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The determination of liothyronine and thyroxine in thyroid preparations

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A procedure is described for determining liothyronine and thyroxine in thyroid substances. This involves stepwise hydrolysis with barium hydroxide, extraction of combined iodinated amino-acids by n-butanol, separation and isolation of liothyronine and thyroxine by paper chromatography, subsequent ignition of their separated chromatograms by oxygen flask combustion and estimation of evolved iodine in benzene solution by spectrophotometric absorption at 295 mμ. The method is suitable for routine quality control of thyroid products.

THE United States Pharmacopeia and British Pharmacopoeia both contain monographs on thyroid preparations which specify a total organic iodine or thyronine iodine assay, the inadequacies of which are generally recognised. The advisability of analysing thyroid preparations for liothyronine and thyroxine content has been noted by Mandl & Block (1959), Devlin & Stephenson (1962), and Backer (1964). Meister, Williams & Florsheim (1963) have developed a method for detecting clinically ineffective thyroid preparations which gives a good correlation between chemical assay and biological potency although the liothyronine and thyroxine contents are not individually determined. Meister found Blau's (1935) method of measuring thyronine iodine to be inadequate for the detection of defective thyroid preparations.

Devlin & Stephenson (1962) determine the two amino-acids separately. Their method, which uses enzymatic hydrolysis, appears to liberate maximum amounts of liothyronine and thyroxine from the proteinaceous gland extracts and we have found it to be an excellent research tool. However, the method has some disadvantages which precluded its adoption by us for routine control in the manufacture of thyroid preparations. Five days are required for complete enzymatic hydrolysis and a uniformly potent and stable enzyme preparation must be assured if reproducible hydrolysis conditions are to be achieved. Also the ceric sulphate-arsenious acid reaction used gives variable results (Custer & Natelson, 1949). Backer's method (1964) uses enzymatic hydrolysis, paper chromatography, oxygen flask ignition of the amino-acid areas of the papergrams and determination of the liberated iodine by the ceric sulphate-arsenious acid reaction. While this method has the disadvantages already described, the oxygen flask ignition was considered to be superior to elution. We sought a procedure having the precision, accuracy and ease of handling required for the routine quality control of thyroid preparations.

In the method we describe, Blau's procedure (1935) for the hydrolysis and extraction of the thyroid substance is subsequently coupled with paper chromatography. The procedure uses 80 μg of total iodine on each paper and triplicate papers are combined for the assay thus allowing direct observation of developed chromatograms by viewing in ultraviolet

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light, or by conventional spraying techniques. Oxygen flask ignition of the relevant areas of developed chromatograms is similar to that described by Backer (1964), and direct determination of the resulting iodine is made in a total inorganic environment.

Experimental

REAGENTS

All chemicals used for the preparation of solutions were analytical grade, except where specified. Common laboratory solutions not listed were prepared from analytical grade reagents.

Barium hydroxide solution. Prepared carbonate-free to contain 7·8–8·0% $Ba(OH)_2.8H_2O$. *Ammoniacal methanol solution.* To 2,6-di-t-butyl *p*-cresol (60 mg) add concentrated ammonia (1 ml) and anhydrous methanol to 100 ml. *t-Amyl alcohol.* Use the freshly distilled fraction, boiling between 100–103°. *4-Aminoantipyrine solution*, 2%. Dissolve 4-aminoantipyrine (m.p. 107–108°) (2 g) and sodium bicarbonate (1·7 g) in water to 100 ml. *Potassium ferricyanide solution*, 2%. Dissolve $K_3Fe(CN)_6$ (5 g) in water to 250 ml. Prepare fresh daily. *Benzene.* Use thiophene-free benzene.

PREPARATION OF SAMPLE

Tablets. Transfer the equivalent of 60 15 mg U.S.P. thyroid tablets, finely ground and accurately weighed, to a 50 ml glass stoppered centrifuge tube with water (25 ml). Shake for 10 min, centrifuge at 2,000 rpm for 5 min, decant and discard the supernatant. Wash the residue with water (25 ml) and with shaking to ensure complete dispersal. Heat for 5 min on a steam-bath, centrifuge for 5 min and discard the supernatant. Repeat this wash procedure. Add 50% v/v methanol in chloroform (25 ml) to the centrifugate, stopper, and shake for 2 min. Centrifuge and discard the supernatant. Repeat this and dry the residue with nitrogen, while warming on a steam-bath.

Bulk powders. Transfer an accurately weighed quantity of thyroid powder equivalent to 2 mg of iodine to a 50 ml glass stoppered centrifuge tube.

HYDROLYSIS

Pipette exactly 10 ml of barium hydroxide solution into the centrifuge tube and add n-butanol (0·5 ml). Reflux using an air condenser over a steam-bath for 6 hr, shaking vigorously at 15 min intervals, cool to room temperature and adjust to pH 0·8 with 6 N hydrochloric acid.

EXTRACTION

Extract the hydrolysate with n-butanol (8 ml), shake for 2 min, and centrifuge for 1 min. Repeat twice using 5 ml of butanol each time. Transfer the butanol top layers to a 50 ml glass stoppered centrifuge tube using a 10 ml syringe equipped with a $3\frac{1}{2}$ inch No. 17 gauge needle with a

filed or flat ground tip. Wash the combined extracts with 0·1 N sulphuric acid (1 ml), centrifuge, remove and discard the bottom layer with a syringe equipped with the same needle as above. In the same manner, wash with two 1 ml portions of water. Transfer the washed butanol extract to a 50 ml round bottom boiling flask, through a medium sintered glass filter under vacuum. Rinse the centrifuge tube with 3 × 2 ml n-butanol, and add via the filter to the butanol extract. Add concentrated ammonia (0·5 ml) and evaporate to dryness using a rotary film evaporator connected to a vacuum pump through a safety trap cooled with a mixture of dry ice and acetone. Transfer the residue with repeated 1 ml portions of ammoniacal methanol solution to a 5 ml volumetric flask through a fine sintered glass filter and dilute to volume with the ammoniacal methanol solution.

CHROMATOGRAPHY

Equilibrate t-amyl alcohol (150 ml) with 6 N ammonia (150 ml) by shaking for 5 min. Transfer the ammonia layer in a container to the bottom of a developing tank lined with Whatman No. 1 paper. Pour half of the t-amyl alcohol phase around the walls of the tank, cover and allow to equilibrate for 2 hr. Wet Whatman No. 1 chromatographic paper 5 × $1\frac{1}{2}$ inch by 18 inch strips with 0·05% sodium sulphite solution and air dry. Along a starting line located 3 inch from one end, apply 200 μl aliquots of the sample solution in streaks 1 inch wide and $\frac{1}{4}$ inch to $\frac{3}{8}$ inch high. Allow to equilibrate for 2 hr in a tank saturated with water vapour. Transfer the papers to the developing tank, fill the trough with the t-amyl alcohol phase and cover. Develop for 18 hr or until the solvent front is about 1–2 inch from the bottom of the papers. Mark the solvent fronts, and allow to air dry. View the chromatograms in ultraviolet light using an apparatus such as that described in the U.S.P. XVI, p. 923. The thyroxine can be seen as an ultraviolet absorbing zone at Rf 0·6, while the liothyronine can sometimes be seen at Rf 0·7. Confirm by spraying the two end chromatograms lightly with 4-aminoantipyrine solution, allow to dry, then spray with the potassium ferricyanide solution. The pink band occurring at about Rf 0·7 is liothyronine. The band at about Rf 0·6 is thyroxine. Mark and cut out the two bands on the three unsprayed assay papers and prepare for oxygen flask ignition.

OXYGEN FLASK IGNITION

For thyroxine, pipette 20 ml of N sodium hydroxide into a 500 ml Schoeniger ignition flask, add water (10 ml) and flush the flask with oxygen. Ignite one paper by means of a wick of Whatman No. 1 paper ($1\frac{1}{2}$ × $\frac{1}{8}$ inch). Stopper and invert the flask and shake for 2 min. Using the same sodium hydroxide solution, treat the remaining thyroxine papers in the same manner. The liothyronine papers are similarly treated except that 10 ml of sodium hydroxide and 20 ml of water are used. Pipette 3 ml of 0·1 N potassium permanganate into both flasks, and also into a third flask containing 10 ml of N sodium hydroxide, together with

sufficient water to approximately equal the volume in the other flasks. Mix and allow to stand for 5 min. To the flasks add 5 N sulphuric acid—3 ml to the blank and liothyronine, and 5 ml to the thyroxine—and 1% sodium nitrite solution (2 ml). Two min after the sodium nitrite add 5% ammonium sulphamate (1 ml). Transfer the contents of each flask to 125 ml separating funnels and wash the contents with benzene 2 × 25 ml. Discard the benzene and add 3 ml of a 7·5% solution of potassium iodide to each funnel. Mix, and extract the liberated iodine with 1 × 25, 1 × 15, and 1 × 10 ml of benzene. Pass each benzene extract through anhydrous sodium sulphate, collect in a 50 ml graduated cylinder, and dilute to volume with benzene. Determine the absorbance of the liothyronine solution and of the reagent blank solution against benzene at 295 mμ, using 10 cm silica cells. Subtract the reagent blank absorbance. Dilute the thyroxine sample (20 ml) to 50 ml with benzene, and determine its absorbance at 295 mμ. Dilute the reagent blank solution similarly.

Standard iodine calibration curve in benzene. This is prepared by measuring the absorbance at 295 mμ of solutions containing 80, 120, 160 and 200 μg/100 ml reagent grade iodine prepared accurately by dilution.

CALCULATION

From the standard calibration curve, determine the μg of iodine per 50 ml of solution, corresponding to the corrected absorbances found for the amino-acids.

Bulk powder:

$$\text{Liothyronine in mg/g} = \frac{\mu\text{g of iodine} \times 5}{3{\cdot}6 \times 0{\cdot}585 \times 1{,}000 \times \text{sample weight in g}}$$

$$\text{Thyroxine in mg/g} = \frac{\mu\text{g of iodine} \times 50 \times 5}{3{\cdot}6 \times 0{\cdot}655 \times 1{,}000 \times 20 \times \text{sample weight in g}}$$

Tablets:

$$\text{Liothyronine in } \mu\text{g/tablet} = \frac{\mu\text{g of iodine} \times 5}{3{\cdot}6 \times 0{\cdot}585 \times \text{number of tablets}}$$

$$\text{Thyroxine in } \mu\text{g/tablet} = \frac{\mu\text{g of iodine} \times 5 \times 50}{3{\cdot}6 \times 0{\cdot}655 \times 20 \times \text{number of tablets}}$$

Results

Table 1 presents values obtained for the liothyronine and thyroxine contents of various batches of commercial thyroid powders of porcine origin. Information permitting a statistical evaluation is also given in the form of standard deviations. The pooled standard deviation of the 40 analyses of Table 1 is 0·17 for thyroxine and 0·07 for liothyronine. All of the thyroid powders have total iodine contents between 0·8% and 1·07%. Results from the analysis of tablets containing proprietary thyroid powders are given in Table 2.

TABLE 1. RESULTS OBTAINED FOR THE LIOTHYRONINE AND THYROXINE CONTENTS OF VARIOUS BATCHES OF COMMERCIAL THYROID POWDERS OF PORCINE ORIGIN

Sample No.	Number of analyses	$\bar{x}$ Thyronine mg/g	s	v	$\bar{x}$ Liothyronine mg/g	s	v
9449	6	2·31	0·12	5·2	0·85	0·06	7·1
1744EM3	4	1·95	0·18	9·2	0·74	0·02	2·7
3557082	4	1·11	0·09	8·1	1·03	0·16	16·0
1386	4	2·34	0·12	5·1	0·77	0·03	3·9
3832	7	2·30	0·21	9·1	0·82	0·05	6·1
6829	5	2·34	0·25	10·6	0·81	0·05	6·2
6375	4	2·11	0·19	9·0	0·81	0·08	9·9
1507	6	1·45	0·06	4·1	1·20	0·08	6·7

s = standard deviation

v = coefficient of variance $= \frac{s}{\bar{x}} \times 100.$

TABLE 2. RESULTS OF ANALYSIS OF TABLETS CONTAINING PROPRIETARY THYROID POWDERS

Sample	Analysis	Thyroxine µg/tab	Liothyronine µg/tab
15 mg tablets. Lot 01063–21..	1	6·20	2·07
	2	6·45	2·18
	3	6·28	2·50
Average		6·31	2·25
s		± 0·13	± 0·23
v		± 2·1%	±10·0%
60 mg tablet. Lot 6832 ..	1	20·7	9·60
	2	20·6	10·90
	3	21·2	10·60
Average		20·8	10·37
s		± 0·3	± 0·7
v		± 1·4%	± 6·8%

s = standard deviation

v = coefficient of variance $= \frac{s}{\bar{x}} \times 100.$

Discussion

Since the analysis of thyroid preparations for liothyronine and thyroxine consists of several analytical procedures, it is convenient to discuss it in terms of the following critical parameters.

Sample preparation. Bulk thyroid powder requires no sample preparation. Thyroid tablet formulations usually contain excipients such as lactose, gelatin or magnesium stearate, which frequently comprise the major fraction of the total mass. It is therefore desirable to remove these ingredients before analysis. No appreciable loss of iodine occurs in the wash procedure described. Of the samples studied, the thyroid component of tablets was sufficiently denatured to be completely insoluble in cold or hot water. The results of the analysis of tablets, when correlated with results for the bulk powders from which they were prepared, showed no loss of liothyronine or thyroxine.

Hydrolysis. To determine if the Blau method of hydrolysis liberated maximum amounts of the two amino-acids, samples were analysed by using a modification of Devlin and Stephenson's procedure for hydrolysis

and extraction. This modification consisted of increasing by a factor of 5 their quantities of sample and reagents for hydrolysis and extraction. The extracted hydrolysate was then analysed for liothyronine and thyroxine by the method previously given. A comparison of enzyme hydrolysis with barium hydroxide hydrolysis using one sample of material gave 2·16 and 2·31 mg/g of thyroxine for the enzyme hydrolysis and the hydroxide hydrolysis respectively. For liothyronine, the figures were 0·73 and 0·85 mg/g. The Blau method of hydrolysis is retained for analysis of the two amino-acids because good agreement with enzyme hydrolysis exists, and because the hydrolysis time is only 6 hr compared with 5 days for enzymes.

Extraction. In recovery experiments, with barium hydroxide hydrolysis, n-butanol would not quantitatively extract the amino-acids at pH 3·5, as given in Blau's procedure. To obtain quantitative extraction, it was necessary to reduce the pH to 0·8. Ammoniacal methanol would not quantitatively dissolve the amino-acids if barium salts were present; these were removed from hydrolysis extracts by washing the butanol extracts with 0·1 N sulphuric acid. By using the above modifications to Blau's procedure, it was possible to recover 97–100% of the standards from the 6 hr barium hydroxide hydrolysis and n-butanol extraction.

Chromatography. Morreale de Escobar, Llorente Rodriquez, Jolin & Escobar del Ray (1963) indicated that liothyronine and thyroxine undergo deiodination during paper chromatography and subsequent handling of paper chromatograms. To minimise this effect, chromatographic papers are treated with sodium sulphite, and the solutions to be chromatographed contain 0·06% of 2,6-di-t-butyl *p*-cresol. This combination of anti-oxidants gave the best recovery values. The sulphite also caused the amino-acids to migrate much farther from the iodine band on the paper chromatogram and acts as a combustion accelerator in the oxygen flask ignition.

The solvent system 6 N ammonia:t-amyl alcohol is used because it not only separates the amino-acids from each other, but also removes them from other iodine-containing species. It also has the advantage of confining liothyronine and thyroxine to small areas. Moisture equilibration of the chromatographic papers is necessary because separation is dependent on the amount of moisture in the papers before development. If the sample spots are not saturated with water before development, developing solvent accumulates and appears held in the sample areas until the excessive amount spills down the chromatogram.

Location of the amino-acids on paper chromatograms. The method of locating liothyronine and thyroxine on paper chromatograms is described in the test for sodium thyroxine in sodium liothyronine of the sodium liothyronine monograph of U.S.P. XVI. Sufficient liothyronine is present in most thyroid preparations to allow visualisation. However, if this proves difficult, 5–10 μg of the pure amino-acid can be spotted *with* the sample solution on papers which are used for spot detection. This is because in the presence of the large amount of sample material the amino-acids do not migrate with the same Rf values as they do when pure.

Determination of liothyronine and thyroxine from developed chromatograms. The oxygen flask technique was found to be rapid, convenient, accurate and more sensitive than other methods. After ignition, all the iodine present is oxidised to iodate by potassium permanganate. Excess permanganate is quickly and easily removed with sodium nitrite which is itself destroyed by ammonium sulphamate. Sodium nitrite immediately reduces permanganate to manganous ion in acid solution but since some manganic ion may be present, a 2 min reaction period is required to ensure complete conversion. No loss of iodate has been observed during the 2 min that nitrite is present.

This procedure for determining the iodine content is sufficiently sensitive to permit the measurement of the low levels of both amino-acids found in thyroid preparations. With normal precautions, the reagent blanks are low and a precision of $\pm 2\%$ is obtained when standard solutions are spotted on paper, ignited by the oxygen flask method and the iodine determined as described. A recovery of 97·9% is obtained for 2-iodobenzoic acid (National Bureau of Standard reference sample), with a standard deviation of 1·0% for five determinations.

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