol) and isopropanol–ammonia–water (17:4:3, by vol) gave good separations of XA from other metabolites, the Rf for XA being 0.8 and 0.3, respectively; however, only about 30% of the XA was recovered from silica gel. The cellulose thin-layer chromatographic method of Walsh (5), which we finally adopted, results in good separation of tryptophan metabolites, and 70% or more was eluted. To correct for losses of XA during purification, urinary solutions containing known amounts of XA were analyzed simultaneously with each set of specimens.

We noted that the XA used as a reference material contained traces of an impurity with a slightly higher Rf than XA, which had a bright blue fluorescence and did not react with DSA or CNA reagents. On the basis of fluorescence characteristics and Rf values, this impurity is presumably the 8-methyl ether of XA, described by Price and Dodge (18).

After separation by cellulose chromatography, tryptophan metabolites were characterized by Rf, color of fluorescence on the plate, color formed with DSA and CNA reagents, and ultraviolet spectra of the eluates. The results (Table 4) show that only xanthurenic acid forms a color with the CNA reagent, and that it is effectively separated from the other tryptophan metabolites.

Measurement. A number of reported colorimetric methods were tested to measure XA. Ferric chloride (12, 13) was insufficiently sensitive for use with small amounts of urine. DSA (5) was more sensitive, but the color complex formed in an ice bath is unstable. The stability data (Figure 4) demonstrate...
that absorbance progressively declines even at 0°C. When the reaction took place in KOH (10 mol/liter) (6), the color could be stabilized at the expense of sensitivity—the optimal pH for maximal color development is about 7 (Figure 5).

In the method finally adopted we used CNA for diazotization; it has a structure similar to that of sulfanilic acid, but the additional NO₂ group increases chromogenicity and specificity. As noted in Table 4, CNA does not react readily with other tryptophan metabolites tested, whereas sulfanilic acid reacts with hydroxykynurenine and anthranilic acid.

Results and Discussion

The described procedure for the measurement of xanthurenic acid in urine was tested for specificity, accuracy, reproducibility, and sensitivity as well as for efficiency in general use.

Specificity. Specificity is enhanced at several steps: First, the isobutanol extracts XA quantitatively, but leaves behind such other urinary substances as the tryptophan metabolites, kynurenine and hydroxykynurenine. XA is further purified by back-extraction with ammonium hydroxide. Thin-layer chromatography separates XA from the few remaining contaminants (Table 4). Use of the CNA reagent confers additional specificity, since other common tryptophan metabolites that may be present in urine do not react with it (Table 4). The specificity of the entire method is such that spectrophotometric analyses of urinary XA extracts carried through the entire procedure usually resulted in almost symmetrical gaussian curves, with no secondary peaks.

Accuracy and reproducibility. Accuracy of the method was evaluated by measuring XA added to pooled urine in which no endogenous XA was detectable. Figure 6 shows the amounts found after 1 to 5 μg of XA was added per milliliter. The coefficient of the regression line is 1.02 (s.d., ±0.029), which indicates a close correlation between amounts added and amounts measured by this method. The line intercepts the y axis at zero, which indicates negligible background contamination. The mean sample standard deviation is ±0.309 μg/ml; this value primarily reflects the deviations from the regression line at the higher concentrations.

A second series of similar urinary XA reference solutions, which contained 1.2 to 8 μg of XA per milliliter, was analyzed on many different days. The results (Table 5) confirmed the above findings. Mean amounts found were within ±5% of amounts added and the standard deviations, whether calculated on the basis of the amount added (accuracy) or the mean amount measured (reproducibility), were within the 10% range.

When duplicate determinations were made on urine samples collected after a 2-g oral loading dose...
Table 5. Repeated Analyses of Urinary XA Standards

<table>
<thead>
<tr>
<th>XA added, µg/ml</th>
<th>N</th>
<th>Mean, XA determinations</th>
<th>SDigned, XA SDXA SDXA</th>
<th>µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2</td>
<td>21</td>
<td>1.2</td>
<td>0.14</td>
<td>0.16</td>
</tr>
<tr>
<td>2.4</td>
<td>7</td>
<td>2.4</td>
<td>0.27</td>
<td>0.29</td>
</tr>
<tr>
<td>4.8</td>
<td>24</td>
<td>5.0</td>
<td>0.49</td>
<td>0.53</td>
</tr>
<tr>
<td>8</td>
<td>8</td>
<td>7.9</td>
<td>0.52</td>
<td>0.55</td>
</tr>
</tbody>
</table>

where XA = concentration added, and XAD = concentration determined.

Table 7. Sensitivity of Method for the Determination of XA in Urine

<table>
<thead>
<tr>
<th>XA added, µg/ml</th>
<th>XA measured µg/ml</th>
<th>Exogenous XA recovered, µg/ml</th>
<th>XA recovered, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.24</td>
<td>0.18</td>
<td>...</td>
</tr>
<tr>
<td>0.1</td>
<td>0.29</td>
<td>0.13</td>
<td>0.01</td>
</tr>
<tr>
<td>0.2</td>
<td>0.56</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>0.4</td>
<td>0.92</td>
<td>0.03</td>
<td>0.68</td>
</tr>
<tr>
<td>0.8</td>
<td>0.92</td>
<td>0.03</td>
<td>0.68</td>
</tr>
</tbody>
</table>

where XAD = concentration determined, and XAD = arithmetic mean of concentrations determined.

A graph illustrating the urinary excretion of tryptophan load

Of tryptophan (Table 6), the standard deviations at each level were within 5% of the mean.

Sensitivity. The sensitivity of the entire procedure was tested by adding small increments of XA (0.1 to 0.8 µg/ml) to urine and analyzing the usual 6-ml sample in triplicate. The results (Table 7) indicate that the method cannot reliably detect concentrations of XA less than 0.4 µg/ml. The sensitivity of the method could be enhanced by using microcuvets and more concentrated reagents, or by extracting larger volumes of urine. None of these modifications is necessary for analyzing urines collected after tryptophan administration.

The method was applied to urine collected from a man before and at various intervals after he ingested 0.5 and 4 g of tryptophan on two different days. The urinary excretion pattern of XA after the administration of standard loads of tryptophan is depicted in Figure 7: Peak excretion occurs within 3 h and is essentially completed after 8 h (19). Consequently, our clinical studies are based on 8-h urine collections.

Although we made no extensive laboratory comparison of the available methods for the determination of xanthurenic acid in urine, Table 8 compares our data with those reported by others for XA concentrations in urine, with and without tryptophan loading. The values reported in Table 8 presumably reflect the characteristics of the various methods of measurement. In column chromatographic methods the xanthurenic acid is so diluted that the analysis must be done fluorometrically, with the attendant problems of quenching, nonlinear concentration-fluorescence curves, and non-specific fluorescing materials. Separation by paper chromatography probably involves losses comparable to those of the thin-layer method which we adopted, and might explain the somewhat lower values reported by Benassi et al. (8).

The procedure is convenient for general use, for several reasons: Only 6 ml of urine is necessary when subjects have been given loading doses of L-tryptophan. Larger amounts of urine can be extracted to determine XA excretion when subjects have not received loading doses. Multiple
samples may be determined conveniently and efficiently and the colorimetry step, at least, is suitable for automation. The chromatography requires little special skill in application and elution. Many plates can be run simultaneously and the chromatographic procedure requires only an hour; one technician can easily analyze six duplicate urine samples and a set of standards in a working day. In our laboratory, a high school graduate, with no special training, does these determinations routinely.

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References