Chemical Determination of the Potency of Thyroid Preparations

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Several methods for the chemical analysis of thyroid have been reported in recent years, but none has been adopted by the official compendia. In an attempt to meet the need for a new official chemical assay, a procedure has been devised, and is reported in this paper for the quantitative determination of triiodothyronine (T_3) and thyroxine (T_4). Results for some of the samples tested are compared to the biological assay. The thyroid is hydrolyzed with barium hydroxide and the digest is extracted with *n*-butanol. The iodoamino acids are separated by thin-layer chromatography on cellulose plates and the T_3 and T_4 zones are scraped separately from the plates and ignited in oxygen flasks. The iodine is subsequently released and determined spectrophotometrically in benzene. Commercial thyroid preparations with different biological activities were analyzed and a chemical potency determined for each. The results correlated well with those obtained by biological assay.

The USP requires that thyroid contain not less than 0.17% and not more than 0.23% of iodine in thyroid combination. This 50-year-old standard does not truly reflect the physiological activity of the drug (1–3). Numerous samples of thyroid meet the official iodine requirement but have little or none of the desired activity.

Many attempts have been made to develop an analytical procedure for thyroid that will more accurately reflect its physiological activity. The complexity of this proteinaceous substance has until recently defeated these efforts. Hydrolysis of thyroid yields a number of iodinated derivatives of tyrosine and thyronine (4–7). However, only four of these, thyroxine, 3,3′,5-triiodothyronine, 3,3′,5′-triiodothyronine, and 3,3′-diiodothyronine, have biological activity. A quantitative method for thyroxine and 3,3′,5-triiodothyronine would suffice to estimate the potency of thyroid since the diiodo compound is present in only trace amounts and the 3,3′,5′-triiodothyronine has negligible activity (8).

Recently several methods have been presented for the determination of thyroxine (T_4) and 3,3′,5-triiodothyronine (T_3) in thyroid (2, 7, 9–14). Most of these procedures use the following scheme: (*a*) hydrolysis of the thyroprotein with sodium hydroxide or barium hydroxide, or by enzymatic action; (*b*) extraction of the released iodoamino acids with *n*-butanol; (*c*) separation of these iodoamino acids by paper, thin-layer or column chromatography, or by gel filtration; (*d*) quantitation of the separated active compounds either by direct determination or by destruction of the compound and determination of the released iodine.

Some of these published methods of thyroid assay have been studied in this laboratory, but the consistent results necessary for routine analysis could not be achieved. Lemieux and Talmage (11) discuss many of the disadvantages and inadequacies of some of the earlier methods. The general scheme of their procedure contains some steps which, with modifications, were adapted to our method. Enzymatic hydrolysis with pronase and alkaline hydrolysis with barium hydroxide were compared in the present study. Devlin and Watanabe (12) reported that hydrolysis with pronase yielded more T_3 and T_4 in a shorter time than hydrolysis with the other enzyme studies. The present data agree with their report with regard to enzymatic hydrolyses, but show that more T_4 is released by hydrolysis with barium hydroxide.

In our procedure the released components of the hydrolyzed thyroprotein are separated by thin-layer chromatography on cellulose plates, with a solution of *p*-toluenesulfonic acid and formic acid as the developing solvent. This procedure releases a significant amount of a component between T_3 and T_4 on the chromatogram which is not usually reported. The compound has been shown to be 3,3′,5′-triiodothyronine.

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The areas of the chromatogram containing T_3 and T_4 are scraped from the plate and ignited in an oxygen flask to convert the iodine, after intermediate treatment, to iodate. The final step in the procedure is the spectrophotometric measurement of iodine in benzene.

EXPERIMENTAL

Apparatus—A Cary 14 or Beckman DK recording spectrophotometer was used for these studies. The 10-cm. cylindrical silica cells for the absorption measurements and the Thomas-Ogg oxygen ignitor were purchased from Arthur H. Thomas Co., Philadelphia, Pa. Glass chromatography tanks were 30 × 30 × 10 cm.; the chromatoplates were 20 × 20 cm.

Reagents—All reagents and solutions were prepared from analytical grade chemicals and with water redistilled from an all-glass still. The following reagents were used: $Ba(OH)_2.8H_2O$; 30% formic acid, diluted from 90% analyzed reagent; *n*-butanol; *n*-butanol equilibrated with 0.1 *N* sodium thiosulfate solution; ammoniacal methanol, 5% v/v solution of ammonium hydroxide in methanol; phosphoric acid solution, 260 ml. of 85% H_3PO_4 diluted to 1 L. with water; benzene, "distilled in glass," b.p. 80–81°, Burdick and Jackson Laboratories, Inc., Muskegon, Mich.; bromine water, USP; *p*-toluenesulfonic acid, 40% w/v; NaOH, 2% w/v; freshly prepared potassium iodide, 7.5% w/v; sodium carbonate, half saturated.

To prepare the diazotized sulfanilamide (15), mix 5 ml. of a 1% sulfanilamide solution in 10% v/v hydrochloric acid with 5 ml. of 5% w/v sodium nitrite solution in a 50-ml. glass-stoppered graduated cylinder for 1 min. Add *n*-butanol to 50 ml. and shake for 1 min. Let stand 4 min. and decant the butanol layer to be used as the spraying solution. Prepare just before use.

The cellulose was MN cellulose powder from Macherey Nagel and Co. (distributed by Brinkmann Instrument Co., Great Neck, N. Y.). The gelatin capsules (No. 0 and 00) were obtained from Eli Lilly & Co., Indianapolis, Ind.

To facilitate transferring the cellulose containing the T_3 nd T_4 to capsules, a funnel rack and a capsule holder were devised (Fig. 1).

Fig. 1—Device to transfer cellulose from chromatoplates to capsules for oxygen ignition.

Preparation of Samples—*Tablets*—Weigh 20 tablets and grind to a fine powder. Transfer the equivalent of 200 mg. of USP thyroid, accurately weighed, to a 50-ml. centrifuge tube or appropriate test tube to fit a centrifuge and heating block. Add 6 ml. of water, mix well on a vortex mixer, and centrifuge for 15 min. Remove the supernatant with a syringe fitted with a large blunt needle and discard the washings. Repeat with a second 6 ml. of water. Fit the tube with an air condenser at least 10 in. long.

Bulk Powders—Place an accurately weighed sample of thyroid powder equivalent to about 400 mcg. of iodine in a test tube of appropriate size that is fitted with an air condenser.

Total Iodine Determination—Weigh accurately a No. 0 gelatin capsule and a sample of ground tablets or bulk powder equivalent to about 8.4 mg. of USP thyroid. Ignite the capsules as described under *Oxygen Ignition* and continue the procedure from there to completion. Calculate as follows:

$$\% \text{ total iodine} = \frac{(\text{mcg. iodine from standard curve})\ (100)}{(\text{wt. of thyroid in sample in mcg.})\ (6)}$$

Hydrolysis and Extraction—To the tubes containing the accurately weighed samples add 3 Gm. of $Ba(OH)_2.8H_2O$ and 6 ml. of water (16). Mix well and place the air condensers in the tubes. Heat the tubes in an aluminum heating block at 100° or in a boiling water bath for 16–18 hr. Swirl the tubes occasionally to mix the contents. After the hydrolysis, allow the mixture to cool and add about 3.5 ml. of dilute hydrochloric acid (1:1) to a pH of 0.8 or 0.9 as determined with a pH meter. Transfer the acidified solution to a 60-ml. separator. Wash the hydrolysis tube with three 5-ml. portions of water and add the washings to the separator. Extract the hydrolysate solution with 10, 5, and 5 ml. of *n*-butanol previously equilibrated with 0.1 *N* sodium thiosulfate. Wash the combined butanol extracts with 25-ml. portions of butanol-saturated water until the washings are neutral to pH indicator paper. Filter the butanol through a medium sintered-glass funnel into a 50-ml. round-bottom flask. Rinse the separator with three 2-ml. portions of butanol and pass the rinsings through the separator. Add a small quantity of ethanol to the filtered butanol solution to dissolve any droplets of water present. Remove the butanol at 55° under reduced pressure. The dried residue may be stored for at least 1 week in the cold under nitrogen (13).

Chromatography—To prepare five 20 × 20 cm. plates homogenize 15 Gm. of cellulose powder in a fast blender with 90 ml. of water. Spread the cellulose slurry on the plates in a 0.6 mm. (±0.05 mm.) layer, and dry the plates overnight at room temperature. Scratch a line across a plate about 3 cm. from the top. Make two lines, each 6 cm. long, of pin-point dots 0.5 cm. apart, about 1.5 cm. from the bottom of the plate and separated by 3 or 4 cm.

Dissolve the hydrolysate residue in 1.0 ml. of ammoniacal methanol, and transfer as much of the solution as possible to a 1-ml. stoppered flask or tube to reduce evaporation. Spot 200 µl. of the ammoniacal methanol solution uniformly along each of the 6-cm. dotted lines. Scrape a vertical band of

cellulose from top to bottom with a narrow flat-edged spatula on both sides of the spotted areas in parallel lines almost touching the lines of sample application (see Fig. 2). These borders will aid in the development of straight horizontal zones.

Line glass chromatography tanks on one side with filter paper. Pour a mixture of 50 ml. of the 40% *p*-toluenesulfonic acid solution and 50 ml. of the 30% formic acid solution down the paper into the tank. Place the chromatoplate in the developing tank leaning on the unlined side and cover the tank with a glass plate. Allow the chromatograms to develop until the solvent front reaches the line across the top of the plate (about 2¹/₂ hr). Air dry the plates in a fume hood.

Spray the plate with the freshly prepared diazotized sulfanilamide solution (15). Dry the plate in a current of air for 5 min. and then spray with half-saturated sodium carbonate solution. The zones containing the amino acids will be visible as pink areas on the chromatogram. Allow the plate to air dry in a fume hood.

Place the plate in a chromatogram viewing cabinet and observe with a long-wave ultraviolet light. The pink areas will appear as dark zones (Fig. 3). Mark the T_3 and T_4 zones with a dissecting needle or other sharp instrument. Scrape the marked T_3 and T_4 zones from the plate and place the cellulose in No. 0 or 00 gelatin capsules. Clean the glass in the scraped zones and the instruments used with small pledgets of cotton and add the cotton to the capsule.

Oxygen Ignition—All glassware used from this point on should be extensively rinsed with the redistilled water. Oxygen ignition is carried out in a Thomas-Ogg oxygen igniter. Ignite the capsules in 1-L. flasks containing 30 ml. of 2% sodium hydroxide solution as the scrubbing solvent, and a small strip of black ignition paper as the igniting point. After the flasks are cooled, invert and shake them for 5 min. Quantitatively transfer the solution to well rinsed 250-ml. conical flasks, collecting a total of about 150 ml. from repeated rinsings. Run a reagent blank starting with the addition of 30 ml. of the sodium hydroxide solution to a 250-ml. conical flask and continuing through the assay.

Assay—Slowly add 5 ml. of the dilute phosphoric acid solution to each flask, then 1 ml. of USP bromine water. Do not reverse this order of addition of reagents; otherwise high and irregular blanks and samples may result. Mix by swirling the flasks gently, and add a few boiling chips. Boil the solutions on a hot plate until all the excess bromine is removed as shown with starch-iodide test paper, and until the solutions are between 75 and 100 ml. Do not let the solutions boil down below 75 ml. Wash down the sides of the flasks with a stream of water and boil for an additional 5 min. Cool the solutions to room temperature and transfer to well-rinsed 125-ml. separator. Extract the solutions with 25 ml. of benzene and discard the benzene. Add a second 25-ml. portion of benzene to each separator and 3 ml. of the freshly prepared 75% solution of potassium iodide. Stopper the separators and shake for 1 min. Allow the layers to separate and collect the benzene layer in benzene-rinsed 50-ml. volumetric flasks. Extract the samples with additional 15-ml. and 9-ml. portions of benzene and add the extracts to the volumetric flasks. Bring the solutions to volume with benzene and mix well. Record the absorbance of the benzene solutions at the minimum (400 mμ) and the maximum (295 mμ) in the 10-cm. cylindrical cells with benzene as the reference. Subtract the absorbance at the minimum wavelength from the absorbance at the maximum wavelength. Subtract the difference, if any, in absorbance at the maximum and minimum of the blank from the sample value to give the corrected absorbance. Prepare a standard curve in the same manner for solutions of iodine in benzene of at least 5 different dilutions ranging from about 20 to 100 mcg. in 50 ml. of benzene (Fig. 4).

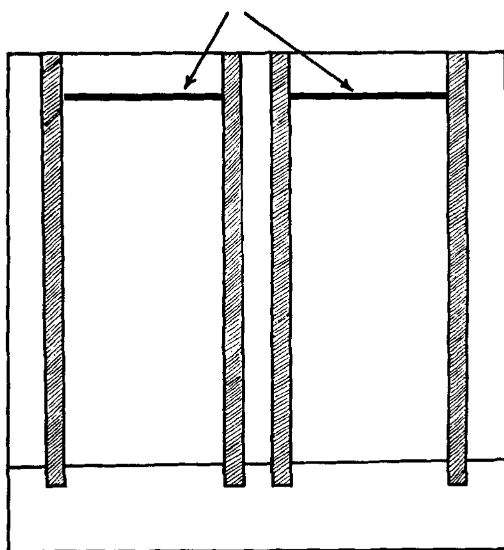

Fig. 2—Diagram of chromatoplate before development. Arrows point to lines of sample application.

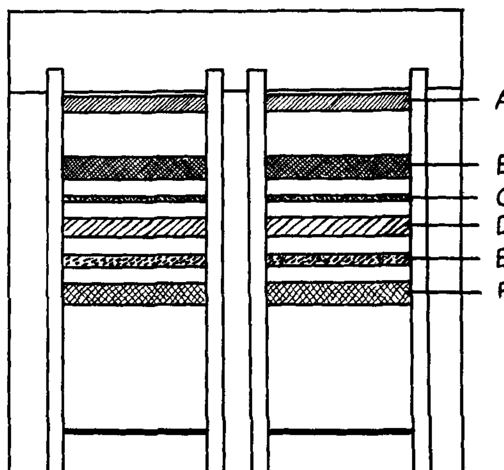

Fig. 3—Diagram of chromatoplate after development. A = solvent front; B = DIT, MIT, T_2; C = iodide; D = T_3; E = T_3-iso; F = T_4.

Calculations—Determine the mcg. of iodine in the 50-ml. benzene solutions from the standard curve, using the corrected absorbances of the sample solutions.

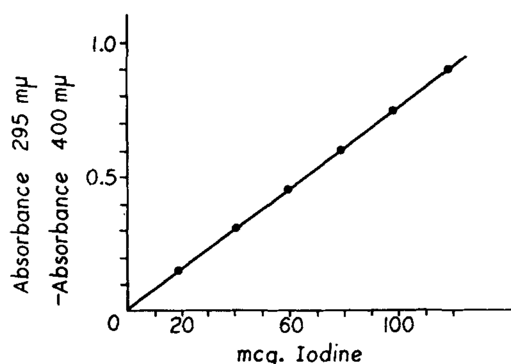

Fig. 4—Standard curve of iodine in 50 ml. benzene.

$$\% \ T_3 \text{ in sample} = \frac{(\text{mcg. of I})\ (1.425)\ (100)}{\text{wt. of sample in mcg.}}$$

$$\% \ T_4 \text{ in sample} = \frac{(\text{mcg. of I})\ (1.275)\ (100)}{\text{wt. of sample in mcg.}}$$

The factors (1.425 and 1.275) in the calculation equations are derived from the percentages of iodine in T_3 and T_4, respectively, as given below. The full equation without using factors is:

$$\% \ T_3 \text{ in sample} = (\text{mcg. of I from std. curve}) \times \frac{(5)}{(6)} \times \frac{(100)}{(58.48)} \times \frac{(100)}{(\text{wt. of sample in mcg.})}$$

where: (5) = multiplication factor due to the use of 200 μl. from 1 ml. of solution for spotting plates;

(6) = oxidation of iodide to iodate and release of iodine with potassium iodide to give six times the iodine in the original iodide; and

$\frac{(100)}{(58.48)}$ = percent factor for iodine in T_3.

$$\frac{(5)}{(6)} \times \frac{(100)}{(58.48)} = 1.425$$

Similarly for T_4: $\frac{(100)}{(65.34)}$ = percent factor for iodine in T_4.

$$\frac{(5)}{(6)} \times \frac{(100)}{(65.34)} = 1.275$$

RESULTS

Table I shows the T_3 and T_4 content of several commercial thyroid powders and tablets as determined by this method. Some of the powders were marketed as USP thyroid and some as triple strength thyroid. The tablets were all labeled as 1-grain USP thyroid tablets.

The total iodine content in thyroid combination agreed with the labeling as to USP and triple strength for all samples tested. Sample A is a USP thyroid powder that was used as the standard.

In Table II, the chemical potency and biological activity are set at 100% for the standard and the other results are compared to it. Assuming T_3 to have five times the antigoitrogenic activity of T_4, a chemical potency was derived for each sample, using the formula $5 \times (\% \ T_3) + \% \ T_4$. These figures were compared with the one calculated for sample A, the standard, to obtain the percent chemical potency. A preliminary biological assay showed that samples B, G, I, J, and K have less activity than the standard. Samples C, D, and F show greater activity. The biological activity was not determined for samples E and H. Sample E is an undiluted porcine thyroid powder purchased directly from the manufacturer. The total organically bound iodine content of this sample is 3.7 times that of the standard sample and the percent chemical potency found is 3.6 times that of the standard. Sample H is a 1-grain tablet from the same manufacturer as sample E and the total organically bound iodine and the chemical potency are, respectively, 1.10 and 1.07 times the corresponding figures for the standard.

Table II shows that the chemical potency gives a good indication of the physiological activity of the samples tested. The biological potency figures are based on a single determination.

A representative sampling of several brands of thyroid tablets was collected from various areas of the United States and the tablets were assayed by this procedure. The results are compared to our standard thyroid powder and are shown in Table III.

The accuracy of the assay procedure was checked with National Bureau of Standards 2-iodobenzoic acid. Recovery results starting with the oxygen ignition step were 101.5% and 99.96%. Recovery results starting with spotting 200 μl. of a 2-iodobenzoic acid solution on a cellulose plate and scraping the area of application from the plate were 101.1% and 100.7%.

TABLE I—T_3 AND T_4 CONTENTS OF SOME COMMERCIAL THYROID POWDERS AND TABLETS

Strength	Sample	No. of Samples	Av. % T_3	Av. Deviation	Av. Deviation from Mean	Av. % T_4	Av. Deviation	Av. Deviation from Mean
USP	A	21	0.016	0.002	0.0004	0.048	0.002	0.0004
USP	B	8	0.015	0.001	0.0004	0.044	0.003	0.001
USP	C	7	0.019	0.0007	0.0003	0.054	0.002	0.0008
USP	D	10	0.022	0.0009	0.0003	0.041	0.002	0.0006
3X	E	17	0.056	0.002	0.0005	0.177	0.007	0.017
3X	F	6	0.061	0.001	0.0004	0.162	0.003	0.001
3X	G	7	0.028	0.002	0.0008	0.046	0.004	0.0015
1-Grain tablet	H	7	0.017	0.003	0.0011	0.052	0.003	0.011
1-Grain tablet	I	8	0.005	0.001	0.0004	0.012	0.001	0.0004
1-Grain tablet	J	4	0.009	0.000	0.000	0.017	0.001	0.0005
1-Grain tablet	K	4	0.008	0.001	0.0005	0.016	0.0003	0.0002

TABLE II—COMPARISON OF THE CHEMICAL POTENCY AND BIOLOGICAL ACTIVITY OF SOME COMMERCIAL THYROID POWDERS AND TABLETS

Sample	% Total Iodine	% T_3	% T_4	5 (% T_3) + % T_4	% Chemical Potency	% Biological Potency
A USP (std.)	0.19	0.016	0.048	0.128	100	100
B USP	0.19	0.015	0.044	0.119	93	85
C USP	0.20	0.019	0.054	0.149	116	164
D USP	0.23	0.022	0.041	0.151	118	132
E 3X	0.7	0.056	0.177	0.457	357	...
F 3X	0.63	0.061	0.162	0.467	122[a]	155
G 3X	0.62	0.028	0.046	0.186	48[a]	30
H 65 mg. (1 gr.)	0.21	0.017	0.052	0.137	107	...
I 65 mg. (1 gr.)	0.20	0.005	0.012	0.037	29	25
J 65 mg. (1 gr.)	0.19	0.009	0.017	0.062	48	44
K 65 mg. (1 gr.)	0.19	0.008	0.016	0.058	45	48

[a] Divided by three for comparison to single strength thyroid.

TABLE III—ANALYSIS OF COMMERCIAL THYROID TABLETS COLLECTED FROM SEVERAL AREAS OF THE UNITED STATES

Product	% Total Iodine	% T_3	% T_4	5 (% T_3) + % T_4	% Chemical Potency
Standard thyroid powder	0.19	0.016	0.048	0.128	100
32 mg. (0.5 gr.) tablets	0.19	0.013	0.053	0.118	92
	0.20	0.018	0.053	0.143	112
	0.20	0.019	0.053	0.148	116
	0.21	0.014	0.052	0.122	95
	0.21	0.009	0.021	0.066	51
65 mg. (1 gr.) tablets	0.20	0.010	0.054	0.104	81
	0.19	0.016	0.037	0.117	92
	0.19	0.019	0.042	0.137	107
	0.24	0.017	0.051	0.136	106
	0.23	0.016	0.047	0.127	99
	0.21	0.016	0.053	0.133	104
130 mg. (2 gr.) tablets	0.19	0.012	0.050	0.110	86
	0.22	0.020	0.042	0.142	111
	0.21	0.019	0.057	0.152	119
	0.19	0.014	0.032	0.102	80
	0.23	0.014	0.046	0.116	91

DISCUSSION

Sample Preparation—The ground tablets are prewashed with water to remove water-soluble tablet ingredients, especially lactose. Lactose, which is present in many tablets, caramelizes during the alkaline hydrolysis, and the resulting dark color obscures the chromatogram. The use of a second wash with chloroform-methanol (1:1) as reported by Lemieux and Talmage (11) was investigated but resulted in considerably lower recoveries of T_3 and T_4.

Hydrolysis—The pronase hydrolysis was an improvement over the earlier reported trypsin-erepsin treatment (13), because a better yield of T_3 and T_4 was obtained and the time required for hydrolysis was considerably reduced. In general, more T_4 was released by the 50% barium hydroxide solution than by pronase, while the quantity of T_3 released was about equal for both procedures. Some samples of thyroid powder yielded very little T_3 or T_4 with pronase hydrolysis, but gave a good yield of these compounds when hydrolyzed with 50% barium hydroxide. Hydrolysis with an 8% solution of $Ba(OH)_2 \cdot 8H_2O$ for 6 hr. was attempted but was less complete and the extracts gave diffused and dirty chromatograms.

Chromatography—Roche *et al.* (17) reported that the 3,3′,5′ isomer of T_3 is a normal constituent of thyroid protein. The majority of systems used in the separation of the components of thyroid digests disregard this isomer. As shown in Fig. 2, this compound is located between T_3 and T_4 on the chromatogram developed by our procedure. T_3, T_3-iso, and T_4 were chromatographed by some paper chromatographic procedures. With formic acid and water (1:5) as the developing solvent, the T_3-iso accompanies the T_3, while with 6 *N* ammonium hydroxide and tertiary amyl alcohol, the T_3-iso accompanies the T_4.

Roche *et al.* (8) compared the antigoitrogenic activity of some of the iodothyronines in propylthiouracil-treated rats to that of thyroxine. DL-3,3′,5′-Triiodothyronine (T_3) was 5–10 times as active as DL-thyroxine and the 3,3′,5′-triiodothyronine (T_3-iso) was only about 5% as active as DL-thyroxine. The extremely low activity of the T_3-iso makes it imperative that this compound be separated from T_3 and T_4 before quantitation.

The cellulose must be removed from the plate on

both sides of the sample line if horizontal zones and good resolution of the components are to be obtained. Without this treatment, the solvent front curves around the ends of the sample line and produces a cone-shaped chromatogram because the developing solvent moves up the plate faster in areas where there is no sample. The plates must be dried thoroughly before spraying to dispel as much formic acid as possible. The color development will occur only when the plate is made basic, and if too much Na_2CO_3 is needed to neutralize the formic acid, some of the color formed is obscured by a coating of sodium carbonate.

Quantitation of T_3 and T_4—The oxygen-ignition procedure has proved to be a direct and accurate method for quantitatively transforming organic iodine to inorganic iodide for subsequent assay. The oxidation of iodide to iodate with bromine does not require the addition of other chemicals to remove excess oxidant as is necessary when potassium permanganate is used.

Some very important precautions must be observed in the procedural operations. The iodide solutions must always be acidified before the bromine is added. If the bromine is added to the alkaline solution, hypobromites, bromates, *etc.*, may be formed (18). High and irregular blanks and samples result from the liberation of iodine if residual amounts of these bromine compounds are present. When the bromine is added to the acidified solutions, these compounds are not formed.

The data in Table II show that the samples marketed as USP all have the necessary amount of iodine in thyroid combination to meet the official requirements. The triple strength samples have a proportional amount of iodine. Many of these samples, though passing the official assay, do not have a corresponding physiological activity. The chemical potency determined for these samples follows the same pattern as the biological activity. The authors believe that this chemical assay procedure can be used to establish standards for ranges of T_3 and T_4 content and ratios of these two compounds that will be meaningful in expressing relative biological activity.

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Keyphrases

Thyroid preparations—analysis
Thyroxine—quantitative determination
Triiodothyronine—quantitative determination
Hydrolysis and extraction
TLC—separation, identity
Oxygen ignition—iodine release
UV spectrophotometry—iodine analysis