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# DETERMINATION OF URINARY THIAMINE BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY UTILIZING THE THIOCHROME FLUORESCENT METHOD

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## SUMMARY

A sensitive, reproducible, and specific method for the determination of urinary thiamine has been established. Unique to this method is the use of high-pressure liquid chromatography (HPLC) to separate the fluorescent thiamine derivative from interfering fluorescent compounds. Urine samples were passed through a Decalso cation-exchange column, washed with 0.5 *M* KCl to remove some interfering compounds, and eluted with 3.4 *M* KCl. The eluted thiamine was converted to the fluorescent derivative, thiochrome, by reaction with alkaline potassium ferricyanide. The reaction mixture was extracted with isobutanol and subjected to HPLC monitored by a fluorescent detector.

Within-day and day-to-day coefficients of variation proved to be 2.5% and 1.2%, respectively. Recovery of added thiamine (range 0.04 to 2.0 μg/ml) averaged 99.9 ± 5.3%. The sensitivity of this method was 0.03 μg/ml.

## INTRODUCTION

The evaluation of possible thiamine deficiency has been attempted by several methods. Erythrocyte transketolase assays [1] have been used even though the assay is difficult and its results considered inadequate by some investigators. The determination of urinary thiamine excretion has been employed to evaluate

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the nutritional status of thiamine in human surveys [2—4]. Other studies suggest that thiamine-to-creatinine ratios might provide a better index of thiamine deficiency [5, 6].

The urinary thiamine assays incorporate conversion of thiamine to thiochrome, which was first described by Jansen [7] in 1936. The early studies incorporating this reaction [8, 9] suffered from low recoveries and non-specific interfering substances. Subsequent investigations attempted to eliminate these problems by modifying the early procedures. Cyanogen bromide has been used in place of potassium ferricyanide to optimize the oxidation of thiamine [10]. Burch et al. [11], using whole blood and blood cells, corrected for non-thiochrome fluorescence by measurement before and after ultraviolet destruction of thiochrome. Haugen [12] eliminated the chromatographic separation [8, 9] by incorporating a blank tube containing benzenesulfonyl chloride which destroys the thiamine[13]. Leveille [14] modified Haugen's procedure by adding a third recovery tube to correct for quenching effects. Schultz and Natelson [15] used Amberlite CG-50 resin, adjusted the pH to 9.8—10.0 for the thiochrome reaction and subsequent extraction, and incorporated a urine blank containing no potassium ferricyanide in an attempt to eliminate non-specific fluorescence.

This study describes a procedure that eliminates urinary compounds which inhibit the thiochrome reaction. The utilization of HPLC further eliminates non-specific fluorescence.

## EXPERIMENTAL

### *Reagents*

Distilled, deionized water was used to prepare all of the reagent solutions. All compounds, unless noted otherwise, were reagent grade and purchased from commercial sources.

Isobutanol was Spectra AR grade from Mallinckrodt (St. Louis, Mo., U.S.A.) Thiochrome was purchased from Pfaltz & Bauer (Stamford, Conn., U.S.A.) and was dissolved in isobutanol to a final concentration of 0.1 μg/ml.

Thiochrome Decalso (Permutit-T), 50—80 mesh cation-exchange resin, was washed with water, 0.5 *M* acetic acid, 3.4 *M* potassium chloride, and finally with water until the final wash did not yield a precipitate with 2% silver nitrate. The resin was dried at 90°for 24 h and prepared as a water slurry when the columns were prepared.

Alkaline potassium ferricyanide was prepared immediately prior to use by diluting 4.0 ml of 1% potassium ferricyanide to 100 ml with 3.8 *M* NaOH; it was stored refrigerated in a brown bottle.

Thiamine hydrochloride (M.W. 337.3) was dried at 90° for 24 h before preparing standard solutions in 0.1 *M* HCl. The working thiamine solutions were 0.15, 0.30, 0.60, and 1.20 μg/ml (1 mg of thiamine hydrochloride equals 0.79 mg of thiamine). An intermediate thiamine standard solution of 10 μg/ml in 0.1 *M* HCl was prepared also. All thiamine solutions were stored refrigerated in brown bottles. The 0.5 *M* sodium acetate buffer was prepared by adding 20 ml of 2.5 *M* sodium acetate solution to 35.5 ml of 1.0 *M* HCl and diluting to 100 ml with water; the pH, if necessary, was adjusted to 4.2.

Methanol was glass-distilled over magnesium turnings and subsequently degassed and filtered through a sintered-glass funnel. Diethyl ether was similarly prepared except no magnesium was used when distilling. The HPLC mobile phase was methanol—diethyl ether (22:88 v/v) and was prepared daily.

*Apparatus*

Bio-Rad 10 × 0.7 cm I.D. glass-barrel Econo-Columns were used for the ion-exchange columns. The columns were filled to 9 cm with resin as an aqueous slurry and lightly tapped while settling.

An Altex 250 × 3.2 mm I.D. stainless-steel column containing stainless-steel frits and packed with LiChrosorb 5 -μm particles was used for HPLC. The column was initially washed by pumping methanol for 1 h. An Altex six-port sample injection valve equipped with a 25-μl external sample loop was used. A Milton-Roy controlled-volume mini-pump and a No. 1309 LDC fluoro-monitor equipped with a 360/400 + filter kit were employed. A Model No. 161 Linear Corp. chart recorder was used.

A Buchi Rotavapor >R< with water-aspired vacuum was used for all sample concentrating. An International Equipment Co. centrifuge, Model HN-S, was used in all sample preparations.

*Samples*

The urine samples were 24-h collections from healthy males and either 24-h or 2 × 12-h collections of pediatric patients (Cleveland Clinic Hospital), some of whom were receiving thiamine supplements. In all cases the urine was made acidic (0.1 *M*) with concentrated hydrochloric acid and stored frozen (—5°).

Rat urine samples from a control group of a study involving induced thiamine deficiency were used for recovery experiments only. These urines were made acidic (0.1 *M*) and stored frozen.

*Procedure*

*HPLC method.* Human urine (6.0 ml) in duplicate, or 6.0 ml of working thiamine solutions, or 6.0 ml of 0.1 *M* HCl (blank) was added to 2.0 ml of the sodium acetate buffer and 0.20 ml of 3.8 *M* NaOH. For recovery samples, an aliquot of the intermediate thiamine standard solution was added to the urine mixture. For rat urines, 2.0 ml of urine plus 4.0 ml of 0.1 *M* HCl or 4.0 ml of the working thiamine standard solutions (recovery samples) was used. In all cases, the pH was between 4.5 and 5.0. The samples were mixed and centrifuged.

The samples were added to the ion-exchange columns and eluted; the eluates and subsequent 2 × 8.0 ml water washings were discarded. Then 2 × 5.0 ml of dilute acid potassium chloride (0.5 *M* KCl — 0.1 *M* HCl) was eluted and discarded. Thiamine was then eluted with 15.0 ml of 3.4 *M* KCl — 0.1 *M* HCl. Alkaline potassium ferricyanide (5.0 ml) was added to the thiamine eluate and allowed to react for 20 min. The reaction was stopped by adding 15 ml of isobutanol and swirling for 15 sec to reduce excess ferricyanide. The samples were then extracted by shaking for 1 min. The isobutanol layer was then washed with 20 ml of water. Sodium sulfate was added and the isobutanol concentrated (35°—40°) on the Rotavapor to about 1—2 ml. The samples were

adjusted to a final volume of 3.0 ml with isobutanol and then centrifuged.

For HPLC, the mobile phase was pumped at 0.85 ml/min until a steady baseline was achieved. About 0.5 ml of the sample was loaded on the sample loop and then injected onto the column and the "zero time" marked. The peak height method was used for quantitation. In some cases, the thiochrome peak was collected to compare its fluorescent spectrum with that of authentic thiochrome.

For precision studies, a 24-h urine from a healthy male was collected and made 0.1 *M* with hydrochloric acid. This sample was analyzed ten times for within-day precision. For day-to-day precision, 15-ml aliquots were frozen and duplicates run daily for five successive days.

The ion-exchange resin was regenerated by adding 3 × 8.0 ml of 3.4 *M* KCl—0.1 *M* HCl solution followed by water washes until the eluate showed no precipitation with silver nitrate. The resin was used three times before repacking new columns. The used resin was washed three times with 0.0001 *M* NaOH and then treated as described previously and reused. The HPLC column was washed by pumping methanol for 30 min and inverted to remove any contaminants trapped on the upper frit.

*Conventional method.* After the samples were placed on the column, 2 × 8.0 ml water washes followed by 3 × 8.0 ml hot water washes were discarded. The thiamine was then eluted with hot 15.0 ml of 3.4 *M* KCl—0.1 *M* HCl and treated as described in the literature [6] using a 20-min reaction time. The samples were quantitatively determined on an Aminco-Bowman SPF spectrofluorimeter and, in some cases, fluorescent spectra were recorded and compared with that of authentic thiochrome.

*Modified conventional method.* This procedure was identical to the conventional method except that, after the hot water washes, 2 × 5.0 ml of the dilute acid potassium chloride was added to the columns and discarded. The

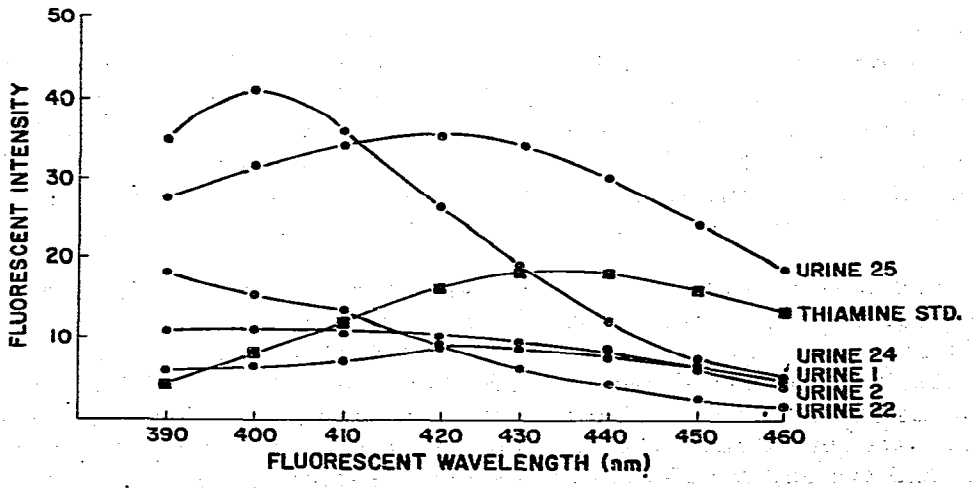


Fig. 1. Fluorescent spectra of urines and thiamine standard analyzed by the conventional method after subtracting respective blanks. Aminco-Bowman SPF; excitation wavelength, 365 nm.

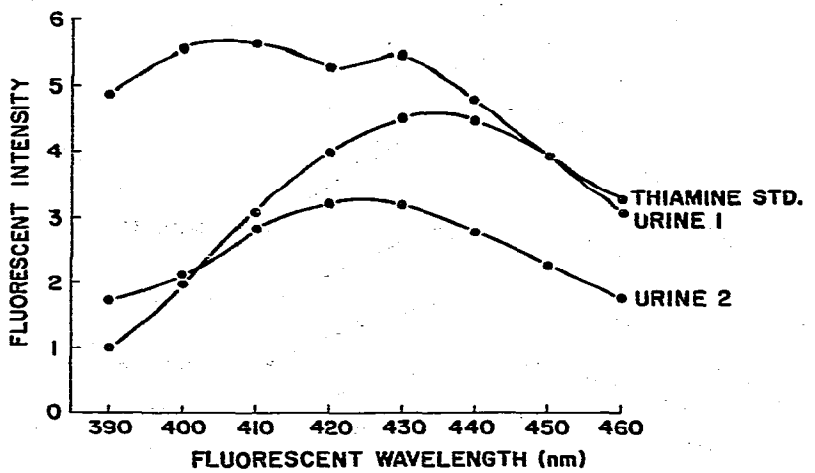


Fig. 2. Fluorescent spectra of urines and thiamine standard analyzed by the modified conventional method after subtracting respective blanks. Aminco-Bowman SPF; excitation wavelength, 365 nm.

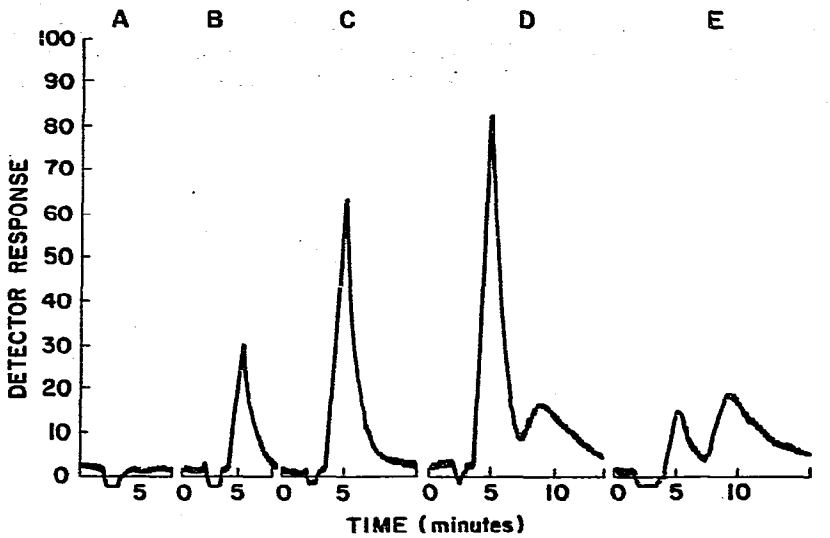


Fig. 3. HPLC chromatogram of: (A) reagent blank; (B) 0.30 μg/ml thiamine-HCl standard; (C) 0.60 μg/ml thiamine-HCl standard; (D) urine No. $7_1$; (E) urine No. $24_1$. Flow-rate, 0.85 ml/min; chart speed 8 in./h; range = 16; mode A on fluoro-monitor.

thiamine was then eluted with hot acid potassium chloride and treated as described under the conventional method.

## RESULTS AND DISCUSSION

Initial attempts to assay for urinary thiamine by the conventional method [6] were unsuccessful, mainly because of low recoveries. Complete reduction of ferricyanide occurred in some cases and precipitation formed in almost

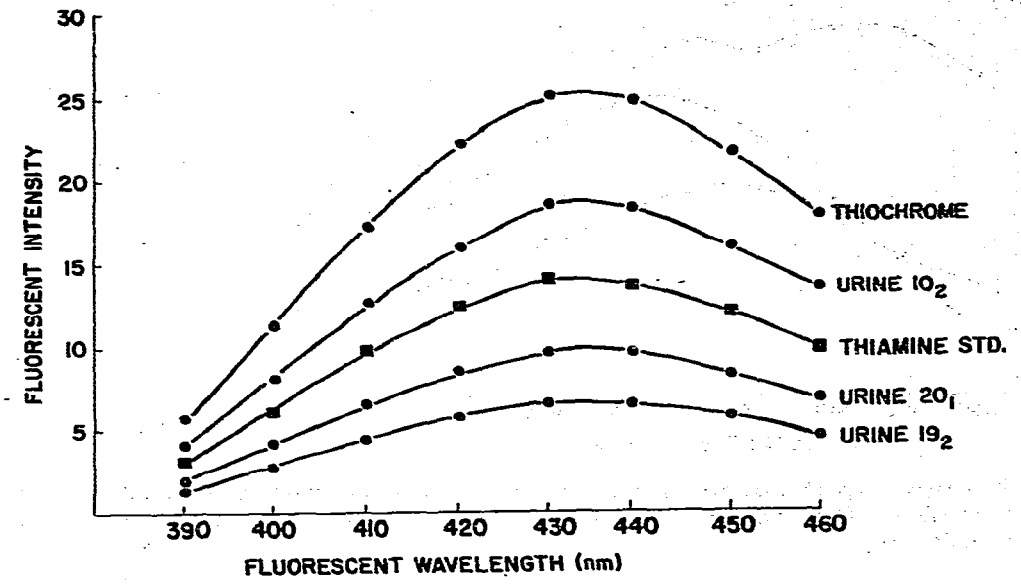


Fig. 4. Fluorescent spectra of samples eluted from the HPLC column at retention time corresponding to thiochrome; read against HPLC mobile phase except for thiochrome (0.10 μg/ml in isobutanol) which was read against an isobutanol blank. Aminco-Bowman SPF; excitation wavelength, 365 nm.

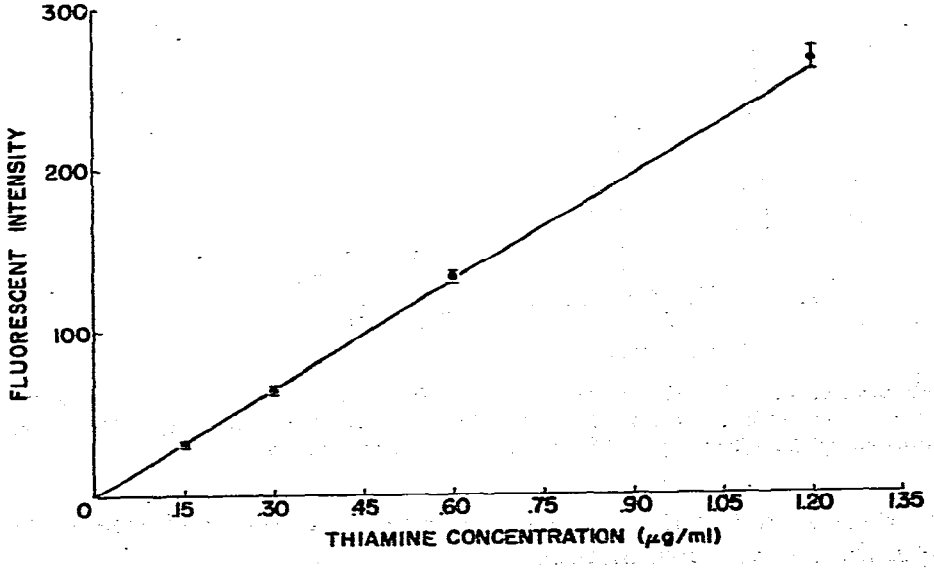


Fig. 5. Thiamine standard curve from a HPLC analysis.

all samples as the thiochrome reaction proceeded. The fluorescent spectra of some urines analyzed by the conventional method are shown in Fig. 1. Obviously, some nonspecific fluorescence was interfering with the assay.

It was found that 0.5 *M* KCl—0.1 *M* HCl solution did not elute thiamine

from the column, but did elute those compounds that reduced ferricyanide and which caused lowered recoveries. A modified conventional method was employed incorporating this solution prior to thiamine elution with hot 3.4 *M* KCl—0.1 *M* HCl solution. Recoveries proved quantitative, but the fluorescent spectra still indicated urinary interference as shown in the spectra of two urines in Fig. 2. It was concluded that the modified conventional method removed urinary inhibition but did not isolate thiochrome sufficiently from non-specific fluorescence.

Excellent results have been achieved using HPLC to analyze for nucleotides [16—19] which are cyclic nitrogenous compounds like thiochrome. Therefore, the HPLC method was set up in an attempt to resolve the thiochrome from the other non-specific fluorescence. A typical HPLC chromatogram is shown in Fig. 3; the urine samples clearly indicate that thiochrome is not the sole

TABLE I

RECOVERY OF THIAMINE ADDED TO URINE BY HPLC METHOD

| Type of urine | Concentration of added thiamine·HCl (μg/ml) | Recovery (%) |
|---|---|---|
| *Human urine* | | |
| 1 | 0.40 | 104 |
| 2 | 0.50 | 98 |
| 3 | 0.60 | 102 |
| 4 | 0.80 | 109 |
| 5 | 0.80 | 95 |
| 6 | 1.20 | 108 |
| 7 | 1.20 | 100 |
| 8 | 1.20 | 100 |
| 9 | 2.00 | 88 |
| | Mean | 100.4 |
| | S.D. | 6.5 |
| *Rat urine* | | |
| 1 | 0.04 | 100 |
| 2 | 0.13 | 103 |
| 3 | 0.16 | 96 |
| 4 | 0.16 | 103 |
| 5 | 0.16 | 100 |
| 6 | 0.30 | 95 |
| 7 | 0.40 | 110 |
| 8 | 0.40 | 98 |
| 9 | 0.50 | 99 |
| 10 | 0.50 | 90 |
| 11 | 0.60 | 102 |
| 12 | 0.67 | 98 |
| | Mean | 99.5 |
| | S.D. | 4.8 |
| Average mean | | 99.9% |
| Average S.D. | | 5.3% |

species being eluted. One prominent non-thiochrome peak was seen in 16 of the 25 human urines tested; some urines had one or two additional non-thiochrome peaks being eluted. The thiochrome peaks were collected and their fluorescent spectra recorded. These spectra are shown in Fig. 4 and, in all cases, confirmed its authenticity as thiochrome. It was evident that the HPLC eliminated the non-specific fluorescence found in the other two methods. A typical standard curve is plotted in Fig. 5 demonstrating the linearity of the HPLC method to 1.20 μg/ml. The actual urinary thiamine concentrations were obtained by the factor method.

The recovery data, demonstrated in Table I, cover a 50 fold range of added thiamine (0.04 to 2.0 μg/ml). The average recovery, 99.9±5.3%, demonstrates

TABLE II

PRECISION STUDIES OF HPLC METHOD

| Type of precision | Concentration thiamine-HCl (μg/ml) |
|---|---|
| *Within-day* | |
| 1 | 0.88 |
| 2 | 0.83 |
| 3 | 0.83 |
| 4 | 0.86 |
| 5 | 0.82 |
| 6 | 0.83 |
| 7 | 0.84 |
| 8 | 0.84 |
| 9 | 0.80 |
| 10 | 0.82 |
| Mean | 0.84 |
| C.V. | 2.5% |
| *Day-to-day* | |
| Day 1 | |
| 1 | 0.86 |
| 2 | 0.83 |
| Day 2 | |
| 1 | 0.86 |
| 2 | 0.84 |
| Day 3 | |
| 1 | 0.84 |
| 2 | 0.86 |
| Day 4 | |
| 1 | 0.85 |
| 2 | 0.84 |
| Day 5 | |
| 1 | 0.85 |
| 2 | 0.85 |
| Mean | 0.85 |
| C.V. | 1.2% |

TABLE III

EFFECT OF POTASSIUM FERRICYANIDE CONCENTRATION ON THIOCHROME REACTION

| Potassium ferricyanide concentration (%) | Fluorescence(%)* | |
|---|---|---|
| | Thiamine·HCl standard** | Urines*** |
| 0.5 | 99 | 99 |
| 1.0 | 100 | 100 |
| 2.0 | 97 | 99 |
| 5.0 | 84 | 74 |

*1.0% potassium ferricyanide used as the base comparison.
**Average of two determinations.
***Average of four human urines.

TABLE IV

EFFECT OF REACTION TIME ON THE THIOCHROME REACTION

| Reaction time (min) | Fluorescence(%)* |
|---|---|
| 5 | 87 |
| 10 | 96 |
| 15 | 100 |
| 20 | 100 |
| 30 | 96 |

*Average of two determinations; 20-min reaction time used as the base comparison.

that urinary inhibition was eliminated. The precision data for both the within-day and day-to-day runs, expressed as the coefficient of variation (C.V.), are displayed in Table II; values of 2.5% and 1.2% were obtained, respectively. The sensitivity of the HPLC method was 0.03 μg/ml using three times the height of baseline fluctuation as the minimum detectable peak height.

Two aspects of the thiochrome reaction, ferricyanide concentration and reaction time, were further investigated. Stock solutions of 0.5—5.0% potassium ferricyanide were diluted with 3.8 *M* NaOH and reacted with a thiamine standard solution diluted with 3.4 *M* KCl—0.1 *M* HCl. Four human urines were also analyzed, using the HPLC—ion-exchange separatory procedure, using the same ferricyanide solutions. For both urine and thiamine standards, as shown in Table III, only at the 5% concentration was there significant interference. The reaction time was tested by diluting a thiamine standard solution with 3.4 *M* KCl—0.1 *M* HCl, reacting with alkaline ferricyanide, and stopping the reaction after 5, 10, 15, 20, and 30 min. No significant differences were found from 10—30 min reaction time as shown in Table IV.

The urinary values obtained by the three methods are summarized in Table V. The values obtained by the conventional method are higher or lower than

TABLE V

COMPARISON OF URINARY THIAMINE LEVELS OBTAINED BY THE THREE METHODS

| Urine sample No. | Concentration of thiamine (μg/ml) HPLC method* | Conventional method | Modified conventional method |
|---|---|---|---|
| 22 | 0.09 | 0.38 | 0.40 |
| 5 | 0.12 | 0.09 | 0.13 |
| 23 | 0.15 | 0.14 | 0.17 |
| 2 | 0.20 | 0.28 | 0.33 |
| 11 | 0.27 | 0.38 | 0.38 |
| 1 | 0.29 | 0.41 | 0.52 |
| 4 | 0.35 | 0.38 | 0.41 |
| 17 | 0.41 | 0.49 | 0.49 |
| 18 | 0.45 | 0.38 | 0.49 |
| 24 | 0.52 | 0.88 | 1.01 |
| 7 | 0.60 | 0.87 | 0.88 |
| 13 | 0.84 | IV** | IV |
| 12 | 0.85 | 0.79 | 0.92 |
| 14 | 1.07 | IV | IV |
| 3 | 1.72 | 1.67 | 1.96 |
| 25 | 7.01 | 11.63 | 12.01 |
| 15 | 10.24 | 9.16 | 10.27 |
| 21 | 10.82 | 10.43 | 11.69 |
| 19 | 11.49 | 9.48 | 11.22 |
| 20 | 14.45 | 13.90 | 14.85 |
| 16 | 17.23 | 17.06 | 17.22 |
| 6 | 19.35 | 18.33 | 19.28 |
| 9 | 21.71 | 21.80 | 21.80 |
| 10 | 113.21 | 104.28 | 115.34 |

*Average of duplicates.
**Insufficient volume.

the HPLC method values depending on which factor is greatest: the urinary inhibition (lowering the thiamine readings) or the non-specific fluorescence (elevating the thiamine readings). In the HPLC method, both factors have been eliminated. Since the modified conventional method removes inhibition, but not non-specific fluorescence, it was expected that these values would be higher than the HPLC values. Such was the case, with the exceptions only occurring in those patients receiving thiamine supplements where non-specific fluorescence becomes less significant.

## CONCLUSIONS

A sensitive, reproducible, and specific method for urinary thiamine has been established using ion-exchange chromatography, thiamine oxidation to fluorescent thiochrome with potassium ferricyanide, isobutanol extraction, and HPLC. Urinary inhibition, non-specific fluorescence, and the need for

urine blanks have been eliminated. This procedure is being employed in an attempt to further define effective thiamine deficiency in humans.

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