Improved fluorometric quantification of urinary xanthurenic acid

MINSHU LIU,1 GONG-REN WANG,2 TSAN-ZON LIU,3 and KAN-JEN TSAI3*

Measurement of urinary xanthurenic acid (XA) has been used clinically to study a variety of disorders caused by vitamin B6 deficiency. To obviate some cumbersome steps of current methods for measuring XA in human urine, we have developed a simple fluorometric method. We apply the urine sample to a solid-phase extraction column containing trimethylaminopropyl group bound to silica, which enables us to purify and concentrate the XA from the urine without contamination from various tryptophan metabolites. The XA in the acidic eluate can then be quantified fluorometrically. The linearity of the proposed method extends from 0.2 to 10.0 mg/L. The method is precise, yielding day-to-day CVs for two pooled control specimens (1.08 and 1.90 mg/L) of 1.2% and 2.6%, respectively. Correlation studies with an established HPLC method and with a spectrophotometric procedure showed correlation coefficients of 0.99 and 0.98, respectively. Interference from vitamin C, uric acid, salicylate, acetaminophen, vanillylmandelic acid, and homovanillic acid was insignificant. The proposed method for urinary XA is rapid, simple, and suitable for routine use in the clinical laboratory.

INDEXING TERMS: vitamin B6 • kynureninase • homocysteine • screening • tryptophan loading test

3-Hydroxykynurenin, an intermediary product of tryptophan metabolism, is converted to 3-hydroxanthranilate by the catalytic action of the enzyme kynureninase (EC 3.7.1.3). The vitamin B6 coenzyme, pyridoxal 5'-phosphate, is an obligatory cofactor for the enzymatic action of kynureninase [1]. Either subclinical deficiencies of vitamin B6 or a genetic defect on kynureninase can lead to the accumulation of xanthurenic acid (XA) in plasma and urine [2, 3]. Thus, measurement of urinary XA concentrations has often been used as an indicator for vitamin B6 deficiency. Along these lines, intracellular homocysteine is metabolized by either the transsulfuration pathway or by remethylation to methionine [4]. The condensation of serine and homocysteine, catalyzed by cystathionine-β-synthase (EC 2.1.22) in the first reaction of the transsulfuration pathway, is also dependent on pyridoxal 5'-phosphate as cofactor. Little is known about the effect of vitamin B6 deficiency on circulating homocysteine concentrations. Park and Linkswiler [5] reported that urinary homocysteine excretion increased considerably when six male volunteers consumed a diet depleted of vitamin B6, whereas several studies on experimental animals suggested that a vitamin B6 deficiency results in homocysteine accumulation [6–8]. Taken together, these studies indicate that vitamin B6 deficiency can lead to a combined accumulation of XA and homocysteine in urine. However, XA is considered to be a more sensitive indicator than homocysteine for the evaluation of vitamin B6 status, because the latter compound can be diverted to other pathways even in cases of vitamin B6 deficiency.

Measurement of urinary XA has been used clinically to study vitamin B6 deficiency [1, 2, 9], febrile disorder [10], theophylline-induced asthma [11], drug-induced diabetes [12], and the effect of tryptophan and six of its metabolites on the nicotinic acid pathway [3]. Furthermore, because of widespread vascular disorders in patients with cystathionine-β-synthase deficiency [13–15], it has been suggested that hyperhomocysteinemia increases the risk for premature occlusive vascular diseases. Circulating homocysteine concentrations have now been shown to be increased in coronary heart disease [16–20] and in cerebral vascular [21–24] as well as peripheral vascular diseases [23–24]. Because high concentrations of either XA or homocysteine in urine are both indicative of vitamin B6 deficiency, however, the question as to whether these two urinary metabolites can be utilized interchangeably as an indicator of high-risk potential for heart diseases has not been delineated. This question warrants further investigation.

Among the methods developed for quantifying urinary XA

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* Nonstandard abbreviations: XA, xanthurenic acid; TPTZ, 2,4,6-tris(pyridyl)-s-triazine; and DAFR, direct acid ferric reduction.
one quite tedious and lengthy assay involves the extraction of XA from urine with isobutanol, isolation by thin-layer chromatography, and spectrophotometric determination of the concentration of XA [26]. The first fluorometric quantitation of urinary XA was devised by Satoh and Price [27], based on the separation of XA by Dowex 50(H+), followed by measurement of its fluorescence in strong alkali; kynurenic acid was simultaneously determined fluorometrically in strong H2SO4. This method was subsequently modified by Cohen et al. [28], who separated XA from other fluorescent substances in urine by a pH- and NaCl-dependent extraction with isobutanol and then determined the fluorescence of XA in alkaline solution so that potentially interfering compounds, e.g., kynurenic acid, could be obviated. These methods involve multiple procedural steps, which are rather tedious and time-consuming. In addition, several HPLC methods have also been devised for quantifying urinary XA [29, 30]. However, the expensive instruments required might not be readily available in a general clinical laboratory. For this reason, we have developed a rapid and simple fluorometric method for measuring urinary XA that is sensitive, free of interference, and easily adopted for routine use in the clinical laboratory.

Materials and Methods

REAGENTS
Unless otherwise stated, reagents of the highest quality available were obtained commercially. XA (4,8-dihydroxyquinolanic acid), kynurenic acid (4-hydroxyquinoline-2-carboxylic acid), 2,4,6-tris-(pyridyl)-s-triazine (TPTZ), ferric chloride hexahydrate, vitamin C, uric acid, salicylate, acetaminophen, vanillylmandelic acid, and homovanillic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Solid-phase anionic-exchange resin (trimethylaminopropyl group bound to silica) was purchased from Analytichem (Harbor City, CA).

PROCEDURES

Urine purification. Routinely, 24-h urine specimens are collected in brown bottles with 6 mol/L HCl as preservative and processed for analyses without delay. The sample should be well mixed and an aliquot frozen if the sample cannot be analyzed within 48 h after collection. Before analysis, the urine specimen (after thawing, if necessary) is filtered through Whatman no.1 filter paper, and 5.0 mL of urine is applied to an anion-exchange solid-phase extraction column (100 mg/column). After the urine sample has passed through the extraction column, the adsorbed XA is eluted with 2.0 mL of 0.1 mol/L HCl.

Fluorometric measurement of XA. The eluate containing XA from urine is dissolved into potassium phosphate buffer, pH 4.0. The fluorescence of XA is then determined at 460–470 nm (after excitation at 305 nm) with a Turner spectrophotofluorometer.

Correlation studies. The results obtained by the proposed method were compared with those by an established HPLC method [25] and with a spectrophotometric method recently established in our laboratory [31]. Briefly, XA was purified as described above, followed by quantification with the direct acid ferric reduction (DAFR) method, in which XA reduces the Fe2+-TPTZ complex to its corresponding Fe3+-TPTZ form. The latter compound can be measured spectrophotometrically at 593 nm, as described elsewhere [32, 33].

Results

ANALYTICAL VARIABLES

pH optimum for XA fluorescence. Appropriate amounts of XA were dissolved into potassium phosphate buffer at various pH values, ranging from 1.0 to 9.0. The final concentration of XA in buffer solution was 2.0 mg/L. Relative fluorescence intensities of all preparations were then measured with a spectrophotofluorometer. As indicated in Fig. 1, the optimum pH for fluorescence production of XA is ~4.0. Investigation of the stability of XA fluorescence indicated that XA fluorescence remained stable for at least 1 week when the specimens were stored at 4 °C (Fig. 1).

Linearity. Relative fluorescence intensities of calibrators were linearly related to XA concentrations from 0.2 to 10 mg/L (Fig. 2).
Analytical recovery. To determine the accuracy of the procedure, we performed a recovery study; the results are tabulated in Table 1. The percentage recovery represents the measured value expressed as a percentage of the expected value. The mean percentage recovery for 10 samples was 99.8%.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Expected</th>
<th>Measured</th>
<th>Recovery, %</th>
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<tr>
<td>1</td>
<td>1.00</td>
<td>0.95</td>
<td>95</td>
</tr>
<tr>
<td>2</td>
<td>1.00</td>
<td>1.02</td>
<td>102</td>
</tr>
<tr>
<td>3</td>
<td>2.00</td>
<td>1.95</td>
<td>98</td>
</tr>
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</tr>
<tr>
<td>10</td>
<td>5.00</td>
<td>5.04</td>
<td>101</td>
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</table>

Correlation studies. We compared results by the proposed method with those by an established HPLC method [25] for 39 samples (Fig. 3, left). Comparison of the proposed method with a spectrophotometric DAFR method [31] for 39 samples is shown in Fig. 3, right.

**Table 1. Analytical recovery results.**

<table>
<thead>
<tr>
<th>Sample</th>
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<tr>
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<tr>
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<td>5.00</td>
</tr>
<tr>
<td>10</td>
<td>5.00</td>
</tr>
</tbody>
</table>

**Precision.** Reproducibility as reflected by the day-to-day and within-run precision data was excellent (Table 2). Five repetitive determinations on two pooled XA-supplemented urine controls had a mean value of 1.08 and 1.90 mg/L, respectively, with CVs of <1.0%. The CVs for the same set of controls assayed on 5 consecutive days were 1.2% and 2.6%, respectively. Again, the day-to-day reproducibility was also excellent.

**Table 2. Precision studies results.**

<table>
<thead>
<tr>
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<th>Level II</th>
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<td>Mean, mg/L</td>
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<td>1.901</td>
</tr>
<tr>
<td>sD, mg/L</td>
<td>0.003</td>
<td>0.006</td>
</tr>
<tr>
<td>CV, %</td>
<td>0.28</td>
<td>0.33</td>
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**Discussion**

The described method for the measurement of XA in urine was tested for specificity, accuracy, and reproducibility in general use. First, the solid-phase extraction column (trimethylaminopropyl group bound to silica) not only concentrates the desired XA, but also leaves behind such urinary substances as the tryptophan metabolites kynurenine and hydroxykynurenine. This purification step for XA confers a unique specificity to the proposed method. The subsequent fluorometric measurement of XA further narrows the specificity of the method.

![Correlation of results for urinary XA concentration (in mg/L) obtained by the proposed method and (left) those determined by an established HPLC method or (right) those determined by a spectrophotometric direct acid ferric reduction method.](image)

Fig. 3. Correlation of results for urinary XA concentration (in mg/L) obtained by the proposed method and (left) those determined by an established HPLC method or (right) those determined by a spectrophotometric direct acid ferric reduction method.

If 39 urine specimens in each comparison were human urine with various concentrations of added XA. The regression line equations are (left) $y = 0.99x + 0.04$ ($S_{xy} = 0.07$, $r = 0.99$) and (right) $y = 0.98x ± 0.03$ ($S_{xy} = 0.07$, $r = 0.98$).
During the course of this developmental work, we considered incorporating a hydrolytic step to account for the possible presence of conjugated forms of XA. Rothstein and Greenberg have previously reported [34] that urinary XA is conjugated as the glucuronide in the rat, and as the sulfate in the rabbit. In contrast, Wallace et al. [26] showed that the amount of XA in human urine remained steady after hydrolysis with acid or glucuronidase, indicating that very little conjugated XA was present. To confirm this observation, we performed tryptophan-loading tests with six volunteers who gave informed consent. The volunteer individuals took 2.0 g of tryptophan orally, and urine specimens were collected 8 h after ingestion. A portion of the urine specimen from each individual was treated with \( \beta \)-glucuronidase before XA quantification. The mean ± SD concentrations for the \( \beta \)-glucuronidase-treated aliquots (8.01 ± 2.73 mg/L) and the untreated controls (8.08 ± 3.04 mg/L) were not statistically different (\( P > 0.2 \)). Therefore, we decided not to include a hydrolysis step for urine in our proposed method.

The accuracy of the method, evaluated by measuring XA added to pooled urine in which no endogenous XA was detectable, is indicated in Table 1, which shows that the mean percentage recovery for 10 samples was 99.8%. Additionally, reproducibility as reflected by the day-to-day and within-run precision data was also excellent (Table 2).

Our proposed method for urinary XA quantification is also convenient for general use for several reasons: (a) In comparison with the method of Wallace et al. [26], which calls for an initial solvent extraction of XA, isolation by thin-layer chromatography, and spectrophotometric quantification, our procedure is considerably faster and simpler. Overall, the number of procedural steps is minimized, and thus the turnaround time should be improved greatly. (b) In comparison with the fluorometric method of Satoh and Price [27], which called for the separation of XA by Dowex 50(\( H^+ \)), followed by measuring its fluorescence in strong alkali so as to measure kynurenic acid simultaneously, our method avoids the substantial loss of sensitivity in this manipulation. Furthermore, the fluorometric method of Cohen et al. [28], which called for a pH- and NaCl-dependent extraction of XA with isobutanol to obviate potentially interfering substances, involved multiple, rather time-consuming, procedural steps. In contrast, our procedure for purifying XA is much simpler. (c) Owing to the stability of XA in acidic potassium phosphate buffer, the specimen can be stored in batch and run later so that technologists can more efficiently manage their time and not be rushed to finish the analysis.

Patients with asthma excrete excessive amounts of XA in urine after oral tryptophan loading [35, 36]. However, the mechanism that leads to vitamin B\(_6\) deficiency in patients with asthma remains unclear. Furthermore, the elderly population may have deficiencies of several micronutrients, including folate, pyridoxine, and cobalamin; if not treated in time, these deficiencies can lead to an accumulation of both XA and homocysteine in urine and present a serious health risk to these individuals. We think a large-scale screening to identify individuals from an elderly population who are deficient in these micronutrients is urgently needed. Our proposed method is simple and rapid and can be adopted as one of the tests for routine use in the clinical laboratory for this screening purpose.

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References


