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Thiamine Whole Blood Pharmacokinetics in Rats Using Both a Specific ^{35}S-Thiamine Liquid Scintillation Assay and the Thiochrome Fluorescence Assay

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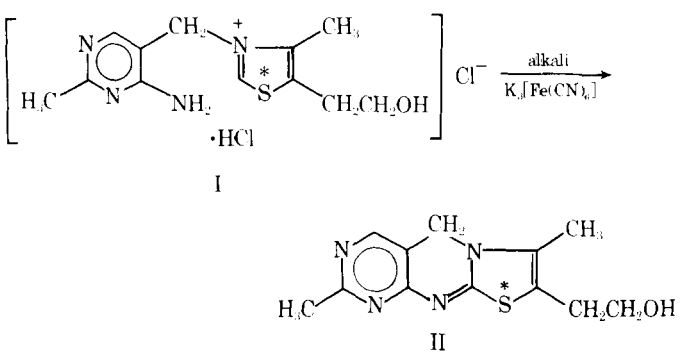
Abstract □ The study of factors altering the CNS and GI absorption of thiamine in rats required the development of a specific assay for thiamine from 100-μl samples of blood and plasma and small quantities of tissue. The specific thiochrome fluorescence assay for thiamine was modified to handle microsamples and to use ^{35}S-thiamine. This sensitive and specific radioassay using ^{35}S-thiamine gave pharmacokinetic parameters for 4-mg/kg iv doses of thiamine in rats equivalent to those using the less sensitive thiochrome fluorescence assay. The new assay, because of its lower limit of detection, allowed the study of the time profile of thiamine after a 1-mg/kg iv dose in rats. Such a time profile could not have been followed using the standard thiochrome fluorescence assay.

Keyphrases □ Thiamine—radiochemical analysis in blood, compared to fluorometric analysis, pharmacokinetics in rats □ Radiochemistry—analysis, thiamine in blood, compared to fluorometric analysis □ Pharmacokinetics—thiamine in rats □ Vitamins—thiamine, radiochemical analysis in blood, compared to fluorometric analysis, pharmacokinetics in rat

The thiochrome (II, Scheme I) fluorescence assay for thiamine (I) in biological fluids was described previously (1–12). Specific assays for thiamine with either ^{14}C- or ^{35}S-thiamine utilized electrophoric separations (12) or the ion-exchange system as in the thiochrome fluorescence assay (1). The study (1) using ion exchange did not, however, determine assay specificity in an *in vivo* system.

The present paper reports the modification of the specific thiochrome fluorescence assay for thiamine in whole blood to its use with microsamples and ^{35}S-thiamine and demonstrates its use in following the pharmacokinetics of intravenously administered thiamine at low doses to rats.



Scheme I

Quantitative studies of factors altering the central nervous system (CNS) and GI absorption of thiamine (vitamin B_1) have been limited. Thiamine, being a very water-soluble compound with a quaternary nitrogen, is poorly absorbed into the CNS (13) and poorly absorbed from the GI tract (14–17). Thiamine passes through those barriers because it is actively transported (13–17). However, as with other active transport systems, thiamine absorption is both saturable and/or easily inhibited (13–17). These absorption characteristics have been implicated in Wernicke's encephalopathy (14–18) in alcoholics, in the terminal children's disorder Leigh's disease (19–27), and in polioencephalomalacia in feedlot cattle (28, 29).

A study of thiamine pharmacokinetics in whole blood as a preliminary to the study of the effects of lipid-soluble thiamine prodrugs (30) in altering thiamine distribution required the development of a specific and sensitive assay for free thiamine (nonphosphorylated) and total thiamine (thiamine plus its mono-, di-, and triphosphate esters) in 100-μl samples of whole blood and plasma and small quantities of tissue, *e.g.*, brain tissue.

EXPERIMENTAL

Reagents and Materials—All reagents were analytical grade, and all aqueous solutions were prepared using glass-distilled deionized water. The primary source for reagent preparation and storage was the review of Mickelsen and Yamamoto (6). Reagent preparation and storage also were discussed elsewhere (1–12).

The ^{35}S-thiamine was obtained commercially[1] as two different lots of varying specific activity. Radiochemical purity was determined by paper chromatography and TLC, and chemical purity was checked by IR spectrophotometry. Thiamine hydrochloride was obtained commercially[2] with its purity confirmed by bioassay.

[1] Amersham/Searle Corp. Arlington Heights, Ill., lot A05006, specific activity 176 mCi/mmole and lot E06046, specific activity 233 mCi/mmole.
[2] Sigma Chemical Co., St. Louis, Mo.

Thiamine hydrochloride solution for injection was prepared in the following manner. ^{35}S-Thiamine hydrochloride, specific activity 176 or 233 mCi/mmole, and unlabeled thiamine hydrochloride were dissolved in an isotonic sodium chloride solution to final concentrations of 1 and 0.25 mg/100 μl, corresponding to 57 and 110 μCi/100 μl, respectively. These solutions were such that injection of 100 μl to 250-g rats resulted in thiamine dosing of 4 and 1 mg/kg, respectively.

***In Vivo* Animal Studies**—Four male Sprague–Dawley rats[3], 340–400 g, were used for the 4-mg/kg study; five rats, 200–250 g, were used for the 1-mg/kg study. The rats were maintained in stainless steel cages for 1–3 weeks prior to use and were fed standard rat food[4], with food and water allowed *ad libitum*.

For the pharmacokinetic study, the rats were anesthetized with urethan[2] (1 g/kg ip). The animals were then placed on an operating table maintained at 37°. Each animal then received the appropriate dose of thiamine hydrochloride administered intravenously *via* the dorsal vein of the penis. Serial blood sampling into 100-μl micropipets[5] (pretreated with an anticoagulant solution[6]) was from the severed tail vein at the appropriate times.

Sample Handling and Extraction—*Free and Total Whole Blood Assay*—Blood samples in the 100-μl micropipets were placed directly into 15-ml conical centrifuge tubes[7] containing 5 ml of 0.1 *N* HCl. The micropipets were then crushed to release the 100 μl of blood. After thorough vortex mixing[8], the centrifuge tubes were placed in a boiling water bath for 30 min to release membrane-bound free thiamine. The tubes and their contents were then cooled, 2 ml of 10% freshly prepared metaphosphoric acid[9] was added, and the contents were diluted to 10 ml in the calibrated tubes. The tubes were thoroughly mixed and allowed to stand for 10 min. They were then centrifuged[10], and 8 ml of the protein-free supernate was removed and placed in a 15-ml centrifuge tube containing 0.6 ml of 2.5 *M* sodium acetate[11]. The contents of the tubes were then diluted to 10 ml with water.

To prepare samples for the total thiamine (free thiamine plus its phosphorylated esters) assay, an aliquot of the above final solution was incubated overnight at 37° with 3 ml of a 2% amylase[12] suspension in a shaking water bath. If a plasma assay was desired, plasma, after separation from red blood cells, was treated in an identical manner to the whole blood.

Free and Total Thiamine Tissue Assay—Brain tissue was used as an example of tissue from which thiamine can be assayed. The excised brain of rats was maintained in refrigerated isotonic physiological buffer for 1–2 hr prior to assay. The brain was patted dry with tissue paper, and the wet weight was determined. The entire tissue was then placed in a calibrated homogenizing tube, heated on a boiling water bath for 30 min, homogenized in 10 ml of 0.1 *N* HCl, and then returned to the boiling water bath for another 15 min. A 5-ml aliquot of a 10% metaphosphoric acid solution was then added, and the suspension was diluted to 20 ml and allowed to stand for 10 min. The suspension was then centrifuged for 10 min, and 15 ml of the supernate was removed and placed in a 25-ml volumetric flask containing 1.1 ml of 2.5 *M* sodium acetate. The solution was diluted to 25 ml with water.

To prepare the sample for total thiamine determination, 8 ml of the 25-ml solution was added to 5 ml of a 2% amylase[12] suspension and incubated overnight at 37° in a shaking water bath. On the following day, this solution was diluted to 25 ml with pH 4.5 acetate buffer, and 8 ml of this solution was used for further analysis.

Standards Preparation—For the 4- and 1-mg/kg iv thiamine hydrochloride studies, three 100-μl blood samples were drawn prior to injecting thiamine hydrochloride. These samples were treated identically to postdosing samples, except that 100 μl of diluted stock thiamine hydrochloride solution was added to the centrifuge tubes containing the 5 ml of 0.1 *N* HCl to give concentrations of thiamine in the expected concentration range to be assayed. Similar procedures were used for preparing standard curves for tissue samples.

Thiamine Isolation and Oxidation Step—The blood and tissue extracts were applied to columns prepared with a cation-exchange resin[13]. (See Ref. 9 for a description of column preparation and elution techniques.) After eluting with acidified potassium chloride, 5 ml of the collected 25-ml eluate (8 ml for the 1-mg/kg study and for brain samples) was slowly released into a 50-ml conical centrifuge tube[3] containing 3 ml of an aqueous solution, which was 0.03% in potassium ferricyanide and 15% in sodium hydroxide. The solution was mixed continuously on a vortex mixer during the addition. Immediately after the addition was complete, 15 ml of isobutyl alcohol[14] was rapidly added *via* an automatic pipet[15]. The two-phase system was vigorously mixed on a vortex mixer for 90 sec and then centrifuged for 60 sec. The isobutyl alcohol fraction was removed immediately after the centrifugation step.

Detection Methods—*Fluorometric Analysis*—Approximately 9 ml of the isobutyl alcohol fraction was collected and dried over ~2 g of anhydrous sodium sulfate. The sample was clarified by centrifuging for 5 min at 1500 rpm. An aliquot of the dried isobutyl alcohol was analyzed fluorometrically[16] at 370 (excitation wavelength) and 420 (emission wavelength) nm. The excitation and emission slit widths were adjusted to 10 nm, and the instrument was used in the ratio record mode. Blanks for fluorometric analysis were of the same composition as the oxidized samples, but 3 ml of 15% NaOH was substituted for the 3 ml of potassium ferricyanide–sodium hydroxide solution.

The relative intensity of the sample due to thiochrome (and thiamine) was calculated by subtracting the blank intensity from the sample intensity. The relative intensity was then related to the concentration of endogenous and/or exogenous thiamine in micrograms per milliliter of blood or plasma or micrograms per gram of fresh brain *via* the standard curve, which was linear and was resolved by least-squares analysis[17].

Liquid Scintillation Detection—Five milliliters of the isobutyl alcohol extract was pipetted into a scintillation vial[18] containing 10 ml of an appropriate scintillation solvent[19]. The vial was stored in a dark cool place for 24–48 hr prior to counting. Radioactivity counting was performed in a liquid scintillation counting system[20] using the carbon-14 and tritium optimized window. External standard ratio was recorded to confirm counting efficiencies. Blanks as previously described also were used for the scintillation counting.

Relative counts per minute were calculated by subtracting the blank counts from the sample counts. The relative counts were related to the concentration of exogenous thiamine hydrochloride per milliliter of blood *via* the standard curve, which was linear and was resolved by least-squares analysis.

Procedure Modifications—The procedures described were used to determine free and total thiamine in whole blood, plasma, and brain tissue. The techniques for determining endogenous thiamine (free and total) were the same as described, except that the fluorometric assay obviously had to be used and 200-μl samples were needed for whole blood. Endogenous levels were estimated from standard curve plots of net relative fluorescence intensity[21] *versus* added exogenous thiamine using the standard addition technique. The fluorescence in the absence of added thiamine (corrected for solvent blank, *etc.*) was attributed to endogenous thiamine.

The procedures outlined were used for a study of thiamine pharmacokinetics after thiamine hydrochloride administration of 1–4 mg/kg. With doses greater than 4 mg/kg, a straight fluorometric assay could be used. The procedures were identical to those described, except that only cold thiamine hydrochloride was administered, standard curves were adjusted appropriately, and thiamine was determined fluorometrically.

Thiamine Pharmacokinetics Analysis—The pharmacokinetics of thiamine hydrochloride in rats were fit to a two-compartment model (4, 31, 32). This model predicts that the data for blood thiamine hydrochloride levels *versus* time in rats after rapid intravenous injection should show a biexponential decay. The decay curve should be described by:

$$C_B = Ae^{-\alpha t} + Be^{-\beta t} \qquad \text{(Eq. 1)}$$

[3] ARS/Sprague–Dawley, Madison, Wis.
[4] Wayne Lab-Blox, Allied Mills, Chicago, Ill.
[5] Corning microsampling glass disposable capillary pipets, Scientific Products, Kansas City, Mo.
[6] Edetate disodium, 10% aqueous solution, Fisher Scientific Co., Fair Lawn, N.J.
[7] Pyrex tubes, No. 8062.
[8] Vortex Genie, Scientific Products, McGaw Park, Ill.
[9] Mallinckrodt Chemical Works, St. Louis, Mo.
[10] Dynac II centrifuge, Clay-Adams, Parsippany, N.J.
[11] J. T. Baker Chemical Co., Phillipsburg, N.J.
[12] Diastase, a nonspecific enzyme mixture, also known as Clarase, used to cleave phosphorylated thiamine to thiamine, Fisher Scientific Co., Fair Lawn, N.J.
[13] Decalso, an artificial zeolite available through Fisher Scientific Co., Fair Lawn, N.J. Some lots showed low recoveries. New batches should be thoroughly tested.
[14] Certified 99 mole % pure. Fisher Scientific Co., Fair Lawn, N.J.
[15] Repipet, Labindustries, Berkeley, Calif.
[16] Hitachi Perkin-Elmer model MPF-2A spectrofluorometer, Perkin-Elmer Corp., Norwalk, Conn.
[17] Hewlett-Packard model 9810A.
[18] LSC vials, 20-ml capacity, Wheaton Scientific, Millville, N.J.
[19] Aquasol, New England Nuclear, Boston, Mass.
[20] LS-150, Beckman, Fullerton, Calif.
[21] Net relative fluorescence intensity (that due to added thiamine only) equals total relative fluorescence intensity minus (relative fluorescence intensity due to endogenous thiamine plus background).

Table I—Endogenous Free and Total Thiamine Levels in Fresh Excised Rat Brain Determined Fluorometrically

Parameter	Present Study (n), $\mu g/g \pm SEM$	Literature Values (References), $\mu g/g$
Free thiamine in fresh brain	0.186 ± 0.101 (7)	0.26–0.40 (35–37)
Total thiamine in fresh brain	3.30 ± 0.12 (5)	3.0–3.1 (37, 38)

where C_B is the blood level at any time, t, and A, α, B, and β are parameters that were adequately described elsewhere (31, 32). The data for each animal and each analytical method were fit to Eq. 1 utilizing the simplex optimization method[22] (33, 34) for nonlinear curve fitting. The mean values for these parameters using each analytical method were determined and statistically compared using the Student t test. The mean values for A, α, B, and β were used to generate the lines representing mean blood levels *versus* time decay curves for thiamine hydrochloride in rats.

RESULTS AND DISCUSSION

The thiochrome fluorometric technique measures the total fluorescence of a particular sample. This fluorescence has contributions from exogenous as well as endogenous thiamine. Endogenous and/or exogenous thiamine is determined by direct extrapolation from a plot of net relative fluorescence intensity[21] *versus* added thiamine. The liquid scintillation assay described, on the other hand, measures only exogenous thiamine.

Normal endogenous levels of free and total thiamine levels in excised rat brains were determined using the described fluorometric assay. The values found in the present study (Table I) compare favorably with previously reported values (35–38).

These procedures (fluorometric and liquid scintillation detection) required considerable time and effort. Numerous procedures that purport to shorten the assay time for thiamine were attempted. For example, a recently published procedure (11) was attempted but gave inconsistent as well as low fluorescence readings compared to the described procedure.

Variables (6, 39) affecting the sensitivity and reproducibility of the fluorometric procedure require attention to reagent purity, preparation, and storage. Of particular importance were the purity of the isobutyl alcohol and the mesh size of the cation-exchange resin.

The isobutyl alcohol used[14] was satisfactory because it gave low background fluorescence. Other sources of isobutyl alcohol obtained were not satisfactory. Attempts made to purify and recycle the isobutyl alcohol using the procedure described by Friedemann and Kmieciak (8) failed. The purification of the used isobutyl alcohol was not only time consuming but the isobutyl alcohol still gave high and unsatisfactory reagent fluorescence after purification. Similarly, some lots of the cation-exchange resin gave low recoveries of thiamine after elution. It was determined that the mesh size of the unsatisfactory lots was below the optimum required for efficient elution. New lots of the cation-exchange resin were tested for their exchange efficiency prior to routine use.

Timing was critical in the oxidation step. The time schedule must be strictly followed to obtain both consistent and reproducible results. Throughout the assay, reagents and working solutions must be protected from dust and other particulates.

Liquid scintillation samples counted immediately after the addition of isobutyl alcohol to the scintillation cocktail showed somewhat inconsistent results. This problem was traced to the presence of small amounts of aqueous sodium hydroxide carryover into the isobutyl alcohol, leading to the phenomenon of chemiluminescence. The increase in counts due to chemiluminescence was overcome by storing the samples for 1–2 days in a cool, dark environment prior to counting.

The pharmacokinetics of thiamine administered at 4 mg/kg iv to rats were previously fit to a two-compartment model (4). In the present experiment, administration of a 4-mg/kg iv thiamine hydrochloride dose was found to follow two-compartment model behavior over the limited time period studied. The two-compartment model predicts that the thiamine blood level *versus* time curve in rats after a rapid intravenous injection should show a biexponential decay and be described by Eq. 1.

[22] The program was developed by Dr. W. White and various graduate students in the Department of Pharmaceutical Chemistry, University of Kansas. The program was run on a Hewlett-Packard model 2100 computer. For copies of the program and further information, contact Dr. W. White.

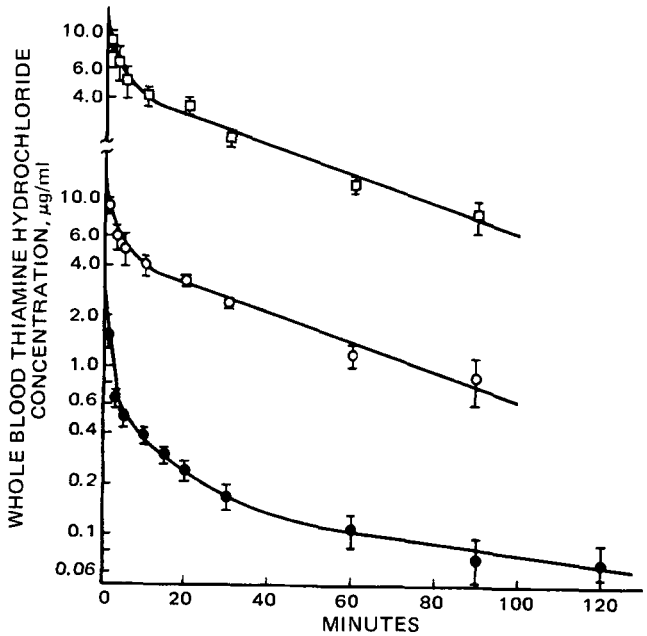


Figure 1—*Time course of whole blood concentration* ± SEM *of thiamine hydrochloride in rats administered 1- and 4-mg/kg iv doses of thiamine hydrochloride. Each point is the mean of four (4 mg/kg) or five (1 mg/kg) animals. The analytical procedures were the liquid scintillation assay (●, 1 mg/kg; and ○, 4 mg/kg) and the thiochrome fluorometric assay (□, 4 mg/kg). Lines for the 4-mg/kg dose were generated utilizing the pharmacokinetic parameters in Table II.*

Figure 1 shows the blood level *versus* time plots for exogenous thiamine decay in rats administered 4 mg/kg iv. Table II lists the pharmacokinetic parameters derived from the nonlinear curve fitting of the blood level–time plots for both the fluorometric and liquid scintillation procedures. The mean values were compared using the Student t test for the two detection methods. Table II also lists the calculated t values and the appropriate t values at the $2p = 0.05$ significance level and the indicated degrees of freedom.

The mean parameters for the fluorometric assay did not differ significantly from the mean parameters using the liquid scintillation procedure at the significance level indicated. The solid lines in Fig. 1 were drawn by a fit to Eq. 1, utilizing the mean values of A, α, B, and β determined for each detection method.

The liquid scintillation technique was used in following the whole blood

Table II—Pharmacokinetic Parameters for the Apparent Disappearance of Thiamine Hydrochloride from Whole Blood in Rats Administered a 4-mg/kg iv Dose of Thiamine Hydrochloride

Animal	A, μg/ml	α, min^{-1}	B, μg/ml	β, min^{-1}	$t_{1/2}$, min
	Fluorometric Assay				
1	6.90	0.258	6.44	0.0315	22
2	8.42	0.494	4.30	0.0192	36
3	6.72	0.666	3.83	0.0172	40
4	7.94	0.217	4.32	0.0164	42
Mean	7.50	0.41	4.72	0.0211	35
SEM	0.41	0.10	0.58	0.0035	
	Liquid Scintillation Assay				
1	7.22	0.369	6.83	0.0336	21
2	9.13	0.524	4.67	0.0226	31
3	7.90	0.710	3.53	0.0133	52
4	7.36	0.180	3.79	0.0138	50
Mean	7.90	0.45	4.70	0.0208	39
SEM	0.43	0.11	0.75	0.0048	
t statistic	0.683	0.240	0.018	0.042	
Degrees of freedom (df)	6	6	6	6	
$t_{0.05}$[a], $df = 6$	2.447	2.447	2.447	2.447	

[a] Two-tail criterion, significance level $2p = 0.05$.

time profile of exogenous thiamine after administration of a 1-mg/kg iv dose of ^{35}S-thiamine hydrochloride (Fig. 1). The fluorometric procedure could not have been used to follow the time profile of exogenous thiamine blood levels, because considerable data in the blood level–time profile would have been collected at levels that would have fallen below accurate detection by the fluorometric method. This result is a consequence of increasing reagent blank fluorescence intensity and increasing noise in the reagent blank and sample fluorescence intensity recorded signals. Of course, some of the profile could have been followed using the fluorometric method, and it could also have been used if larger blood samples were taken. However, the latter alternative would entail sacrificing individual animals at appropriate times. Consequently, much larger numbers of animals for each dose would have been required, as well as the loss of proper controls available with serial blood sampling over an extended period from single animals.

The lower level of detection for thiamine for 100-μl blood samples using the fluorometric assay is 0.3 μg/ml, while the lower level of detection using the liquid scintillation assay (using ^{35}S-thiamine of specific activity 233 mCi/mmole) is 0.05 μg/ml. It appears from Fig. 1 that thiamine whole blood pharmacokinetics is dose dependent. These differences will be discussed in a later paper where data for the time profile of thiamine hydrochloride in the dose range of 1–36 mg/kg will be presented.

In conclusion, the advantages of the liquid scintillation assay are its ability to determine exogenous thiamine in the presence of endogenous thiamine and the low level of detection it affords. This last point, of course, will be dependent on the specific activity of ^{35}S-thiamine that can be purchased or prepared.

Another important advantages of the liquid scintillation assay is that once the thiamine (free and/or total) has been isolated *via* oxidation to thiochrome and extracted into the isobutyl alcohol layer, the labeled samples can be counted at the investigator's convenience (keeping in mind the relatively short half-life of sulfur-35 of 87.2 days). In the fluorometric assay, fluorescence intensity from isobutyl alcohol-extracted thiochrome deteriorates with time. Chemical quenching and chemiluminescence with the liquid scintillation assay can be problems if unrecognized. Balanced against the advantages of the liquid scintillation are disadvantages of not being able to determine endogenous thiamine directly and the added expense of purchasing the ^{35}S-thiamine.

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