(2) L. J. Honkomp, R. P. Halliday, and F. L. Wessels, *Pharmacologist*, 12, 201(1970).

(3) K. O. Ellis and J. F. Carpenter, *Fed. Proc.*, 30, 670(1971).

(4) R. D. Hollifield and J. D. Conklin, *Arch. Int. Pharmacodyn. Ther.*, 174, 333(1968).

(5) P. L. Cox, J. P. Heotis, D. Polin, and G. M. Rose, *J. Pharm. Sci.*, 58, 987(1969).

(6) R. Herman, N. Mayer, and S. Mecomber, *Amer. J. Phys. Med.*, in press.

ACKNOWLEDGMENTS AND ADDRESSES

Received June 5, 1972, from the *Unit of Drug Distribution, Eaton Laboratories, Norwich Pharmacal Co., Norwich, NY 13815*

Accepted for publication September 22, 1972.

The authors express their appreciation to T. Schwan and R. White, Jr., for providing Metabolite A and to D. Barry for the solvent admixture used in the fluorometric measurement of acetylated dantrolene.

▲ To whom inquiries should be directed.

DRUG STANDARDS

Determination of Liothyronine and Thyroxine in Dried Thyroid by GLC

ROMAN BILOUS▲ and JOHN J. WINDHEUSER*

Abstract—The analysis reported here was successful in the quantitative determination of liothyronine and thyroxine in driedthyroid. Three basic steps were employed: (*a*) hydrolysis of the dried thyroid to release the iodoamino acids from their protein linkage, (*b*) separation of liothyronine and thyroxine from the interfering substances by extraction and column chromatography, and (*c*) detection and quantitation by GLC.

Keyphrases □ Thyroid, dried—separation, GLC analysis, liothyronine and thyroxine □ Liothyronine and thyroxine—separation, GLC analysis in dried thyroid □ Thyroxine and liothyronine—separation, GLC analysis in dried thyroid □ GLC—analysis, liothyronine and thyroxine in dried thyroid

The thyroid gland contains a number of iodoamino acids, of which the principal hormones secreted are thyroxine and liothyronine. Small amounts of 3,3′,5′-triiodothyronine, diiodotyrosine, and monoiodotyrosine are also present (1). However, biological activity appears to be present only in thyroxine and liothyronine, with little or no activity in 3,3′,5′-triiodothyronine (2, 3). The original method of standardization was done by the determination of total organic iodine (4) or by the "thyronine" iodine content determination of Harington and Randall (5). Although this method is highly nonspecific, it is still being utilized, with slight modifications, as the official USP XVIII method.

GLC appeared to hold a great deal of promise in the determination of thyroid hormones. The results would be specific and would differentiate between the compounds found useful in therapy, namely liothyronine and thyroxine. Other compounds of lesser activity such as 3,3′,5′-triiodothyronine would also be separated and quantitatively determined.

The high molecular weight and the lack of volatility necessitated the formation of a volatile derivative. Derivatives prepared by Stouffer *et al.* (6) and Richards and Mason (7) required a two-step reaction. Zimmerer and Grady (8) used a one-step method to form methyl derivatives by means of diazomethane, but their derivatives eluted as a pair of peaks for both liothyronine and thyroxine and this did not appear suitable for our approach. A one-step derivatization method using a silylating agent appeared to be the method of choice. Although Zimmerer and Grady (8) noted the difficulty in obtaining reproducible silylation, this was not noticed in our work when suitable precautions were taken. Since thyroxine and liothyronine are found bound in a peptide linkage to thyroglobulin, which is present in the colloid material in the follicles of the thyroid gland (1), it is necessary to break this peptide linkage either chemically or enzymatically to obtain the free iodoamino acids prior to GC.

Some authors used alkaline hydrolysis to break the peptide linkage (9–11) while others used various enzymes to achieve the same result (12–15). Generally, the method involved the refluxing of dried thyroid with various bases or the utilization of a number of proteolytic enzymes. The hydrolysates, obtained by either method, were extracted with 1-butanol.

However, the quantitation of liothyronine and thyroxine in the 1-butanol extract by GLC was not feasible due to the presence of interfering substances

which necessitated further purification of the extract. It was reported in a previous article (16) that the interference was probably due to charring of nonvolatile material on the GLC column.

To accomplish this purification, a number of different methods were used: column chromatography (14, 17–22), paper chromatography (10, 12, 13, 15), TLC (11, 23), and gel filtration (24). Although all methods achieved limited success, they were still not totally specific for liothyronine and thyroxine. Quantitation was based on the determination of iodine from the separated fractions. This approach suffered from the obvious disadvantage of being only partially specific, because iodine could come from physiologically active and nonactive iodoamino acids.

The clean separation of the physiologically active thyroid hormones from the other constituents, present after hydrolysis and extraction, is the main problem that has defied many workers in this field. Without this separation, the specific determination of the amounts of liothyronine and thyroxine in dried thyroid is difficult to achieve.

Although GLC has been successfully used for synthetic mixtures of iodoamino acids, it has not been successfully applied to dried thyroid (6, 8, 24–30). This article reports the successful separation and quantitation of liothyronine and thyroxine from dried thyroid by GLC.

EXPERIMENTAL[1]

Reagents and Chemicals—The following were used: thyroxine (T_4, 3,5,3′,5′-tetraiodothyronine) recrystallized from 80% (v/v) aqueous ethanol and 5 ml. 2 *N* hydrochloric acid (25); liothyronine (T_3, 3,5,3′-triiodothyronine) USP reference standard; 3,3′,5′-triiodothyronine[2] (T_3', reverse T_3, or T_r-iso); Sephadex G-10[3]; dimethyl silicone[4]; diatomaceous earth[5], silane treated, 60–80 mesh; and *N,O*-bis(trimethylsilyl)acetamide[6]. All other chemicals were reagent grade.

Solvents and Solutions—All solvents were of reagent grade or as noted. The following were used: pyridine, anhydrous, reagent grade, distilled and stored over potassium hydroxide, b.p. 113–115°; 1-butanol, redistilled, b.p. 114°; ammonia–methanol solution, 5% v/v: ammonium hydroxide in anhydrous methanol; aqueous ammonia–methanol solution, 10% v/v: ammonium hydroxide and water in anhydrous methanol (10:40:50); acid–methanol solution, 5% v/v: hydrochloric acid in anhydrous methanol; and 1-butanol equilibrated with 0.1 *N* sodium thiosulfate.

Preparation of Sephadex Column—Thirty-three grams of Sephadex G-10 was mixed with 100 ml. of aqueous ammonia–methanol solution, 10% v/v, and allowed to solvate overnight. A column (27 × 2.1 cm.) was then prepared and allowed to stand overnight. It was foil covered.

[1] The instrumentation consisted of a Nuclear-Chicago Research gas chromatograph, series 5000, with hydrogen-flame ionization detector and U-shaped column oven; a Barber-Colman strip chart recorder, series 8000, equipped with a disk chart integrator, model 205; a Beckman Expandomatic pH meter, and a Mettler Micro Gram-atic balance, high-speed M-5 S/A. The apparatus used were a Buchler portable flash evaporator; a Burrell wrist-action shaker (Burrell Corp., Pittsburgh, Pa.); Hamilton microliter syringes, #701-N (10 μl.) and #701-NCH (500 μl.); U-shaped borosilicate glass columns, 1.83-m. (6-ft.) heavy wall 3-mm. i.d.; and liquid chromatography columns, 27 × 2.1 cm., with Teflon stopcocks.

[2] Warner-Lambert Research Institute, Lot 9, Feb. 1970, through Dr. Daniel Banes, Director, Office of Pharmaceutical Research and Testing, Bureau of Drugs.

[3] Pharmacia Fine Chemicals, Piscataway, N. J.

[4] OV-1, Applied Science Laboratories, Inc., State College, Pa.

[5] Gas-Chrom Q, Applied Science Laboratories, State College, Pa.

[6] Pierce Chemical Co., Rockford, Ill.

PROCEDURE

Preparation of Sample—Mix approximately 0.3 g. of dried thyroid, accurately weighed, with 3 g. of barium hydroxide [$Ba(OH)_2 \cdot 8H_2O$] and 6 ml. of water in a foil-covered round-bottom flask. Flush with nitrogen and air flux for 6 hr. at 96°. When reflux temperature is reached, flush again with nitrogen and lightly seal the apparatus. Cool and acidify with approximately 3.5 ml. of dilute hydrochloric acid (1:1) to pH 0.8–0.9 as determined by a pH meter. Extract the acidified solution with 10, 5, 5, and 5 ml. of 1-butanol saturated with 0.1 *N* sodium thiosulfate while protecting from light. Wash the combined 1-butanol extracts with three 25-ml. quantities of distilled water saturated with 1-butanol. The final washing is to be neutral to pH indicator paper. Filter through a medium sintered-glass filter. Rinse the separator with 1-butanol and, after passing the rinsings through the filter, combine with the original extract. Evaporate to dryness under reduced pressure with a rotary evaporator in foil-covered flasks. Add ethanol to aid in removal of moisture and evaporate to dryness.

Transfer the residue quantitatively, using a total of 5 ml. of aqueous ammonia–methanol solution, 10% v/v, to a Sephadex G-10, light-protected, 27 × 2.1-cm. column. Elute with the same solvent. Discard the first 35 ml. of eluate but collect the second fraction of 80 ml. Evaporate the eluate to dryness under reduced pressure in a light-protected flask. Add 5 ml. of acid–methanol solution, 5% v/v, and evaporate to dryness under reduced pressure. Add 5 ml. of ethanol, evaporate to dryness, and flush with nitrogen.

Add 100 μl. of anhydrous pyridine and 300 μl. of *N,O*-bis(trimethylsilyl)acetamide and heat for 2 hr. at 50°. Refrigerate overnight.

Preparation of Standard Derivative—Accurately weigh on a microbalance approximately 1 mg. of liothyronine and 1.5 mg. of thyroxine into a 4-ml. (1-dram) vial with a foil-lined screw cap. Wrap the vial with foil. Add 100 μl. of anhydrous pyridine and 300 μl. of *N,O*-bis(trimethylsilyl)acetamide and heat for 2 hr. at 50°. Store overnight in a refrigerator. Inject 3 μl. onto the GLC column.

GLC—For analysis, use GLC under the following conditions: 1.83-m. (6-ft.) × 3-mm. i.d. heavy wall borosilicate glass U-shaped column, packed with 1% dimethyl silicone coated onto 60–80-mesh diatomaceous earth, silane treated, and conditioned at 300° overnight; initial column temperature, 165°; final column temperature, 285°; programmed rate, 5°/min.; chart speed, 1.3 cm. (0.5 in.)/min.; nitrogen carrier gas flow, 50 ml./min.; injector temperature, 240°; detector temperature, 330°; attenuation for thyroxine, liothyronine, and 3,3′,5′-triiodothyronine, 16×10^{-10} amp.; and attenuation for dried thyroid sample, 16×10^{-10} amp., changed to 2×10^{-10} amp. at 235° (13 min.). Inject 3 μl. of standard derivative and obtain the areas for liothyronine and thyroxine. Then inject 3 μl. of the dried thyroid sample derivative to obtain areas of liothyronine and thyroxine.

Quantitation of Results—Calculate the amount of liothyronine and thyroxine from the integration areas:

$$\frac{A_u}{A_s} \times \frac{C_s}{W} = \text{mg./g.} \qquad \text{(Eq. 1)}$$

where A_u = integrated area of unknown, A_s = integrated area of standard, C_s = milligrams of standard in 400 μl., and W = weight of dried thyroid sample in grams.

RESULTS AND DISCUSSION

Effect of Hydrolysis on Synthetic Mixtures of Liothyronine and Thyroxine and Completeness of Extraction—This portion of the study was undertaken to determine if the hydrolytic conditions caused degradation of the iodoamino acids and if the extraction procedure gave quantitative results. To determine these points, synthetic mixtures of liothyronine and thyroxine were subjected to a 6-hr. hydrolysis as described previously. The hydrolysis flask fitted with an air condenser was protected from light and flooded with nitrogen. The recovery of liothyronine and thyroxine was 100.6% (1.2σ) and 98.92% (2.22σ), respectively, using the described analytical procedure. The results are recorded in Table I.

The study indicated that liothyronine and thyroxine were stable under the conditions of hydrolysis; extraction was complete and could be determined by GLC.

Table I—Recovery of Liothyronine (T_3) and Thyroxine (T_4) after Hydrolysis

Liothyronine (T_3)			Thyroxine (T_4)		
Added, mg.	Found, mg.	Percent Recovery	Added, mg.	Found, mg.	Percent Recovery
0.883	0.885	100.2	1.529	1.450	94.83
0.812	0.792	97.54	1.645	1.585	96.35
0.905	0.932	103.0	1.610	1.604	99.63
1.040	1.058	101.7	1.486	1.559	104.9
	Mean	100.6		Mean	98.92
	σ	1.2		σ	2.22

Table II—Recovery of Liothyronine (T_3) and Thyroxine (T_4) from Sephadex G-10 Columns

Liothyronine (T_3)			Thyroxine (T_4)		
Added, mg.	Found, mg.	Percent Recovery	Added, mg.	Found, mg.	Percent Recovery
1.086	1.111	102.3	1.527	1.619	106.0
1.086	1.084	99.82	1.527	1.569	102.8
1.005	1.041	103.6	1.507	1.534	101.8
	Mean	101.9		Mean	103.5
	σ	1.1		σ	1.3

Direct extraction of the synthetic mixtures without prior hydrolysis was also found to be quantitative.

Purification—Several techniques were attempted to separate the iodoamino acids from interfering substances in the 1-butanol extract. Column chromatography appeared to offer the greatest probability of separation; therefore, columns of aluminum oxide, diatomaceous earth[7], and Sephadex G-10, G-15, and LH-20 were tried using a number of solvents. Recoveries ranged from zero to 95%. The low recoveries could have been due to adsorption on the columns or the failure to remove the charring impurities.

However, when liothyronine and thyroxine were eluted with aqueous ammonia–methanol solution, 10% v/v, from a Sephadex G-10 column, previously prepared with the same solvent, the first fraction of 35 ml. contained the interfering material. The second fraction of 80 ml. contained the liothyronine and thyroxine. The results are contained in Table II.

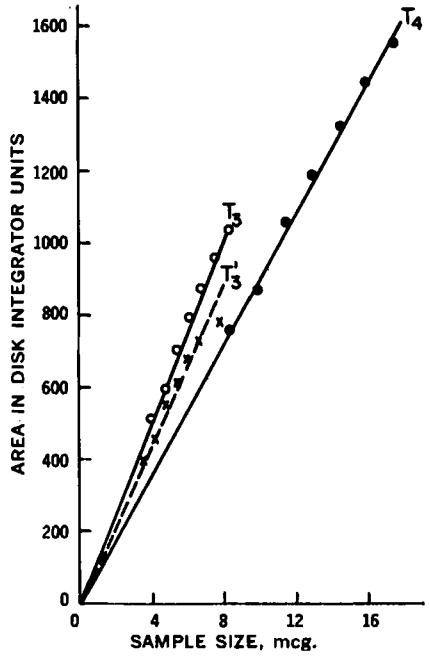

Figure 1—*Detector response for varying amounts of liothyronine (T_3), 3,3',5'-triiodothyronine (T_3'), and thyroxine (T_4).*

[7] Celite 535, Johns-Manville, New York, N. Y.

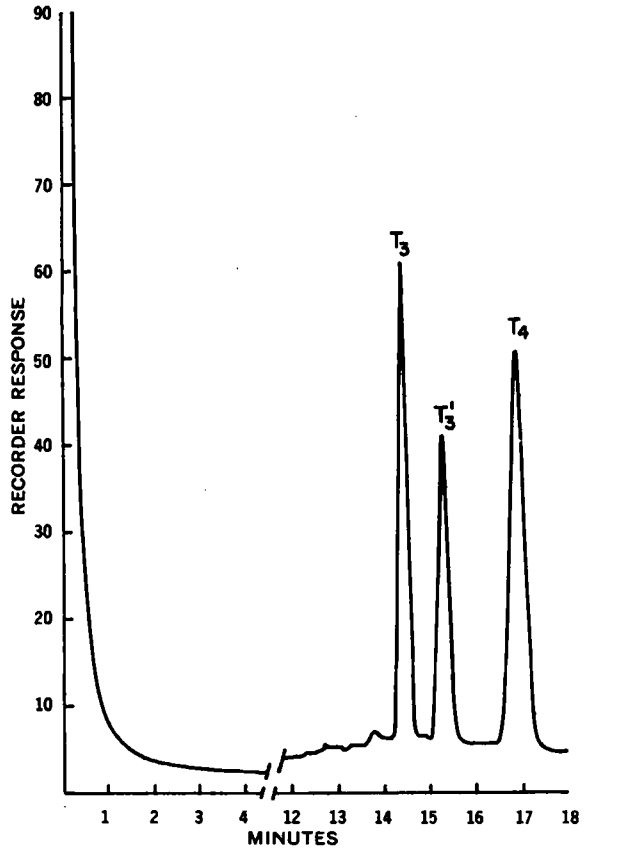

Figure 2—*Temperature-programmed chromatogram of a standard derivative (programmed temperature = 165–285°, programmed rate = 10°/min., and attenuation = 16 × 10^{-10} amp.).*

The success of separation depends on the preparation and packing of the column. Reproducible elution patterns were obtained only if the Sephadex G-10 was solvated overnight and then the packed column was allowed to stand overnight to ensure uniform density. Directly before using, 200 ml. of eluting solvent is passed through the column.

After elution and removal of the solvent from the fraction containing the iodoamino acids, 5 ml. of 5% v/v acid–methanol solution is added to the flask and removed under reduced pressure. This step is vital to the successful preparation of silyl derivatives. The conversion of liothyronine and thyroxine to the acid form is necessary since the corresponding salts will not readily form these derivatives.

Effect of Light—Initially, attempts to elute quantitatively liothyronine and thyroxine from columns gave low results. It was suspected that the low results might have been due to light-catalyzed degradation. Experiments were then carried out where all operations, from elution to the preparation of the trimethylsilyl derivative, were protected from light. Recoveries were reproducible and quantitative. Based on these findings, all other facets of the procedure, from hydrolysis to derivatization, were protected from light and treated with nitrogen gas. The exact nature of the degradation was not studied further.

Preparation of a Trimethylsilyl Derivative—To prepare this derivative, a method similar to that of Alexander and Scheig (25) was used. Pyridine was used to facilitate silylation of the iodoamino acids. The temperature was kept at 50°, because signs of possible degradation appeared when higher temperatures were employed. Greater and more reproducible responses were obtained if the derivatives were refrigerated overnight. As is the case with most trimethylsilyl derivatives, they are prone to hydrolytic degradation, so care must be exercised to prevent contact with moisture. After overnight refrigeration, the sample must be allowed to come to room temperature before being opened.

Table III—Determination of Liothyronine (T_3), 3,3′,5′-Triiodothyronine (T_3'), and Thyroxine (T_4) in Dried Thyroid

Porcine Dried Thyroid[a]		T_3, mg./g.	T_3', mg./g.	T_4, mg./g.
Lot 263 containing 0.89% w/w iodine		0.344	—	1.571
		0.355	—	1.352
		0.331	—	1.249
		0.345	—	1.432
		0.392	0.082	1.421
		0.383	0.073	1.326
		0.360	0.077	1.265
	Mean	0.358	0.077	1.374
	σ	0.008	0.003	0.042
Lot 102		0.298	—	1.348
		0.326	0.090	1.187
	Mean	0.312	0.090	1.267
	σ	0.014	—	0.081

[a] Armour.

Quantitative Results—It was found that there was a linear relationship between the sample size and recorder response for liothyronine, 3,3′,5′-triiodothyronine, and thyroxine as shown in Fig. 1. A typical chromatogram of a standard derivative is shown in Fig. 2.

When the described method, including the purification step, was applied to dried thyroid, the results given in Table III were obtained.

Data are presented on two lots of dried thyroid. These results appear reasonable based on estimates of iodine content. However, it is not possible to state with absolute certainty the total percentage recovery of liothyronine and thyroxine in these samples since the absolute analysis is not known. However, when liothyronine and thyroxine were added to dried thyroid (Lot 263) and assayed according to the described method, quantitative recovery of the added material was obtained when calculated by difference. Although this is not an absolute proof of quantitation, it does lend support to the data.

A chromatogram of the 1-butanol extract of a dried thyroid hydrolysate is shown in Fig. 3. In addition to liothyronine and thyroxine, 3,3′,5′-triiodothyronine and an unknown were separated. The unknown compound is probably an iodoamino acid whose identity has still to be verified.

Other iodoamino acids were not quantitatively determined because it was found in previous work that 3-monoiodotyrosine, 3,5-diiodotyrosine, and 3,5-diiodothyronine were not quantitatively extracted by this procedure; therefore, correlation with the total iodine content of the dried thyroid powder could not be done. Further work to substantiate absolute quantitation must be carried out as well as the correlation of this method with biological activity measurements.

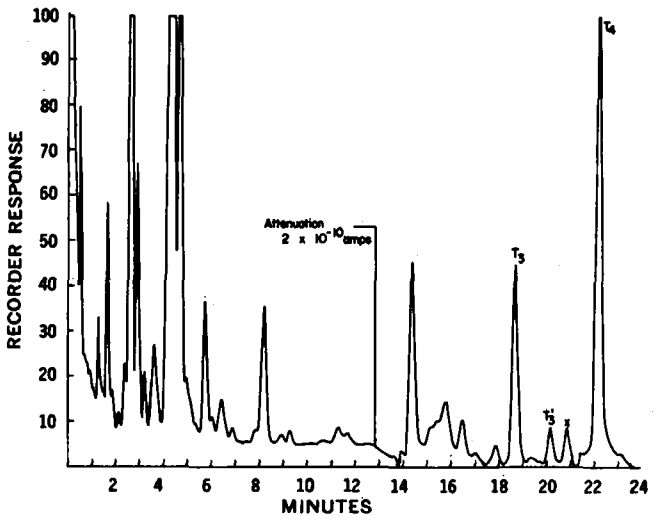

Figure 3—*Chromatogram of dried thyroid hydrolysate. The thyroid hormones were liothyronine (T_3), 3,3′,5′-triiodothyronine (T_3'), thyroxine (T_4), and an unknown, X.*

REFERENCES

(1) W. F. Ganong, "Review of Medical Physiology," 5th ed., Lange Medical Publications, Los Altos, Calif., 1971, pp. 230–233.

(2) J. Roche, R. Michel, W. Wolf, and N. Etling, *Compt. Rend. Soc. Biol.*, **148**, 1738(1954).

(3) N. R. Stasilli, R. L. Kroc, and R. I. Meltzer, *Endocrinology*, **64**, 62(1959).

(4) E. C. Kendall, *J. Biol. Chem.*, **39**, 125(1919).

(5) C. H. Harington and S. S. Randall, *Quart. J. Pharm. Pharmacol.*, **2**, 501(1929).

(6) J. E. Stouffer, P. I. Jaakonmäki, and T. J. Wenger, *Biochim. Biophys. Acta*, **127**, 261(1966).

(7) A. H. Richards and W. B. Mason, *Anal. Chem.*, **38**, 1751 (1966).

(8) R. O. Zimmerer, Jr., and L. T. Grady, *J. Pharm. Sci.*, **60**, 493(1971).

(9) A. D. Williams, L. Meister, and W. H. Florsheim, *ibid.*, **52**, 833(1963).

(10) R. Lemieux and J. M. Talmage, *J. Pharm. Pharmacol.*, **18**, 94(1966).

(11) J. E. Moody, Jr., J. R. Hohmann, and G. B. Kaplan, *J. Pharm. Sci.*, **57**, 634(1968).

(12) W. F. Devlin and N. R. Stephenson, *J. Pharm. Pharmacol.*, **14**, 597(1962).

(13) V. J. Pileggi, O. J. Golub, and N. D. Lee, *J. Clin. Endocrinol.*, **25**, 949(1965).

(14) S. Kologlu, H. Schwartz, and A. C. Carter, *Endocrinology*, **78**, 231(1966).

(15) W. F. Devlin and H. Watanabe, *J. Pharm. Sci.*, **55**, 390 (1966).

(16) R. Bilous, E. Koehler, and J. J. Windheuser, *ibid.*, **60**, 1270 (1971).

(17) J. Gross and R. Pitt-Rivers, *Biochem. J.*, **53**, 645(1952).

(18) J. W. Braasch, E. V. Flock, and A. Albert, *Endocrinology*, **55**, 768(1954).

(19) T. H. Kennedy, *Aust. J. Biol. Sci.*, **11**, 106(1958).

(20) P. Blanquet, R. W. Dunn, and C. A. Tobias, *Arch. Biochem. Biophys.*, **58**, 502(1955).

(21) V. A. Galton and R. Pitt-Rivers, *Biochem. J.*, **72**, 310(1959).

(22) V. J. Pileggi, N. D. Lee, O. J. Golub, and R. J. Henry, *J. Clin. Endocrinol.*, **21**, 1272(1961).

(23) M. A. Faircloth, A. D. Williams, and W. H. Florsheim, *Anal. Biochem.*, **12**, 437(1965).

(24) J. E. Stouffer, *J. Chromatogr. Sci.*, **7**, 124(1969).

(25) N. M. Alexander and R. Scheig, *Anal. Biochem.*, **22**, 187 (1968).

(26) F. Shahrokhi and C. W. Gehrke, *ibid.*, **24**, 281(1968).

(27) K. Funakoshi and H. J. Cahnmann, *ibid.*, **27**, 150(1969).

(28) E. T. Backer and V. J. Pileggi, *J. Chromatogr.*, **36**, 351(1968).

(29) L. B. Hansen, *Anal. Chem.*, **40**, 1587(1968).

(30) P. I. Jaakonmäki and J. E. Stouffer, *J. Gas Chromatogr.*, **5**, 303(1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received May 12, 1972, from the *School of Pharmacy, University of Wisconsin, Madison, WI 53706*

Accepted for publication September 25, 1972.

Presented to the APhA Academy of Pharmaceutical Sciences, Houston meeting, April 1972.

Supported by a grant from Armour Pharmaceuticals, Kankakee, Ill.

The authors acknowledge the technical assistance of Miss E. Koehler.

• Present address: Alza Research, Palo Alto, Calif.

▲ To whom inquiries should be directed. Present address: Faculty of Pharmacy, University of Manitoba, Winnipeg, Manitoba, Canada.