DETERMINATION OF THYROID HORMONES IN SERUM BY MEANS OF A CATION EXCHANGE RESIN AND CHLORIC ACID DIGESTION

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SUMMARY

A simple and specific method for the determination of iodoamino acids (IAA) and hormonal iodine (HI) in serum is presented. 0.5 ml serum is allowed to run through a small column with a cation exchange resin. To isolate IAA, the column is washed with water and eluted with ammonia. To isolate HI, the column is washed with borate buffer, then with water and eluted with ammonia. After removal of the ammonia by heat, the organic iodine compounds are digested with chloric acid. Iodine is assayed by ceric sulphate–arsenious acid colorimetry. Normal values for IAA ranged from 3.6 to 7.2 μg iodine/100 ml serum (mean value 5.10 μg iodine/100 ml), and for HI from 3.0 to 6.0 μg iodine/100 ml serum (mean value 4.30 μg iodine/100 ml).

INTRODUCTION

Measurement of the protein-bound iodine concentration in serum (PBI) is generally considered to be one of the most valuable tests for the detection of thyroid disorders, especially hyperthyroidism and hypothyroidism. The test has become practicable in the routine clinical laboratory by the introduction of the alkaline ashing method[1] and especially the simpler chloric acid technique[2].

The term PBI, currently used, is actually somewhat misleading. It is used to designate the iodine compounds precipitated together with the serum proteins. These include in the first place the thyroid hormones, thyroxine (T_4) and triiodothyronine (T_3), which normally account for about four fifths or more of the total PBI. The remainder presumably consists of iodoproteins and, depending on the nature of the precipitant, a greater or lesser part of the precursors mono- and diiodotyrosine (MIT and DIT).

Determination of the butanol-extractable iodine (BEI) proved more specific than the PBI, but the method is rather cumbersome[3–5]. Specific methods for the determination of the thyroid hormones have been published which incorporate chromatography on paper[6–8], thin layer[9], columns[9], anion exchange resin[10,11], and

the binding of thyroxine to a specific serum protein[12]. They are, however, still rather complicated for use in a routine clinical laboratory.

Blanquet *et al.*[13] briefly described the use of a cation exchange resin, Dowex 50 W×2, for the separation of (radioactive) iodoamino acids from other radioiodinated compounds in serum. This method has since been used by one of us for the concentration of labelled iodoamino acids in serum or urine for paper chromatography[14–16]. The present paper describes its adaptation to the chemical determination of the total amount of iodoamino acids (further referred to as IAA) as well as the concentration of $T_3 + T_4$ (further referred to as hormonal iodine or HI) in serum. The method is thought to compare favourably with existing procedures for its simplicity and reliability. Anticipating a forthcoming report, it can further be said that a number of organic iodine contaminants do not interfere with the method.

APPARATUS

(1) *Glass columns*, 6–7 mm inner diameter, about 12 cm long, tapered at one end. Pasteur pipettes (capillary cut off) will suit the purpose. (2) *Aluminium block*, electrically heated and thermostatically controlled, with 30 holes to accommodate colorimeter tubes and a thermometer, and with adjustable legs so as to permit the block to be tilted at an angle of about 60°. (3) *Mechanical test tube mixer.* (4) *Water bath*, thermostatically controlled. (5) *Colorimeter.*

REAGENTS

All reagents are prepared from analytical grade chemicals with glass distilled water. Demineralized water may or may not be found pure enough.

(1) Dowex 50 *W×2*, 200–400 mesh, H^+-form.

(2) Boric acid 0.4 M: 24.7 g H_3BO_3 per 1000 ml.

(3) Sodium tetraborate 0.1 M: 38.1 g $Na_2B_4O_7 \cdot 10\ H_2O$ per 1000 ml.

(4) Borate buffer pH 8.5: 45 ml soln. (2) plus 55 ml soln. (3).

(5) Ammonia 5 N: 375 ml 25% ammonia per 1000 ml.

(6) Carborundum, or a similar anti-bumping material, is steeped in acid dichromate and thoroughly rinsed with water.

(7) Chloric acid: 600 g $KClO_3$ are dissolved in 1200 ml water in a 3-l beaker by heating in a well-drafted fume hood. To the hot solution are added, with constant stirring, 500 ml $HClO_4$ (70–72%). The resulting green-yellow suspension is allowed to cool and refrigerated overnight. The chloric acid solution is decanted (or filtered through glass wool) while still cold and stored in a refrigerator. The yield is about 900 ml.

(8) Arsenious acid 0.12 N: 1.48 g As_2O_3 are dissolved in a mixture of 16 ml HCl ($d = 1.19$) and about 125 ml water by heating on a water bath. After cooling 60 ml H_2SO_4 ($d = 1.84$) are slowly added and the solution is diluted to 250 ml.

(9) Ceric ammonium sulphate 0.03 N: 4.74 g $Ce(SO_4)_2 \cdot 2(NH_4)_2SO_4 \cdot 2\ H_2O$ are dissolved in a mixture of 8 ml H_2SO_4 ($d = 1.84$) and about 150 ml water, and the solution is diluted to 250 ml.

(10) Standard iodate solution: 84.3 mg dried KIO_3 are dissolved in water to a volume of 100 ml (soln. A); 1 ml soln. A is diluted to 100 ml (soln. B); 1 ml soln. B

is diluted to 250 ml (standard solution). The standard solution contains 0.02 μg iodine/ml (equivalent to 4 μg iodine/100 ml serum). Suitable standards, *e.g.* equivalent to 0, 2, 4, 8 and 12 μg iodine/100 ml serum are run with each batch of sera.

TECHNIQUE

Preparation of columns

A small plug of glass wool is placed in the column. A suspension of the resin in water is poured into the column to a depth (when settled) of about 2.5 cm. After washing with successively 5 ml 1 *N* HCl and 5 ml water, the column is ready for use. The columns do not rapidly dry up, but when not in use for a longer period (more than one day), they are best kept in a beaker filled with distilled water to about the upper edge of the resin in the column. The same resin, which is regenerated by washing with successively 2 ml water, 5 ml 1 *N* HCl and 5 ml water, may be used for at least 50 determinations.

Column chromatography

(1) Isolation of IAA: 0.5 ml serum is allowed to run through the column. The column is washed with 5 ml water and the iodoamino acids are eluted with 2 ml 5 *N* ammonia into a colorimeter tube.

(2) Isolation of HI: 0.5 ml serum is allowed to run through the column. The column is washed with successively 8 ml borate buffer pH 8.5 and 2 ml water. $T_3 + T_4$ are eluted with 2 ml 5 *N* ammonia into a colorimeter tube.

(3) Column "blank": 2 ml 5 *N* ammonia are passed through the column and collected into a colorimeter tube, which is carried through as a reagent blank.

Removal of ammonia

After addition of some carborundum to the colorimeter tubes, the ammonia is completely removed by heating in the aluminium block (tilted at an angle of 60° to prevent spattering) at a temperature rising from 90° to 115° in about half an hour. The tubes are then removed from the block.

Digestion

From this point standards are put through with the samples, prepared as above. Standards are prepared by pipetting 0, 0.5, 1.0, 2.0 and 3.0 ml of the standard iodate solution into tubes, containing some carborundum. To the standards and the samples 1.0 ml chloric acid is added and the tubes are replaced in the block, previously heated to 115°. The temperature is then gradually raised in about 1 h to 135–145°. During the digestion, which needs not be closely watched, the solution first becomes brightly yellow and then colourless. This indicates the end point of the digestion and the tubes, containing a constant volume of about 0.2 ml, are removed from the block.

Iodine determination

After cooling, 4.0 ml water and 0.5 ml arsenious acid are added and the tubes are placed in a 27° water bath for about 10 min. In sequence, at 30-sec intervals, 0.5 ml ceric ammonium sulphate is added to each tube. Immediately after the addition of the ceric reagent to a tube, the contents are carefully mixed and the tube is rapidly

replaced in the water bath. Exactly 15 min after the addition of the ceric reagent, the transmittance is read at 420 nm against a water blank.

Calculation

A standard curve is constructed by plotting the transmittance readings of the standards against the equivalent μg iodine/100 ml. The IAA and HI values of the samples (in μg iodine/100 ml serum) are read from the graph and corrected for the blank.

RESULTS

Development of the procedure

Samples of 0.5 ml serum with added amounts of labelled KI, iodotyrosines or iodothyronines were allowed to run through 2.5×0.6 cm columns of Dowex 50 W $\times$ 2 (H^+-form). Most of the serum components, including iodide which is not adsorbed on the column, were readily washed away with 5 ml water. The iodoamino acids were not displaced and remained on the columns. Both iodotyrosines and iodothyronines were eluted with ammonia. 1 *N*, 3 *N* and 5 *N* ammonia were increasingly effective; with 2 ml 5 *N* ammonia over 98% of the iodoamino acids was recovered. The iodotyrosines could be selectively eluted from the column with borate buffers of pH varying from 7.6 to 8.5. Buffer of pH 8.5 proved most efficient; 8 ml eluted more than 90% of MIT and DIT. Less than 5% of T_3 and T_4, on the other hand, was eluted by this wash. By subsequent washing with 2 ml water (to remove excess buffer) and eluting with 2 ml 5 *N* ammonia, approximately 94% of the labelled iodothyronines was recovered.

Prior to digestion of the ammonia eluates with chloric acid, it was necessary to completely remove the ammonia. Firstly, because most of the chloric acid is precipitated when directly added to the eluates. Secondly, because ammonia was found to exert a marked inhibitory effect on the ceric–arsenite reaction. It was demonstrated that heating the eluates to 115° completely removed the ammonia without concomitant loss of iodine. When ammonia was passed through a column "blank" and further processed in the usual way, only 1 ng iodine (equivalent to 0.2 μg/100 ml) was repeatedly found. Therefore, 0.2 μg iodine/100 ml has since been routinely deducted from all values with an occasional check.

The IAA and HI eluates form a highly purified hormone solution, containing only a few mg of organic material. As the samples are digested with 1 ml chloric acid, a large excess of oxidant will be present throughout the procedure. Therefore, the digestion needs not be closely watched and addition of chromate prior to the digestion or chloric acid during the reaction is unnecessary. The end point of the digestion is clearly indicated by the disappearance of the yellow colour (chlorine), the excess of chloric acid being converted to perchloric acid which is stable at the prevailing temperature (135–145°). Perchloric acid was found to act strongly upon the catalytic activity of iodine, the observed effect being very similar to that described by Hoch *et al.*[17]. As a consequence it is essential that the digests of standards, blank, IAA and HI samples contain equal amounts of perchloric acid. From the weight and the refraction of the digests it was indeed found that a constant volume of approximately 60% perchloric acid by weight was left in all tubes.

The colorimetric reaction has been analyzed in many previous papers[18,19]. This obviates the need for a discussion of the various parameters which determine the sensitivity of the reaction. An S-shaped standard curve is obtained which is linear in the transmittance range from 15 to 70%. Temperature and reaction time were adjusted such, that transmittance readings for 2 to 10 μg iodine/100 ml cover this range and most of the samples could be read from the linear part of the graph.

To study recovery values, 0.5 ml serum samples with or without added amounts of stable MIT, DIT, T_3 and T_4 were processed as described under TECHNIQUE. HI values were not influenced by the addition of 10 μg MIT or DIT per 100 ml, and neither IAA nor HI by 100 μg iodide per 100 ml. Furthermore, 98 to 101% of all 4 iodoamino acids was recovered in the IAA and 92 to 95% of T_3 and T_4 in the HI.

Normal values of IAA and HI

IAA was assayed in samples taken from a group of 100 healthy subjects (hospital personnel); in 77 of the samples HI was simultaneously analyzed. Subjects found to have used iodinated compounds or to take drugs known to influence the circulating thyroid hormone level (such as oral contraceptives) were not included. Nearly all the analyses were done in triplicate. In Fig. 1 the IAA values are plotted against the equivalent HI values, and in Table I the results are summarized.

Over 95% of all values were found to range from 3.6 to 7.2 μg iodine/100 ml serum for IAA and from 3.0 to 6.0 μg iodine/100 ml serum for HI. These values have been adopted as normal ranges. The distributions of the IAA and HI values in the group are shown in Fig. 2. In concordance with earlier observations on PBI, the values were not normally distributed.

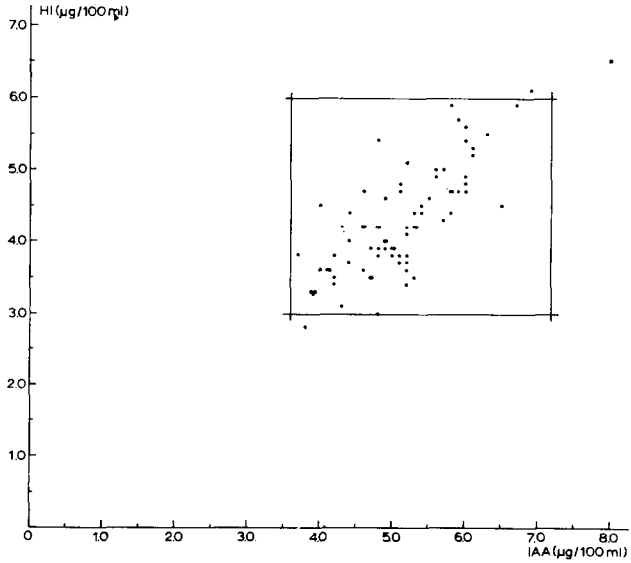

Fig. 1. Correlation between IAA and HI values in a group of healthy subjects. Rectangle indicates adopted normal values.

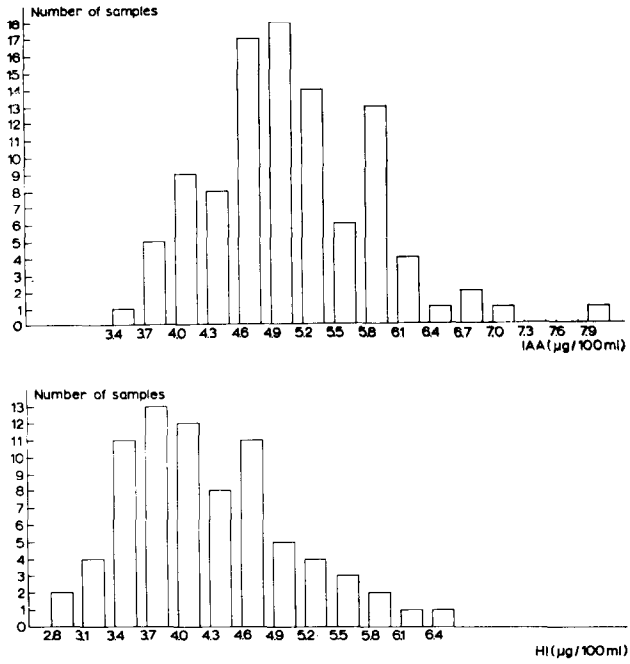

Fig. 2. Distribution of IAA and HI in a group of healthy subjects.

TABLE I

NORMAL VALUES OF IAA AND HI
(μg iodine/100 ml serum).

	*Number of subjects**	*Mean value*	*Range*	*Adopted normal range*
IAA	100 (98)	5.10	3.4–8.0	3.6–7.2
HI	77 (74)	4.30	2.8–6.5	3.0–6.0

* Numbers in brackets denote subjects in adopted normal range.

Comparison of IAA and HI

Fig. 3 shows the distribution of the differences between IAA and HI in the group of 77 subjects, assayed for both IAA and HI. IAA values were slightly higher than HI values, with a few exceptions due to the error of the method. The differences were found to be normally distributed (χ^2 test). The average difference was 0.8 μg iodine/100 ml serum (range −0.6 to +2.0) with a standard deviation of 0.5 μg iodine/100 ml serum and a standard error of the mean of 0.06 μg iodine/100 ml serum. For the group as a whole, therefore, it can be stated that the difference between IAA and HI is significant and amounts to 16% (or 12% when corrected for the loss of HI in the buffer washing) of the IAA value. The difference between IAA and HI is thought to represent iodotyrosines or iodotyrosine-like compounds. The presence, amount, nature and origin of these compounds are still a matter of controversy[15], beyond the scope of this paper to discuss.

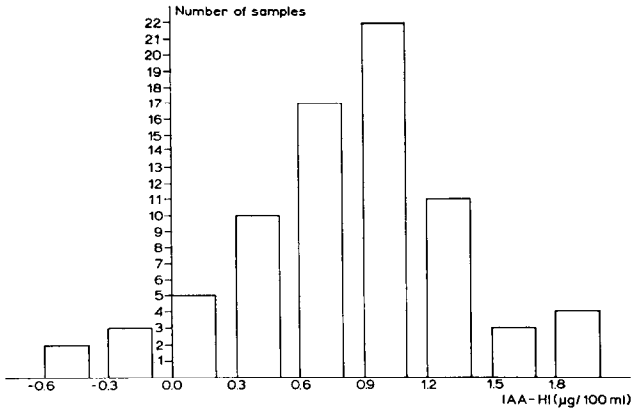

Fig. 3. Distribution of the difference between IAA and HI in a group of healthy subjects.

Comparison of IAA and PBI

IAA was assayed in 101 specimens of 92 patients sent to the routine clinical laboratory for PBI determination (alkaline ashing method[1]). Patients known to have received organic iodine or large amounts of iodide were excluded. PBI determinations were done in duplicate, and IAA, with a few exceptions, in triplicate. In Fig. 4 the IAA values obtained are plotted against the equivalent PBI and in Table II the data are summarized.

TABLE II

COMPARISON OF IAA AND PBI IN PATIENTS WITH LOW (< 4.1), NORMAL (4.1–8.0) OR HIGH (> 8.0) PBI

All values are in µg iodine/100 ml serum.

PBI-group	*Number of samples*	*PBI*		*IAA*		*PBI minus IAA*
		mean	*range*	*mean*	*range*	
low	16	3.24	1.3– 4.0	3.26	1.0– 4.4	−0.01
normal	66	5.79	4.1– 7.7	5.48	2.3–10.1	+0.32*
high	19	10.36	8.1–15.9	9.39	6.0–16.2	+0.96*

* IAA exceeded PBI by more than 1.5 µg iodine/100 ml serum in only 5 out of 101 cases.

Apart from the low PBI group, PBI values were slightly higher than IAA values. The difference is thought to represent protein-like iodine compounds. Especially in hyperthyroidism, the presence of (labelled) iodoproteins in serum of patients, receiving a tracer dose of radioiodine, has been demonstrated[20]. Iodoproteins will not interfere in the determination of IAA (or HI), as they are removed together with the serum proteins in the column procedure. In the determination of PBI, on the other hand, they will be found with the hormones after serum protein precipitation.

Comparison of HI with related methods

In Table III normal values for HI and the ratio of the mean value to PBI are compared with those for BEI and the T_3+T_4 fraction of serum, as found by other authors using specific methods which incorporate chromatography on paper[6–8], thin

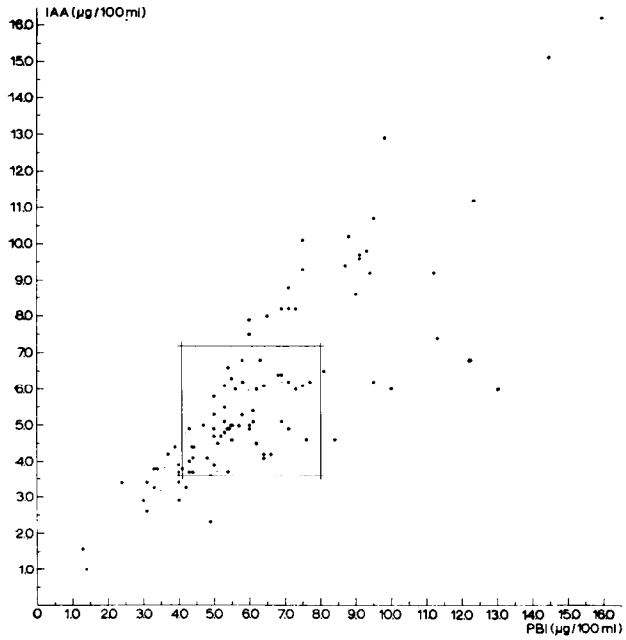

Fig. 4. Correlation between IAA and PBI in random sera. Rectangle indicates normal PBI group.

TABLE III

COMPARISON OF HI AND RELATED METHODS

All values are in *µg* iodine /100 ml serum and are uncorrected for recovery.

Method	*Isolation of hormones*	*Normal range*	*Mean value*	*Mean value as % of PBI*
HI	cation exchange resin	3.0–6.0	4.3	80
BEI	butanol extraction[21]	3.2–6.4	4.3	88
T_3+T_4	paper chromatogr.[8]	3.1–6.5	5.2	91
T_3+T_4	anion exchange resin[10,22]	as BEI	—	90
T_3+T_4	anion exchange resin[11]	—	—	85
T_3+T_4	thin layer and column[9]	3.2–7.4	5.2	83
T_4	binding by TBG[12]	2.6–7.2	4.3	74

layer or columns[9], anion exchange resin[10,11], and the binding of thyroxine to a specific serum protein[12].

HI values are in good agreement with BEI and most of the T_3+T_4 values reported by other authors. It will be seen from the table that the mean values (uncorrected for recovery) generally account for 80 to 90% of the normal PBI level.

Conclusions

It may be concluded that for IAA the specificity is superior to PBI and for HI equal to BEI and the other T_3+T_4 methods. The procedure for determining IAA and HI, however, is thought to compare favourably with the existing methods on behalf of the following points:

(1) the isolation of hormones is simple and time-saving: serum without any

further treatment is used, the washing procedure is performed with small volumes, the columns do not rapidly dry up and are used many times.

(2) the digestion needs not be closely supervised.

(3) minimal sample handling considerably reduces the risk of cross contamination.

(4) 12 assays (or 24, when 2 aluminium blocks are simultaneously put in operation) can easily be performed in duplicate by one technician in about 7 h.

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