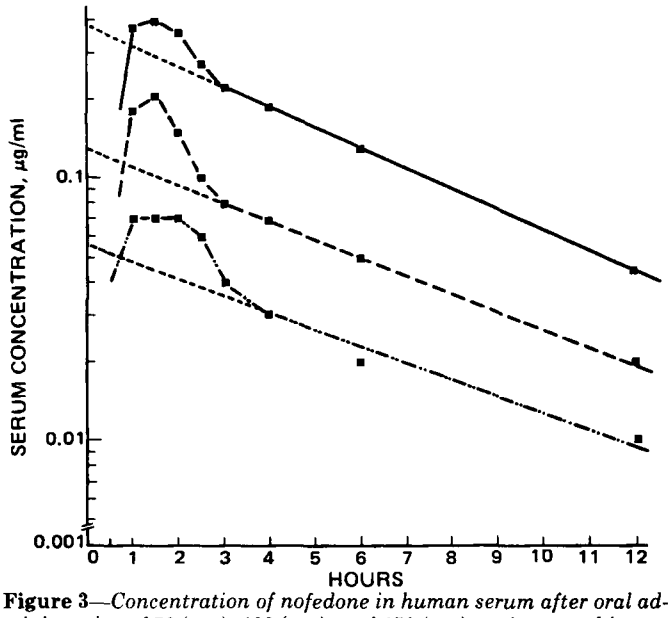

Figure 3—*Concentration of nofedone in human serum after oral administration of 50 (-·-·-), 100 (- - -), and 150 (——) mg in one subject.*

extract had the same chromatographic behavior as the authentic compound. The minimum detectable quantity was 100 pg, and the linearity of the ^{63}Ni-electron-capture detection response was observed from 0.5 to 2 ng in the organic phase injected (attenuation $4 \times 2.5 \times 10^{-10}$).

The theoretical sensitivity initially was 10 ng/ml, but it improved during use. The sensitivity limit for the quantitative determination of I in serum by electron-capture detection was 5 ng/ml of serum after a 2-ml sample extraction. We observed, as did Leitch *et al.* (3), that the sensitivity increased with the number of plasma extracts injected. At the beginning of the GLC analysis, plasma extracts were injected to obtain the plateau of best sensitivity. This plateau was maintained by alternating injections of serum extracts and reference samples. The apparent recovery of I compared with that of the internal standard and the accuracy of the results obtained are shown in Table I. The determinations were repeated seven times for each quantity. The last column in Table I shows the validity of the method. A plot of added drug *versus* found drug had a slope of unity. According to the data provided, a single determination is expected to be within about 12% of the true value, and multiple determinations should vary by ±12% (reproducibility).

The chromatographic analysis of the serum blanks showed that no compound extracted under the conditions described interfered with I or the internal standard (II) (Fig. 1). TLC of derivatized extracts from serum taken from patients treated with nofedone showed that there was no interfering substance with a close R_f value. In the urine of treated patients that contained metabolites, the concentrations of nofedone in the form of bis-derivatives were the same as those measured after elution of a silica plate and GLC analysis.

The sensitivity of this technique allowed determination of serum levels in humans for ~9 hr after an intravenous bolus injection (1 mg/kg in 2 min) and for 12 hr after a single oral dose (50–150 mg in a capsule or tablet). Figures 2 and 3 show the concentrations of nofedone in human serum after an intravenous bolus injection of 1 mg/kg and after an oral dose of 50, 100, and 150 mg in a capsule in another subject. Pharmacokinetic data can be derived from these distribution values. The selectivity of this method allows determination of the compound in saliva, urine, and feces.

Isopentyl alcohol was used because it prevents the molecule from adhering to the glass walls during extraction, derivatization, and GLC analysis. It also prevents tailing peaks and thus increases sensitivity. For serum concentrations below 50 ng/ml, optimal stability of the chromatograph is required to minimize background noise. For higher concentrations, the method provided good reproducibility and allowed calculation of pharmacokinetic parameters up to 9 hr following an intravenous bolus injection (1 mg/kg) and up to 12 and 24 hr after oral doses of 50, 100, and 150 mg.

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ACKNOWLEDGMENTS

The authors thank Mr. C. Cotrel, Centre Nicolas Grillet, Rhône-Poulenc, Vitry, France, and Mr. C. Saturnin and Mrs. M. Besenval, Institut de Biopharmacie, Rhône-Poulenc, Vitry, France, for technical assistance; and Mrs. M. Eiglier, Rhône-Poulenc Santé, Paris, France, for English translation.

Fluorometric Determination of Thiazole-Containing Compounds

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Received August 9, 1979, from the *Merck Sharp & Dohme Research Laboratories, Rahway, NJ 07065.* Accepted for publication October 11, 1979. *Present address: Ethicon, Somerville, NJ 08876.

Abstract □ Fluorescence spectroscopy was applied to the development of sensitive analytical methods for the determination of thiazole and several congeners that contain substituted thiazole rings. Treatment to yield thionine, previously used spectrophotometrically to measure thiazole and fluorometrically only for sulfur determinations in inorganic systems, is further characterized and illustrated with the determination of the antibiotic thiopeptin. This method is selective for submicrogram quantities of thiazole rings in the presence of fused-ring derivatives and reduced analogs. It has a precision of ±2% *RSD* (n = 11) at the 15-ng/ml thiazole concentration level with a signal-to-noise ratio of 3:1. For thiopeptin, this method has an accuracy of 5% mean relative error (n = 8) over the 5–20-ppm range in medicated feed.

Keyphrases □ Thiazole—thiazole-containing compounds, fluorometry □ Fluorometry—analysis, thiazole-containing compounds □ Spectroscopy, fluorescence—thiazole-containing compounds

The thiazole ring is a common structural element found in many compounds exhibiting biological and therapeutic activity and in several molecules used in agricultural products as well as in pharmaceutical formulations (1, 2). Because of these properties, sensitive analytical methods for thiazole-containing compounds are of interest for application in low concentration level formulations, bioavailability studies, and environmental samples.

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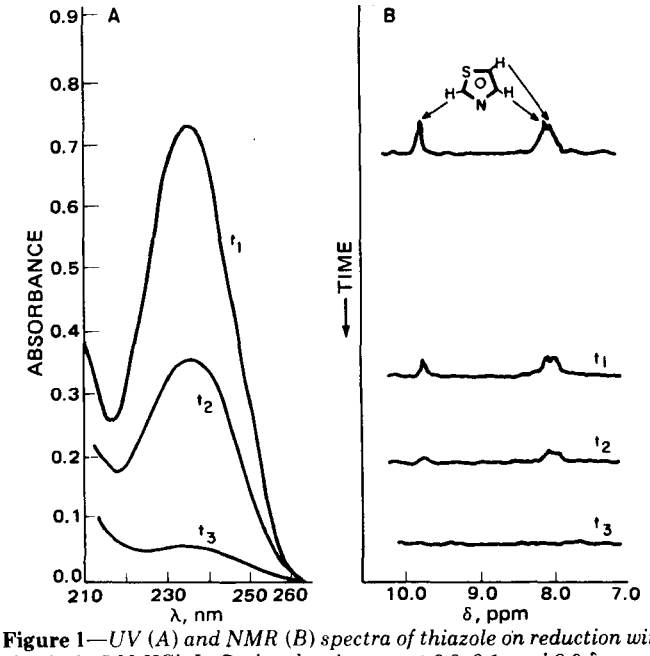


Figure 1—*UV (A) and NMR (B) spectra of thiazole on reduction with zinc in 0.15 N HCl. In B, the absorbances at 9.8, 8.1, and 8.0 δ are assigned to H(2), H(4), and H(5), respectively (20). This reaction was run with deuterium chloride in deuterium oxide.*

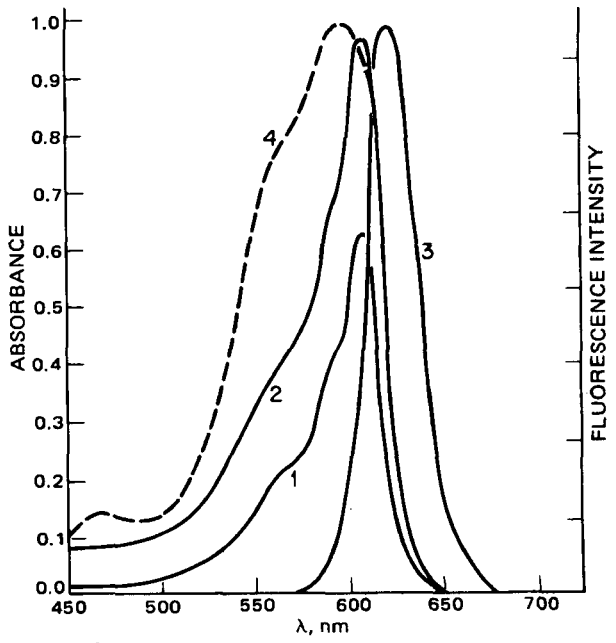


Figure 2—*Spectral properties of the thionine fluorophore in n-butyl alcohol. Key: 1, absorption spectrum of thionine, 10^{-5} M; 2, absorption spectrum obtained from treatment of thiazole according to the text (a solution containing 6 × 10^{-5} M thiazole was treated; the absorbance of this thionine reaction product represents a chemical yield of 26%); 3, uncorrected emission spectrum, λ_{excite} = 595 nm; and 4, uncorrected excitation spectrum, λ_{emit} = 620 nm.*

The condensation reactions of sulfides with *p*-phenylenediamine or its *N,N*-dimethyl derivative to yield thionine or methylene blue, respectively, have been known for many years. Most previous analytical applications of this reaction sequence primarily have been for spectrophotometric determinations of hydrogen sulfide (3, 4) and other sulfides (5, 6) and for sulfur in inorganic systems such as sulfate after reduction to sulfide (7). A prior inorganic application by luminescence measurement involved the determination of traces of sulfate and sulfite in semiconductors (8, 9).

This inorganic reaction sequence has had only one previous analytical application to the thiazole ring. In work in these laboratories, thiabendazole [2-(4-thiazolyl)-1*H*-benzimidazole] was determined spectrophotometrically by the generation of sulfide *via* pretreatment with zinc in acidic solution (10). The present report describes the extension of this technique to the development of a sensitive general analytical method for the determination of thiazole, substituted thiazoles, and other thiazole-containing compounds. The reaction is characterized further through the examination of model compounds, and its sensitivity is enhanced with the first application of fluorometric measurement instead of absorptiometric analysis for the organic system. Included is the determination of thiopeptin (an antibiotic that contains four thiazole rings) at the low parts-per-million concentration level.

EXPERIMENTAL

Apparatus—A spectrofluorometer[1] equipped with a xenon lamp and a 1P21 photomultiplier tube was used for all fluorescence measurements. The excitation and emission slit widths at the cell were both 1.0 mm, and a 0.2-mm slit was used at the entrance beam to the photomultiplier tube. The sample container was a fused quartz fluorescence cell, 10.5 mm i.d. square × 46 mm high.

All emission intensities were read on the microphotometer[1] and standardized relative to the fluorescence intensity (450 nm) of 1.0-ppm quinine sulfate in 0.1 *N* H_2SO_4 (350-nm excitation). Excitation and emission spectra were recorded on an *x–y* recorder[2] and are uncorrected for frequency dependence of source excitation intensity, photomultiplier output, or monochromators. Absorption spectra were recorded on a spectrophotometer[3].

Reagents and Solutions—Ferric ammonium sulfate[4], *n*-butyl alcohol[4], sodium sulfate[4], pyridine[4], *p*-phenylenediamine[5], zinc dust[6], thiazole[7], thiophene[8], thionine[5], thiabendazole[9], cambendazole[9], and the remaining substituted thiazoles[7] were used as received. Other chemicals were reagent grade.

Procedure—The fluorophore was developed according to the procedure described previously (10). To a 15-ml sample of thiopeptin in 0.15 *N* HCl were added 5.0 ml of 0.5-mg/ml aqueous *p*-phenylenediamine and 150 ± 5 mg of zinc dust, and the sample was mixed. After 7 min, 5.0 ml of ~0.65 *M* aqueous ferric ammonium sulfate was added, and the solution was mixed and allowed to remain at room temperature for 15 min.

The reaction vessel was placed in a water bath at 90 ± 3° for 20 min and then cooled in an ice bath for 5 min. An additional 2.0 ml of ferric ammonium sulfate solution was added, and the mixture was mixed again. After 30 min, ~5 g of sodium sulfate (anhydrous) and 5.0 ml of *n*-butyl alcohol were added, the phases were equilibrated for 2 min, and the fluorescence intensity of the *n*-butyl alcohol phase was measured at 620 nm under 475-nm excitation.

RESULTS AND DISCUSSION

In the present reaction sequence, thiazole was treated with zinc in acidic solution to yield an intermediate that might have been the result of two consecutive reactions, *i.e.*, zinc reduction of the ring followed by hydrolysis to sulfide. This unidentified product added 2 moles of *p*-phenylenediamine; in a ring-closing reaction with ferric ion as the oxidizing agent, the central ring was closed to yield thionine according to standard techniques. A boiling water bath was used for heating the reaction mixture. (When *N,N*-dimethyl-*p*-phenylenediamine was used, the product for colorimetric measurement was methylene blue.)

[1] Aminco-Bowman SPF, American Instrument Co.

[2] Aminco J4-8976, American Instrument Co.
[3] Cary model 15, Varian Associates.
[4] J. T. Baker.
[5] Eastman.
[6] Matheson, Coleman and Bell.
[7] Aldrich.
[8] Pfaltz & Bauer.
[9] Merck Sharp & Dohme.

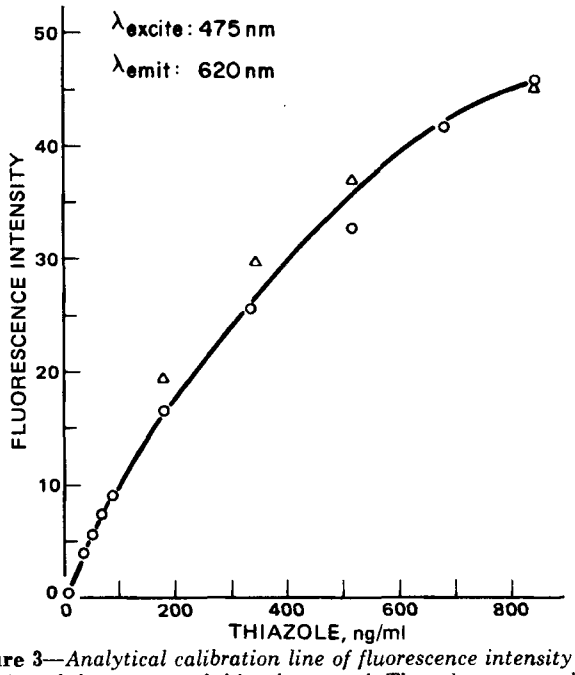


Figure 3—*Analytical calibration line of fluorescence intensity as a function of the amount of thiazole reacted. The solvent was n-butyl alcohol; λ_{excite} = 475 nm, λ_{emit} = 620 nm. Key: O, direct measurement; and Δ, dilutions of a more concentrated reaction product solution. For reaction conditions, see text.*

The identity of the thiazole reaction product was confirmed by chromatographic comparison with authentic thionine in three systems. The R_f values of the reaction product, neutral thionine, and the conjugate acid thionine hydrochloride on silica gel TLC plates[10] were 0.93, 0.91, and 0.17, respectively, with a mobile phase of *p*-dioxane–water (5:1); on silica gel RP-2 with a mobile phase of acetic acid–methanol (100:1), thionine appeared at R_f 0.70, and the reaction product obtained from thiazole appeared at R_f 0.72. This product also eluted with the same retention time as thionine from a silica gel high-pressure liquid chromatographic (HPLC) column[11] with acetic acid–methanol (0.1:200) as the mobile phase.

The first step of this reaction was monitored without the organic coupling reagent and the ferric oxidizing agent by treating thiazole with zinc in acidic solution (Fig. 1). As the reaction proceeded, the single-band, λ_{max} 236-nm electronic absorption spectrum of thiazole decreased in intensity, with no band shifts or new absorption transitions appearing. Eventually, the solution became transparent at λ greater than 210 nm, demonstrating a loss of aromatic π-character. Simultaneously, the intensity of the NMR[12] signals assigned to the three protons of thiazole also diminished and were ultimately lost, with no new NMR signals apparent.

Although proton exchange could have contributed to these lessening NMR signals with time, these results nevertheless were in accord with the UV absorbance spectral changes, confirming that this extremely stable heteroaromatic molecule was broken down by this treatment to furnish the sulfur for the succeeding condensation. This reaction was run with deuterium chloride in deuterium oxide[13]. The ratio of reducing agent to thiazole substrate can be regulated to govern the reaction rate; when microgram quantities of thiazole were treated with milligram quantities of zinc, the reaction proceeded too rapidly to record the UV and/or NMR spectra.

The absorption spectrum of the thionine reaction product is included in Fig. 2. The Beer's law deviation (metachromatic effect) exhibited by this fluorophore has been attributed to self-association to form dimers (or higher polymers) in a stacking, sandwich equilibrium (11–13) that can occur at concentrations as low as 10^{-7} *M* (12). The observed 600-nm λ_{max} in Fig. 2 has been assigned to the monomer, with the dimer evident as the shoulder at 560 nm (12–14).

[10] Whatman LK5DF.
[11] Spectra-Physics model 740B pump, 740C controller, 714 pressure monitor, and SP 8400 UV detector (254 nm) with Spherisorb silica 5-μm column.
[12] Perkin-Elmer R24B.
[13] Merck Sharp & Dohme Canada Ltd.

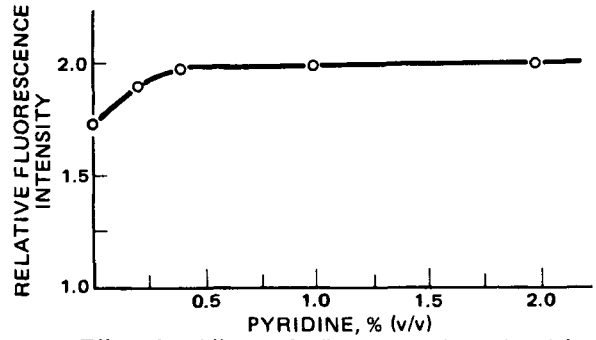


Figure 4—*Effect of pyridine on the fluorescence intensity of the thionine reaction product. The solvent was n-butyl alcohol; λ_{excite} = 475 nm, λ_{emit} = 620 nm. The product was obtained from a solution containing ~0.5μg of thiazole/ml. For reaction conditions, see text.*

The fluorescence spectrum of thionine in *n*-butyl alcohol (Fig. 2) has an emission maximum (uncorrected) at 620 nm, similar to the fluorescence in aqueous and in methanolic solution (13, 15). At least three features of these spectral properties attenuate the analytical signal:

1. The very small Stokes shift and overlap of the absorption and emission spectra cause self-absorption of emitted radiation.
2. Because of the high absorptivity of thionine in butyl alcohol ($\epsilon \sim 6.3 \times 10^4$ liters/mole-cm at 605 nm), the sample is excited at 475 nm so that absorbance at the excitation wavelength is low (Fig. 2) to reduce excitation energy loss near the entrance surface of the cell.
3. Dimerization causes a Beer's law deviation.

Figure 3 presents the fluorescence intensity calibration line as a function of the amount of thiazole treated. This line exhibits the usual attenuation at high concentrations predicted from these spectroscopic properties, although it does furnish a convenient linear range for dilute solutions. Included in Fig. 3 are fluorescence measurements of solutions obtained from dilutions of a more concentrated solution of the reaction product, as in the similar characterization of the Beer's law calibration line by Gustafsson (7). In accordance with the absorbance results, the fluorescence intensity from these solutions near the calibration line confirms that the attenuation results from optical and equilibrium effects and is not the result of decreased reaction yield with larger amounts of thiazole.

Because thionine is not fluorescent in its associated state (12, 13), this dimerization, which also would attenuate the analytical signal, should be avoided over the range of analytical calibration concentrations. To investigate the aggregation of thionine, pyridine was added to the solution to perturb the dimerization equilibrium; similar studies with methylene blue demonstrated that pyridine eliminates the self-association (16). Because of the structural similarity of pyridine to these thiazines, this nitrogen heterocycle can associate with the thionine molecules and thus dissociate the sandwich dimers.

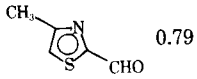
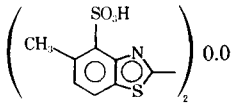
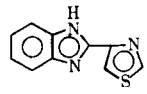
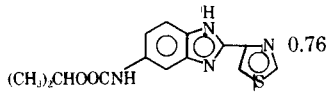
Figure 4 illustrates the small effect pyridine exerts on this system. The increase in fluorescence intensity is only ~15%, suggesting that there is no significant dimerization in this solvent. (Most reports of dimerization have been for aqueous, not alcoholic, solutions.) Moreover, these data also demonstrate that the analytical signal cannot be significantly enhanced by this technique.

Structural Effects—Table I lists a series of model compounds that were examined by this fluorometric procedure using the same reaction conditions. The first column lists single-ring substrates with the fluorescence intensity obtained from thiazole. Thiazole *per se* is not fluorescent (17). Saturation of the aromatic ring inhibits the reaction, as demonstrated by the negative response from thiazolidine. In this aromatic system, the nitrogen heteroatom is necessary for the reaction to proceed; thiophene does not yield the sulfide under these conditions.

The second column of Table I demonstrates that fusing thiazole with a benzene nucleus also inhibits the reaction; neither benzothiazole nor its indicated derivative produced any fluorescence at 620 nm. Each monosubstituted and disubstituted thiazole in Table I gave approximately equimolar (±25%) fluorescence intensity with the parent compound. These compounds are each substituted only in the 2- and/or 4-positions.

Analytical Application—The last disubstituted entry in Table I provides an example of a thiazole-containing compound that was determined by the present method. Thiopeptin is an antibiotic administered to swine and chickens at 10–20 g of drug/ton of feed. The structure of this fermentation product recently was elucidated (18). Thiopeptin has seven

Table I—Relative Fluorescence Intensity from Some Model Compounds[a]

Single Rings	Fused Rings	Monosubstituted	Disubstituted
 1.0	 0.0	 1.03	 0.79
 0.0	 0.0	 0.89	Thiopeptin 0.76/thiazole
 0.0		 0.84	
		 0.76	

[a] Reaction conditions optimized for thiopeptin; see text.

ring systems, including a partially reduced quinoxaline moiety, one piperidine ring, and five five-membered nitrogen–sulfur heterocyclic rings, one of which is a thiazoline nucleus. The remaining four heterocyclic rings are thiazole rings, each bound to the carbon–nitrogen–oxygen skeleton at the 2- and 4-positions.

Because the present reaction sequence is not stoichiometric, it must be optimized (7). Figure 5 presents the effects of four factors on the yield of thionine from thiopeptin, monitored by the absorbance of the fluorophore. Optimal levels for each factor were selected from these data: 2.5 mg of *p*-phenylenediamine was used to minimize the signal from the reagent blank, the amount of zinc and the reaction time were selected at points on the plateaus of each function, and the reaction medium was selected at the peak response for the effect of pH (*i.e.*, 0.15 *M* HCl). The residuals of the linear least-squares calibration line of $A_{605\ nm}$ obtained

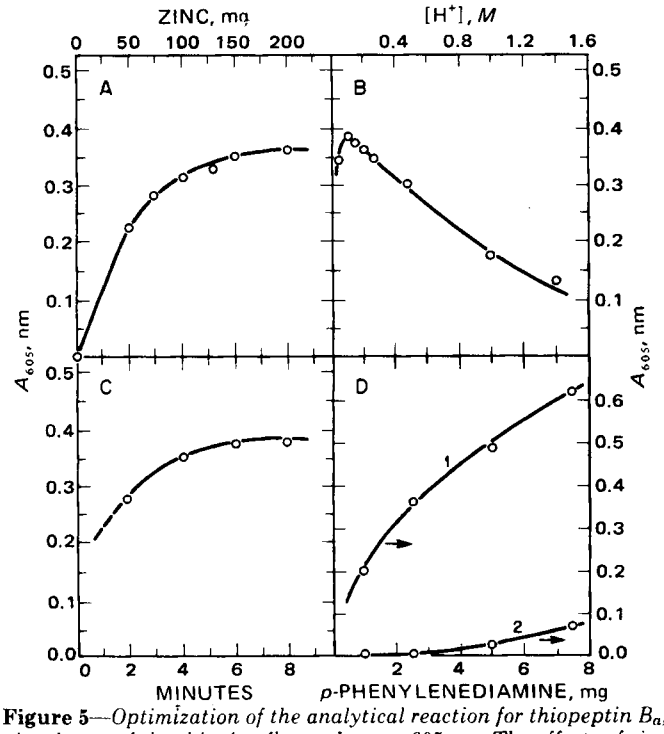


Figure 5—*Optimization of the analytical reaction for thiopeptin B_a; absorbance of the thionine fluorophore at 605 nm. The effects of zinc (A), [H+] (B), time (C), and reagent (D) are shown. Each sample contained ~30 μg of thiopeptin B_a in 15 ml of hydrochloric acid. Zinc, 150 mg, was used in B, C, and D; the reaction mixture was heated 15 min in A, B, and D; 2.5 mg of* p*-phenylenediamine was used in A, B, and C; 0.1 N HCl was used in A; and 0.15 N HCl was used in C and D. In D, line 1 presents the data from thiopeptin and line 2 is the reagent blank response. Each point in this figure is the mean of two, three, or four replicate measurements.*

Table II—Representative Analytical Results for the Determination of Thiopeptin B_a in Feed

Thiopeptin Added, ppm	Fluorescence Intensity	Thiopeptin Found ppm	Thiopeptin Found Relative %
5.0	4.35	5.0	100
	4.30	4.8	96
10.0	5.50	10	100
	4.95	7.6	76
15.0	7.10	17.0	113
	6.45	14.1	94
20.0	7.70	19.6	98
	7.10	17.0	85
Mean			95
Reagent blank	3.20	—	—
Unmedicated feed	3.45	1	—
	3.70	2	—
Analytical standard	5.90	—	—
	5.70	—	—
	5.70	—	—
	5.70[a]	—	—
	5.30[a]	—	—

[a] Processed through the separation scheme.

from seven samples covering the range of 2–15 μg of thiopeptin B_a[14] treated by this procedure averaged 2.9% relative to the line, demonstrating the precision possible with this method.

In the animal feed sample analyzed, thiopeptin was only a trace ingredient in a poorly defined complex matrix. An efficient isolation scheme was required to separate the drug from any other thiazole rings. Furthermore, in addition to thiazole-containing extraneous materials, protein that contains sulfur can also be a significant interference. For instance, these same reactions were used previously for cysteine determinations (19). In the present study, the drug was extracted from the feed into ethyl acetate in the presence of a pH 7 aqueous buffer; the aqueous phase was discarded, and the ethyl acetate extract was charged to the top of a magnesia–silica gel[15] adsorption column. After interfering substances were removed using successive elutions of increasing polarity with petroleum ether, ether, and acetone, the drug was eluted with 5% acetic acid in methanol. The sample was evaporated to dryness under nitrogen and taken up in the 0.15 *N* HCl reaction medium for the fluorogenic reaction, and the thionine fluorophore was partitioned into *n*-butyl alcohol for measurement.

Table II presents some representative analytical results obtained with this procedure. The range of fluorescence intensities from the five analytical reference standards illustrates the precision of replicate measurements. Furthermore, the comparison between the reaction of a pure thiopeptin solution with the same pure solution processed through the analytical isolation demonstrates that there is no loss of thiazole through

[14] Thiopeptin consists of several components, each containing four thiazole rings (18).

[15] Florisil, 100–200 mesh, Floridan Co.

this treatment. The unmedicated feed samples produced fluorescence representing 1–2 ppm of thiopeptin, furnishing sufficient selectivity to determine this drug at the 5-ppm level with an intensity 33% greater than the reagent blank. The mean of the eight analyses covering the 5–20-ppm range in the feed was 95% of the known thiopeptin concentration that was added to the feed. These results demonstrate the use of this treatment for the determination of one thiazole-containing analyte and suggest that this method also should be applicable to other analogous thiazole-containing compounds of pharmaceutical and biological interest.

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ACKNOWLEDGMENTS

Presented in part at the 13th Middle Atlantic Regional Meeting, American Chemical Society, West Long Branch, N.J., March 1979.

Decreased Activity of Proteins Adsorbed onto Glass Surfaces with Porous Glass as a Reference

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Received April 9, 1979, from the *Faculty of Pharmaceutical Sciences, Nagoya City University, Mizuho-ku, Nagoya 467, Japan.* Accepted for publication October 15, 1979.

Abstract □ The biological activity of proteins bound to controlled-pore glass surfaces was studied as a model of denaturation of biologicals upon storage in glass containers. After adsorption onto the glass for 1 week, the activities of alkaline phosphatase, catalase, and horse-radish peroxidase recovered from the glass column were 88, 63, and 97%, respectively. However, the phosphatase activity recovered after adsorption for 3 months was 14% of the total activity loaded onto the column, and the activities recovered of peroxidase and catalase were 48 and 2%, respectively. Insulin had almost full activity after adsorption for 3 months, but calcitonin activity was absent. The scission of peptide bonds of proteins eluted after adsorption for 3 months was not observed, but dissociation to the subunits was found. The proteins were active in the state adsorbed onto glass surfaces.

Keyphrases □ Adsorption—proteins, glass surfaces, activity decrease □ Proteins—adsorption onto glass surfaces, activity decrease □ Activity—proteins, decrease after adsorption onto glass surfaces

The reaction of biological materials on glass is well known (1–4). By use of controlled-pore glass (5) with a large surface area (97 m^2/g), it was shown that 5 μmoles of basic drugs and materials was adsorbed onto 97 m^2 of glass surface and that adsorption of proteins onto the surfaces was caused by amine–silanol bonding and a cooperative aggregative factor between silica and proteins (6–10). Proteins also were adsorbed in detergent solutions such as urea and guanidine hydrochloride (11). From the value of the maximum amount of protein adsorbed (233 mg/97 m^2), the amount of protein adsorbed onto the surfaces of a glass container (50 cm^2, 20 ml) and a glass injector was estimated to be >12 μg. The 12-μg/20 ml concentration must be a marginal point not affected by adsorption, and the biologicals used at lower concentrations would be affected by adsorption to glass surfaces (10).

Some biologicals composed of proteins are stored or maintained in pharmaceutical glass containers in a water medium (12). However, denaturation of proteins by their adsorptive aggregation on surfaces of glass containers upon storage for long periods is not clear. The purpose of this investigation was to show changes in the biological activity and the structure of proteins adsorbed onto glass surfaces upon storage as a model of denaturation of protein biologicals in glass containers. Enzymes and hormones were studied, and controlled-pore glass was used as a reference standard.

EXPERIMENTAL

Materials and Column Operation—The controlled-pore glass[1] was 96% silica and had a surface area of 97 m^2/g and a particle size of 100 μm. After being washed with water, 0.1% sodium dodecylsulfate, water, chromic acid mixture, and water, the glass was packed in columns (0.6

[1] CPG-10, Electro-Nucleonics, Fairfield, N.J.

0022-3549/80/0300-0279$01.00/0