The Determination of 4-Pyridoxic Acid in Human Urine*

SARANYA K. REDDY,† MAY S. REYNOLDS, AND J. M. PRICE‡

From the School of Home Economics and the Cancer Research Hospital Medical School, University of Wisconsin, Madison, Wisconsin

(Received for publication, March 20, 1958)

The existence of a fluorescent compound in human urine after the ingestion of vitamin B₆ was reported by Singal and Sydenstricker (1) in 1941. Huff and Perlzweig (2, 3) isolated a pyridoxine metabolite from human urine which they identified as 2-methyl-3-hydroxy-4-carboxy-5-hydroxymethylpyridine (4-pyridoxic acid). A fluorometric method for the determination of this substance was also described (9).

Using the above method, 4-pyridoxic acid has been shown to be the predominant vitamin B₆ metabolite excreted in urine, both from food alone and following ingestion of supplements of pyridoxine, pyridoxal, or pyridoxamine (4-6). The usefulness of this method has been limited in that there are other compounds in urine which fluoresce under the conditions used for 4-pyridoxic acid estimation so that one obtains 4-pyridoxic acid values which are too high (7-9).

Sarett (9) partially avoided the interference of substances having fluorescence similar to 4-pyridoxic acid by giving a large test dose of pyridoxine to human subjects, which made it possible to dilute the urine samples until the effects of interfering substances became negligible. Although Sarett's method to determine the excretion of 4-pyridoxic acid has been useful following administration of large doses of vitamin B₆, its value in assaying for 4-pyridoxic acid in normal urine appears to be limited.

Since the problem in the estimation of urinary 4-pyridoxic acid appeared to be interference by other fluorescent substances, attempts have been made to free the 4-pyridoxic acid from these unknown urinary constituents. Sarett (9) attempted to remove some of the extraneous fluorescent compounds from urine without success. The present communication deals with a more specific method for the estimation of 4-pyridoxic acid in which ion exchange resins have been used to purify the urinary pyridoxine metabolite.

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation, and by the American Cancer Society. Data in this paper are taken from a thesis submitted by Saranya K. Reddy in partial fulfillment of the requirements for the degree of Doctor of Philosophy with a major in Human Nutrition.

† Present address, 4 Rundall's Road, Vepery, Madras 7, South India.

‡ American Cancer Society, Charles Hayden Foundation Professor of Surgery in Cancer Research.

MATERIALS AND APPARATUS

Ion Exchange Resins—Dowex 1 (Cl⁻), 10 per cent cross-linkage, 200 to 400 mesh, and Dowex 50 (H⁺), 12 per cent cross-linkage, 200 to 400 mesh, were prepared as described by Price (10).

Reagents—Synthetic 4-pyridoxic acid and 4-pyridoxic acid lactone were prepared by the method of Heyl (11).

Chromatography Columns—The chromatographic columns were made by sealing a glass tube of 1.2 cm. outside diameter, and 20 cm. long, to the bottom of 125 ml. Erlenmeyer flasks. A constriction near the bottom of the tube held a plug of glass wool upon which the resin column was placed.

Analytical Method

The 24 hour urines were collected in amber bottles containing about 25 ml. of toluene, and an aliquot of these samples was removed and stored at 0° until analyzed.

Preparation of Analytical Columns—A slurry of the resin was pipetted into the columns to form a packed layer 3 cm. long. The Dowex 50 columns were washed successively with 50 ml. of 5 N HCl, 50 ml. of 2 N HCl and 50 ml. of water. The Dowex 1 columns were washed with 50 ml. of 2 N HCl followed by 50 ml. of water. The columns were operated without added pressure, and were discarded after use.

Chromatography of 4-Pyridoxic Acid in Urine—About 4 per cent of the 24 hour urine was adjusted to a pH of 10.6 with saturated NaOH and filtered through Whatman No. 42 filter paper. An aliquot of this filtrate representing 1 per cent of the 24 hour excretion was pipetted into duplicate 50 ml. graduated centrifuge tubes and 25 μg. of synthetic 4-pyridoxic acid was added to one tube. Then 1.5 ml. of 1.5 N NH₄OH was added to each centrifuge tube and the contents of both tubes were diluted to 40 ml., stirred well, and added to the Dowex 1 (Cl⁻) columns. The centrifuge tubes were rinsed with an additional 20 ml. of water and this water was used to wash the sample through the columns. The 4-pyridoxic acid was then eluted from the Dowex 1 with 50 ml. of 0.05 N HCl and the effluent was passed directly into the Dowex 50 columns. The Dowex 50 columns were washed with 20 ml. of water and the 4-pyridoxic acid was then eluted with 50 ml. of 2 N HCl.

Quantitative Estimation of 4-Pyridoxic Acid—The quantitative estimation consisted of 4 steps: (1) delactonization, (2) lactonization, (3) adjustment of pH and dilution, and (4) reading in the fluorometer.

Delactonization—In the initial studies blank values were
found to be unduly high. This was the result of considerable lactonization which occurred spontaneously at room temperature in the 2 N HCl used to elute the 4-pyridoxic acid from the Dowex 50. A de lactonization step was introduced to eliminate this problem. For de lactonization 4 ml. of the 2 N HCl eluate from the Dowex 50 was pipetted in duplicate into 60 ml. graduated centrifuge tubes. 2 ml. of 5 N NaOH were added to each tube which made the solution 0.33 N and the tubes were heated in a boiling water bath for 5 minutes and then cooled. Several samples of 4-pyridoxic acid lactone were heated in 0.33 N NaOH from 0 to 25 minutes and 5 minutes was found to be adequate for complete de lactonization.

Lactonization—To neutralize the alkali, 2 ml. of 1 N HCl were added to the tube which contained the sample to be lactonized. Then 2 ml. of 5 N HCl were added and the tube was heated in a boiling water bath for 15 minutes and cooled. It was found that 15 minutes of heating was adequate for lactonization. The second tube was not lactonized but was used to prepare the blank as described below.

Adjustment of pH and Dilution—After lactonization the samples were made slightly alkaline by the addition of 11 ml. of 1 N NaOH and were then diluted to 30 ml. with water. From 0.5 to 5 ml. (usually 2 ml.) of this solution was immediately diluted to 10 ml. by the addition of 1 per cent sodium borate solution. The pH of the final solution was 9.0 ± 0.3 which was optimal for the fluorescence measurements (3).

The blanks were handled differently because considerable lactonization appeared to take place in acid solution at room temperature. To prepare the blank, 11 ml. of 1 N NaOH was added to the second of the pair of tubes which had been de lactonized as described above, followed by additions of 2 ml. of 1 N HCl, 2 ml. of 5 N HCl and water to a volume of 30 ml. 2 ml. of this mixture were immediately diluted to 10 ml. with 1 per cent sodium borate for reading in the fluorometer. The fluorometric readings of the samples and blanks did not change for several hours in borate buffer.

Fluorescence Measurements Fluorescence measurements were made with a Coleman model 12 photofluorometer using filters B-1 and PC-1. A solution of 0.0375 μg. of quinine sulfate per ml. of 0.1 N H2SO4 was employed as a reference standard.

The standard curve was obtained by taking dilutions of synthetic 4-pyridoxic acid ranging from 0.007 to 0.201 μg. per tube and plotting the fluorometer readings for the lactone (corrected for blanks) against 4-pyridoxic acid concentration. A straight line graph relating the concentration of 4-pyridoxic acid to fluorometer readings was obtained.

Fluorescence Spectra—An Aminco-Bowman spectrophotofluorometer was utilized to obtain the fluorescence spectra of synthetic 4-pyridoxic acid, urine which had been passed through the columns, and whole urine. The samples were de lactonized and lactonized as described. A typical urine sample was selected from a subject who had not received pyridoxine supplements and was analyzed by the method of Huff and Perlzweig (3) but with de lactonization and with a recovery sample. An aliquot of the same urine sample along with a duplicate sample containing added 4-pyridoxic acid for recovery was fractionated through Dowex 1 and Dowex 50 columns, and then de lactonized and lactonized in the usual manner. The optimum activating wave length for 4-pyridoxic acid lactone was found to be 350 μm and the fluorescence peak was at 450 μm. Silt arrangement No. 5 was used with the meter multiplier set at 0.3 and the resulting curves were recorded photographically.1 The scale of the oscillograph supplied with the Aminco-Bowman spectrophotofluorometer was modified as shown to make a more satisfactory photographic recording of the fluorescence spectra.

EXPERIMENTAL

Fluorescence Removed by Chromatography—To determine the order of improvement attained by the revised method as compared with the method of Huff and Perlzweig (3), the amount of background fluorescence lost through the chromatographic procedure was used as an index. The 4-pyridoxic acid in a number of samples was determined with and without the chromatographic procedure and the results were compared.

Interference of Vanilla—Sarett (9) found that a metabolite of coumarin was responsible for the high 4-pyridoxic acid excretion values reported in some studies in which vanilla flavoring containing coumarin had been used. The revised method was tested to determine whether or not the ingestion of vanilla flavoring would result in similar interference.

The excretion of 4-pyridoxic acid by four subjects on a self-selected diet was determined on 3 successive days. On the fourth and fifth days, subject L. M. was given a supplement of 5 ml. of vanilla extract, and subjects A. W., A. R., and V. V. were given a supplement of 5 ml. of artificial vanilla flavoring and 4-pyridoxic acid determinations were made on the 24 hour urine collections.

Investigation of Existence of 4-Pyridoxic Acid Conjugates—Since Scudi et al. (12) stated that 4-pyridoxic acid was conjugated in part by both man and the dog, evidence for the existence of 4-pyridoxic acid conjugates was sought. For this purpose urine samples were submitted to hydrolysis by refluxing in 0.5 N HCl or 1.0 N NH4OH for 1 hour. The acid-hydrolyzed sample was adjusted to pH 10.6, diluted to the original volume, and fractionated and assayed as usual. The NH4OH in the other sample was removed by boiling. The solution was then adjusted to pH 10.6, diluted to the original volume, and chromatographed and assayed as usual. The samples were run in duplicate and before hydrolysis 4-pyridoxic acid was added to one of the samples in each pair to determine the recovery. Similar samples of the same urine were analyzed without hydrolysis.

Excretion of 4-Pyridoxic Acid Following Ingestion of Single Supplement of Pyridoxine Hydrochloride—In this study two subjects on self-selected diets each collected a 24 hour urine sample after which each subject ingested 48.0 μmoles (10 mg.) of pyridoxine hydrochloride. 24 hour urine collections were obtained for 2 or 3 additional days. All of the urine samples were analyzed for 4-pyridoxic acid by the method of Huff and Perlzweig (3) and by the revised method.

Excretion of 4-Pyridoxic Acid on Constant Daily Intake of Vitamin Be—In the first study four subjects were placed for 5 days on a constant diet containing 8.6 μmoles of vitamin B6 plus 9.7 μmoles of synthetic pyridoxine hydrochloride. The pyridoxine hydrochloride supplement was discontinued for the next 5 days and the ten 24 hour urine samples collected from each subject were analyzed for 4-pyridoxic acid by the revised analytical method. Analysis of a sample of the diet for the second 5 days showed a vitamin B6 content of 8.3 μmoles per daily portion.

In the second study four other subjects were maintained for

The urine samples were analyzed for 4-pyridoxic acid by the ion exchange procedure and by the original method of Huff and Perlzweig (3). The excretion of 4-pyridoxic acid is given in moles per 24 hours and the recoveries are given in per cent. The urine samples were all analyzed by the revised method reported here and by the original method of Huff and Perlzweig (3) for comparison. The pyridoxine supplement was given at the end of Day 1.

The results in Table I provide evidence that the ingestion of capsule excipients or flavoring on values for urinary 4-pyridoxic acid.

Excretion of 4-pyridoxic acid on two levels of vitamin B₆ intake

During the first 5 days the subjects received 8.6 μmoles of vitamin B₆ from a constant diet of natural foods and 9.7 μmoles of pyridoxine hydrochloride. During the next 5 days the subjects received 8.3 μmoles of vitamin B₆ from the foods without any supplement.

The peak of the recovery sample corresponded in magnitude to 100 per cent recovery of the added synthetic material as determined from the standard in Fig. 1. The deactonized samples had more fluorescence than the reagent blank so complete removal of interfering substances was not achieved in the fractionation.

In Fig. 3 the fluorescence peaks of the unfractionated urine of added synthetic 4-pyridoxic acid. The concentration of urine used to obtain the curves in Fig. 3 was half that used for Fig. 2. The peaks obtained in Fig. 3, however, were about twice the magnitude of those in Fig. 2. Thus about 75 per cent of the interfering fluorescence was removed from this urine sample by the chromatographic procedure without any detectable loss of 4-pyridoxic acid.

The results in Table I provide evidence that the ingestion of vanilla flavoring containing coumarin failed to alter the values.
were not affected by hydrolysis. Since the results were un-
hydrolysis as described and the recoveries of 4-pyridoxic acid
questionably negative the data have not been recorded in detail.

4-pyridoxic acid content of the urine following acid or alkaline
jugates revealed that there was no change in the apparent
synthetic diet was 14.6 pmoles daily, essentially all of which was
supplied by a multivitamin capsule, which was administered from
the start on the 10th day of the experiment. The urines were
analyzed only on the days indicated and by the revised method.

The study to ascertain the existence of 4-pyridoxic acid con-
jugates revealed that there was no change in the apparent
4-pyridoxic acid content of the urine following acid or alkaline
hydrolysis as described and the recoveries of 4-pyridoxic acid
were not affected by hydrolysis. Since the results were un-
questionably negative the data have not been recorded in detail.

Table II shows the 4-pyridoxic acid excretion by two subjects
following ingestion of a single supplement of 48.6 μmoles (10
mg.) of pyridoxine hydrochloride. For purposes of comparison
4-pyridoxic acid values obtained by Huff and Perlwaieg's method
are also presented. The corresponding recoveries are included
and clearly indicate the relative efficacy of the two methods.

The percentages of the test dose of pyridoxine accounted for
by the increase in 4 pyridoxic acid excreted on the first day after
supplementation were 37 and 44 per cent for the two subjects.
According to Rabinowitz and Snell (5) 4-pyridoxic acid levels
returned to normal 12 hours after the ingestion of a 100 mg.
dose of any one of the three forms of vitamin B₆. In this study,
however, it was found that by the second day following the in-
gestion of the test dose of pyridoxine the urinary 4-pyridoxic acid
levels had not returned to the basal levels. The sum of the
increased excretion of 4-pyridoxic acid on the first two days
was adequate to account for 51 and 48 per cent of the supplement
administered to the two subjects.

The results of the first study of 4-pyridoxic acid excretion by
the subjects on a constant daily intake of vitamin B₆ (Table
III) showed that within 2 days of the addition of the supplement
of synthetic vitamin B₆ the 4-pyridoxic acid levels increased and
stabilized at a higher level. 2 days following removal of the
supplemental pyridoxine the 4-pyridoxic acid levels decreased
and stabilized at lower levels. The levels at which they stabi-
ized in either case accounted for about 50 per cent of the corre-
spending vitamin intake.

The 4-pyridoxic acid excretion during the second study of
subjects on a constant diet is shown with corresponding vitamin
B₆ intakes (Table IV). With the ingestion of a total of 14.6
μmoles of pyridoxine on the semisynthetic diet the 4-pyridoxic
acid in the urine gradually increased. On the 13th and 14th
days (the 22nd and 23rd days of the experiment) 4-pyridoxic
acid excretion stabilized at a level which accounted almost quan-
titatively for the vitamin ingested. Striking uniformity in values
was observed from subject to subject throughout the experi-
ment.

The 4-pyridoxic acid excretion values following oral and sub-
cutaneous administration of equal amounts of 4-pyridoxic acid
are graphically presented in Fig. 4. Following subcutaneous
administration of 10.5 μmoles of 4-pyridoxic acid the increased
excretion of 4-pyridoxic acid by the four subjects was sufficient
to account for from 89 to 111 (average 98) per cent of the supple-
ment.
When 10.5 μmoles of 4-pyridoxic acid was administered orally, the increase in the 4-pyridoxic acid excretion in the first 24 hours after administration was adequate to account for 50, 55, 37, and 22 (average 41) per cent of the ingested amount. There appeared to be some increase in 4-pyridoxic acid excretion on the second and even on the third day. The ingested 4-pyridoxic acid accounted for in the urine of the four subjects through the entire period of 72 hours after administration of the oral dose was 50, 68, 49, and 31 (average 50) per cent.

**DISCUSSION**

In the method described here ion exchange chromatography was used to remove from 40 to 75 per cent of the fluorescent substances in urine which had fluorescence similar to that of 4-pyridoxic acid in the fluorometric determination of this metabolite, whereas no loss of added 4-pyridoxic acid occurred. As a result, it was possible to assay normal urines for 4-pyridoxic acid with considerably greater precision than was possible previously as a result of the removal of a large part of the interfering substances.

Recoveries with the method of Huff and Perlzweig (3) as modified by Sarett (9) were extremely variable, presumably because of interfering substances. Recoveries with the ion exchange procedure ranged between 85 and 105 per cent. When the lactone of 4-pyridoxic acid was added to urine it was recovered equally well. The fluorescence spectra of the fractionated urine also indicated that reasonably good purification of the urinary 4-pyridoxic acid was achieved without loss of added 4-pyridoxic acid in the chromatographic procedure.

It was possible to analyze satisfactorily only those 24 hour human urines which contained 2 or more μmoles of 4-pyridoxic acid, whereas values obtained with samples which contained less than 2 μmoles were subject to question. It is possible that the method could be modified by additional chromatographic steps to make it suitable for use with samples of urine containing lower levels of 4-pyridoxic acid.

Results obtained by the two methods showed that the 4-pyridoxic acid values with the ion exchange procedure were about a third to a fifth of the values obtained by Huff and Perlzweig's method. High 4-pyridoxic acid values obtained previously may have been responsible in part for data showing excretion in distinct excess of intake (6, 14, 15). From these data it was postulated that there was considerable synthesis of the vitamin in man. Thus Linkswiler et al. (6) found that on a constant intake of 0.76 mg. of vitamin B₆, the daily excretion of vitamin B₆ metabolites amounted to 3.54 mg. On an intake of 2.78 or 15.78 mg. per day the excretion of pyridoxine metabolites was 4.36 or 10.69 mg., respectively. Since the diet was otherwise constant, the level of urinary substances which interfered with the assay may be assumed to be constant. Thus with a low intake of vitamin B₆ the interference of substances other than 4-pyridoxic acid in the fluorometric procedure would be expected to be relatively large, while on an intake almost 20 times as great, interference might be expected to be negligible. It should now be possible to test this possibility with further studies.

Johnson et al. (4) reported that 54 per cent of an 8 mg. supplement of pyridoxine was excreted by way of known metabolic products of vitamin B₆. 45 per cent of a 100 mg. supplement of pyridoxine was accounted for in the urine in the study of Rabino-witz and Snell (5). In the present studies 37 and 44 per cent of a 10 mg. dose of pyridoxine hydrochloride could be accounted for by the increase of 4-pyridoxic acid excretion. Thus the results obtained here were very similar to those previously reported when single large supplements of pyridoxine hydrochloride were given.

The excretion of 4-pyridoxic acid by the subjects on the constant diet of ordinary foods 1 day before (basal) and for 3 consecutive days after a single supplement of 10.5 μmoles of 4-pyridoxic acid. The supplement was given orally in one experiment and subcutaneously in the second experiment.

The differences in the extent to which 4-pyridoxic acid was accounted for in the urine following oral and subcutaneous administration of 4-pyridoxic acid itself cannot be explained. The 4-pyridoxic acid given orally may either be incompletely absorbed or partially destroyed by intestinal microorganisms, or both. The high urinary recovery of injected 4-pyridoxic acid in man would suggest that this product may not be metabolized further to an appreciable extent. If it were oxidized further or even conjugated it would likely have been lost in the chromatographic procedure or might have failed to change in fluorescence during lactonisation.

After the development of this method a similar method by Fujita et al. (16) using ion exchange resins was noted. The method of Fujita et al. has the advantage over the present one

---


---

![Fig. 4. Urinary excretion of 4-pyridoxic acid by four subjects on a constant diet of ordinary foods 1 day before (basal) and for 3 consecutive days after a single supplement of 10.5 μmoles of 4-pyridoxic acid. The supplement was given orally in one experiment and subcutaneously in the second experiment.](http://www.jbc.org/)

Downloaded from http://www.jbc.org/ by guest on August 27, 2017
in that pyridoxal, pyridoxine, and pyridoxamine may be individually estimated along with 4-pyridoxic acid. However, the determination of 4-pyridoxic acid by the method of Fujita et al. appears to be more complicated than the one described here, although recoveries appear to be comparable in the two methods. The revised method reported here was satisfactory to assay urines containing as little as 0.5 μg per ml. Such levels have often been found in normal urine samples. It is not known whether the method of Fujita et al. could be used to assay urines containing less 4-pyridoxic acid than 1.24 μg per ml., the lowest values presented in their report.

SUMMARY

A method has been described for the fluorometric determination of urinary 4-pyridoxic acid after the removal of interfering fluorescent compounds by ion exchange chromatography on Dowex 1 (Cl−) and Dowex 50 (H⁺). From 40 to 70 per cent of the fluorescence of the urine was removed by chromatography while recoveries of added 4-pyridoxic acid were essentially quantitative. The method was suitable for human urine samples containing over 2 μmoles of 4-pyridoxic acid per 24 hours.

Approximately half of a single dose of 48.0 μmoles (10 mg.) of pyridoxine hydrochloride could be accounted for by increased urinary 4-pyridoxic acid excretion. The 4-pyridoxic acid excretion by human subjects ingesting a constant diet of ordinary foods containing 18.6 or 8.3 μmoles of vitamin B₆ was adequate to account for a similar percentage of the vitamin intake. On a constant semisynthetic diet the urinary 4-pyridoxic acid accounted for almost all of the pyridoxine intake.

Ingested 4-pyridoxic acid was accounted for to the extent of about 50 per cent in the urine, but following subcutaneous administration it was excreted almost quantitatively within 24 hours.

REFERENCES

The Determination of 4-Pyridoxic Acid in Human Urine

Saranya K. Reddy, May S. Reynolds and J. M. Price