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Note

Quantification of urinary xanthurenic acid excretion by anion-exchange solid-phase extraction and high-performance liquid chromatography

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The tryptophan load test with subsequent quantification of urinary xanthurenic acid (XA) excretion, is often applied in the assessment of vitamin B_6 nutritional status [1–5]. Methods for urinary XA excretion either employ a simple colorimetric assay [6], which is not very specific at low concentrations [7], or a fluorimetric assay which has been reported to have higher specificity [7]. Although the direct colorimetric and fluorimetric assays are simple, several authors found it necessary to purify urine by either ion-exchange chromatography [7, 8] or thin-layer chromatography [9, 10] to eliminate background interference, resulting thus in more time-consuming procedures. High-performance liquid chromatography (HPLC) has also been described for the separation and quantification of different urinary tryptophan metabolites [11–14]. Although these methods are valuable for the study of tryptophan metabolism in different pathological conditions [15, 16], they are generally not suitable as rapid, simple assays for urinary XA excretion as an index of vitamin B_6 nutritional status. We report a highly selective purification of XA from urine using anion-exchange solid-phase extraction and a rapid, isocratic HPLC method for quantification of XA.

EXPERIMENTAL

Reagents

XA was purchased from Fluka (Buchs, Switzerland), while all other reagents were of analytical grade and supplied by Merck (Darmstadt, F.R.G.).

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Urine purification

Disposable extraction columns packed with 100 mg of strong anion-exchange resin (trimethylaminopropyl groups bonded to silica; supplied by Analytichem, Harbor City, CA, U.S.A.) were washed with 1.0 ml of methanol and 2.0 ml of water. Urine (0.4 ml) was diluted to 1.0 ml with 0.2 *M* potassium phosphate buffer (pH 8.0), and the diluted, buffered urine sample was applied to the extraction column. The extraction column was then washed with 2.0 ml of 0.2 *M* phosphate buffer and subsequently with 0.2 ml of 0.1 *M* hydrochloric acid. Bound XA was eluted from the anion-exchange resin with 1.2 ml of 0.1 *M* hydrochloric acid. This eluate was collected in a silanized 2.0-ml volumetric flask, and the pH was adjusted to 7.0 by adding 1.0 *M* sodium hydroxide. The eluate was then diluted to 2.0 ml using water, and 100 μl were directly injected for HPLC analysis. All solutions were eluted from the extraction column by applying vacuum to a twelve-position Supelco vacuum manifold (Supelco, Bellefonte, PA, U.S.A.).

HPLC analysis

A Beckman (Beckman Instruments, Berkeley, CA, U.S.A.) Model 112 solvent delivery module was fitted with a Whatman (Clifton, NJ, U.S.A.) PartiSphere C_{18} analytical column (110 mm $\times$ 4.7 mm I.D.; particle size 5 μm). To protect the analytical column, a Whatman reversed-phase guard cartridge was installed between the injector and the analytical column. The guard cartridge was replaced every two weeks. A 0.025 *M* potassium dihydrogenphosphate buffer (pH 5.5) containing 5% acetonitrile was used as mobile phase at a flow-rate of 1.2 ml/min. The column eluate was monitored at 340 nm with a Beckman Model 165 variable-wavelength detector, coupled to a Spectra-Physics (San Jose, CA, U.S.A.) 4270 integrator.

Linearity of XA extraction from urine

Different volumes of urine (0.2, 0.4, 0.6, 0.8 and 1.0 ml) were diluted to 1.0 ml with water, then mixed with 1.0 ml of 0.2 *M* potassium phosphate buffer (pH 8.0) and subsequently used for anion-exchange solid-phase extraction of XA as described above.

Standards

Standard solutions containing between 5 and 25 μM XA were prepared and subjected to anion-exchange solid-phase extraction and HPLC analysis as described above.

RESULTS AND DISCUSSION

Fig. 1 shows typical chromatograms obtained after HPLC analysis of (A) an XA standard solution, (B) a urine sample purified by anion-exchange solid-phase extraction and (C) the same urine sample spiked with 20 μM XA. Anion-exchange solid-phase extraction was highly effective in removing possible interfering urinary components. A few unidentified urinary components eluted from the reversed-phase column within 2 min of injecting the sample onto the column,

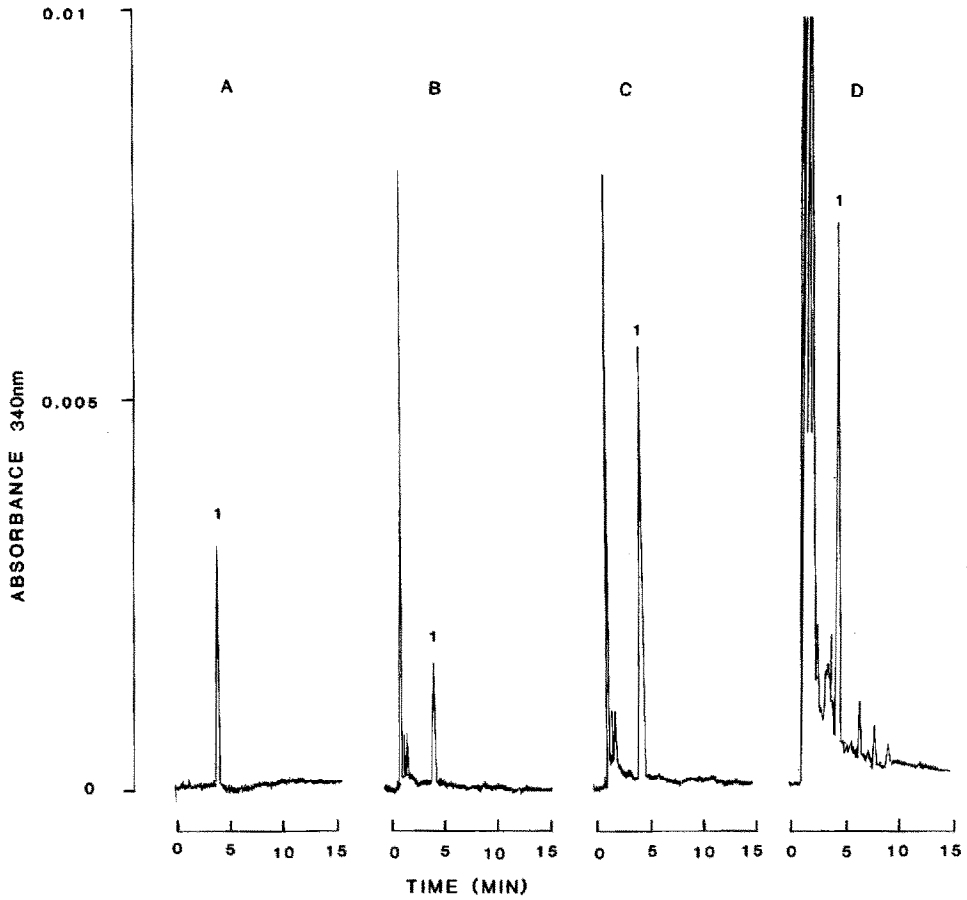

Fig. 1. Determination of xanthurenic acid (XA) in urine, purified by anion-exchange solid-phase extraction, using HPLC and UV detection at 340 nm. (A) A 15 μM XA standard; (B) urine sample from a healthy person containing 7.7 μM XA; (C) the same urine sample spiked with 20 μM XA; (D) the unspiked urine sample analysed with the UV detector set at 243 nm. Peaks: 1 = XA; unmarked peaks are unidentified background components.

with XA eluting after 4.3 min. Due to the fact that relatively few compounds absorb at 340 nm, no background interference was experienced, and the sensitivity was found fully adequate for reliable XA quantification in urine samples. Although detection of XA is more sensitive at 243 nm [17], co-elution of unidentified urinary components also absorbing at 243 nm (Fig. 1D) could result in overestimation of urinary XA levels.

Fig. 2 represents the elution profile obtained when bound XA was eluted from the strong anion-exchange extraction column using 0.1 M hydrochloric acid. Complete recovery of bound XA was achieved after washing the column with 1.4 ml of 0.1 M hydrochloric acid; the first 0.2 ml was routinely discarded since it contained no XA (Fig. 2). Satisfactory recovery was confirmed by adding small amounts (15 μmol) of XA to different urine samples. Analysis of these urine samples for XA indicated that the percentage of XA recovered using this method

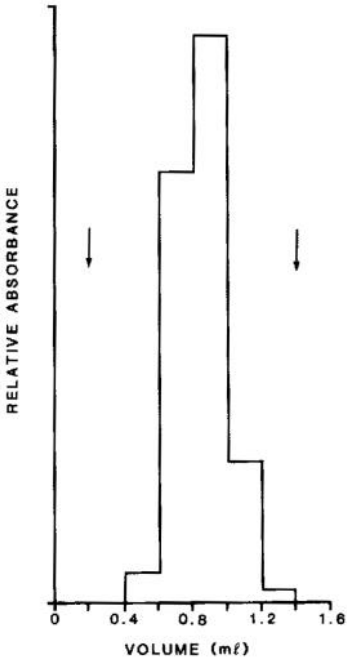

Fig. 2. Elution profile of xanthurenic acid from a 100-mg Analytichem strong anion-exchange extraction column. After absorbtion of an XA standard solution to the column, the column was eluted with 0.1 *M* hydrochloric acid, and 0.2-ml fractions of the eluate were collected for HPLC analysis. The volume eluting between the two arrows was recovered in routine urine analysis.

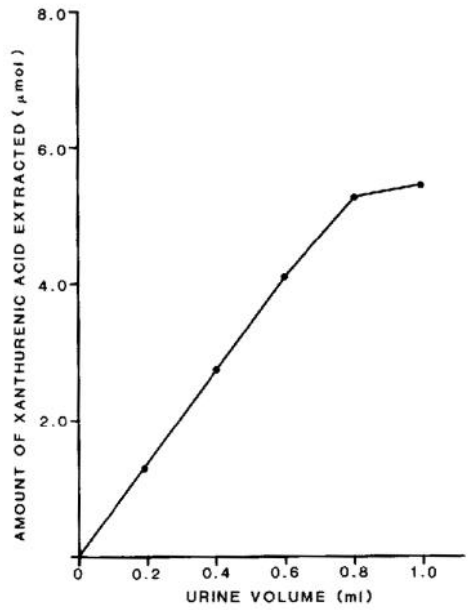

Fig. 3. Relationship between the amount of xanthurenic acid extracted and the urine volume applied to the Analytichem column packed with 100 mg of strong anion-exchange resin.

was $92.3 \pm 2.2\%$ (mean $\pm$ S.D.; $n=10$). Furthermore, the reproducibility of the method was also satisfactory as indicated by a within-batch coefficient of variation of 5.3% ($n=10$).

A major potential limitation of ion-exchange solid-phase extraction is saturation of ionic binding sites by other ionic species, resulting in incomplete XA recovery. This is especially true of urine, since samples from different individuals could vary considerably in both composition and concentration of different solutes. The extraction of XA from five different urine samples, selected for their relatively high osmolarity, was therefore investigated (Fig. 3) by applying different urine volumes to the extraction column. Fig. 3 represents a typical result; in all urine samples tested the amount of XA extracted from urine increased linearly with increasing urine volume up to 0.6 ml. Application of 1.0 ml of urine resulted in incomplete XA recovery, presumably due to column overloading. We therefore decided to use 0.4 ml of urine for routine XA quantification, which is well within the linear range for XA extraction.

Using the Supelco vacuum manifold for solid-phase extraction, twelve different urine samples could be prepared simultaneously for HPLC analysis. Since the HPLC method is also simple and rapid, this method for urinary XA analysis could

be the method of choice in clinical trials where urinary XA excretion before and after oral tryptophan loading is used as an index of vitamin B_6 nutritional status.

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