NOTES

Separation and Determination of Thyroid Hormones in Blood Serum

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Parallel to the determination of the thyroxine (T_4) content of blood serum in the last few years, the determination of the triiodothyronine content has also become increasingly important. While there are already a number of reliable methods of analysis available for the determination of T_4 (*1–4*), there is still a lack of fast and simple methods of determining the T_3 content. The difficulties in the T_3 determination are due to the fact that the content of T_3 in the serum (normal range: 90 to 170 ng/100 ml blood serum) is no more than between 1 and 2% of the T_4 content in the serum.

In the chemical methods of T_3 determination, T_3 is isolated from the blood serum in more or less pure form through chromatographic procedures. The T_3 determination is then achieved by means of competitive protein binding analysis (*5*) or catalytic iodine determination (*6, 7*). For the competitive protein binding analysis, the T_3 which has been separated may have only a very small T_4 content, as the binding protein shows far greater affinity to T_4 than to T_3. The classical catalytic iodine determination, on the other hand, has already reached the limit of its usefulness for the T_3 determination.

The present paper represents an attempt to develop a simple chromatographic method of isolating and separating T_3 and T_4 from the blood serum. In conjunction with a sensitive automated measuring system for the catalytic determination of the thyroid hormones (*8*), this should make possible a routine determination of the content of T_3 and T_4, respectively, in blood serum.

EXPERIMENTAL

Apparatus. For measuring the thyroid hormones labeled with ^{131}I, a Philips scintillation counter was used. The catalytic measurement of the thyroid hormones was done by means of the apparatus described by Knapp and Leopold (*8*).

Reagents. *Ion Exchange Resins.* AG 1 ×2, 200- to 400-mesh (Bio-Rad Laboratories, Richmond, Calif.), was eluted with acetic acid (7*M*). QAE-Sephadex A-25 and SP-Sephadex C-25 were obtained from Pharmacia-Uppsala.

Radioactive Compounds. Liothyronine-^{131}I](3,5,3-triiodo-L-thyronine) and L-thyroxine-^{131}I(Radiochemical Centre Amersham) were purified by means of gel filtration with Sephadex G-25 fine (*9*). All the other reagents used were of analytical grade purity and were used without further purification.

Procedure. *Optimizing the Parameters.* Before discussing in detail the method of separation, it is necessary to briefly deal with the basic factors that make a separation of the thyroid hormones on ion exchange resins possible. Ortner *et al.* (*10*) have thoroughly investigated these parameters.

For the separation of T_3 and T_4 on ion exchangers, two fundamentally different effects are of importance. First of all, the ion exchange properties of the two hormones that may be characterized by their pK values must be considered. T_3 and T_4 differ most in the pK values of their phenolic OH-groups (6.73 for T_4 and 8.45 for T_3), while the pK values of their COOH groups and NH_2 groups, respectively, show practically no differences (*10*). This is why a separation of the two hormones using anion exchangers promises to yield especially favorable results.

The other important effect is the affinity of the exchanger substance for the non-ionogenic areas of the hormone molecules. It is generally known that especially phenols in exchangers with aromatic matrices are subject to strong non-ionogenic powers of adsorption. In ion exchangers with polysaccharide matrices (such as QAE-Sephadex A-25), considerably less non-ionogenic adsorption occurs, which makes it much more likely to achieve separation of the two hormones. In addition, the non-ionogenic adsorption may be influenced by varying the electrolytes used for adjusting the pH and especially by using an organic solvent component.

Given these basic facts, the following parameters were investigated.

Ion Exchangers. The separation of T_3 and T_4 on QAE-Sephadex A-25 has already been mentioned (*7, 10*). Much better results can be achieved, however, by using a mixed-bed exchanger consisting of QAE-Sephadex A-25 (anion exchanger) and SP-Sephadex C-25 (cation exchanger). By carefully choosing the mixing proportions, it is possible to achieve quite specific properties of the mixed-bed exchanger. With increasing amounts of QAE-Sephadex, the hormones become more difficult to elute, but the separation efficiency improves. The flow velocity of the eluent also depends on the composition of the mixed-bed exchanger. It reaches its maximum if the mixing ratio is 50:50, and decreases toward either end.

Eluting Solutions. The eluent consists of an aqueous organic system set at a certain pH by means of an electrolyte. The following organic systems were compared with each other: tetrahydrofuran, tetrahydrofurfuryl alcohol, acetone, propan-2-ol, and ethanol. The separation properties of acetone and ethanol are below those of the other three substances. For reasons of economy, we continued to use propan-2-ol as the organic phase, and a mixing ratio of 30% propan-2-ol in H_2O was found to give optimum separation efficiency.

The electrolyte in the eluting mixture is also of great importance. The substances tested were acetate, phosphate, borate, and maleate. A buffer of maleic acid and tris-(hydroxymethyl)-methylamine resulted in by far the best elution and separation properties.

Figure 1 illustrates the way in which different pH values of the eluent affect the separation of T_3 and T_4. In these experiments, a

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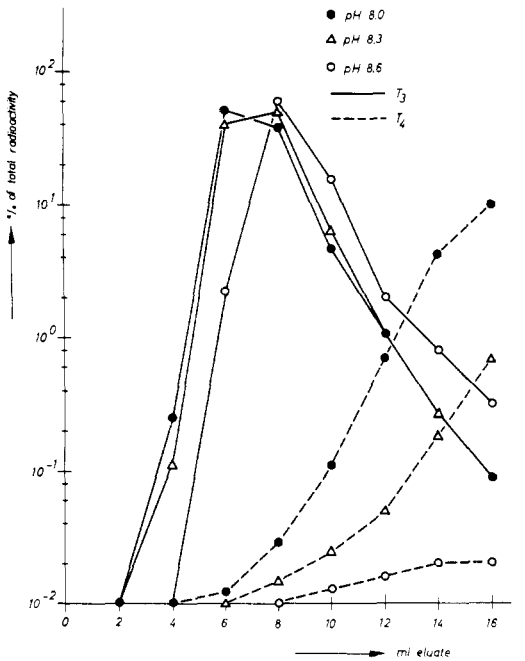

Figure 1. Separation of T_3 from T_4 related to the pH of the eluent

mixed-bed exchanger (QAE:SP = 60:40) was used in a column 10 mm in diameter and 40 mm long. As was to be expected, increased pH slowed down the T_3 elution. At pH values below 8.3, already considerable quantities of T_4 were eluted.

The chromatographic procedure comprises the following steps: the isolation of the hormones T_3 and T_4 from the proteins in the blood serum, the separation of T_3 and T_4 from each other, and the concentration of the separated hormones.

The isolation of the thyroid hormones from the carrier proteins of the blood serum and the separation of the two hormones T_3 and T_4 from each other cannot be accomplished on one single column. When isolating the hormones from the blood serum at pH 12, the T_4 does not remain quantitatively at the upper end of the ion exchanger column, but some of it is carried down into the lower part of the column by the carrier proteins. Thus, when the T_3 is eluted next, there will always be too great a quantity of T_4 eluted with it. In order to eliminate these difficulties, it is necessary to do the isolating on as small a column as possible and to connect to this isolation column a second column, the separation column, before separating the two hormones from each other. Figure 2 shows the elution behavior of T_3 and T_4 when using an isolation column (QAE:SP = 30:70, column dimension 7 mm in diameter by 30 mm in length) and a separation column (QAE:SP = 60:40, column dimension 10 mm in diameter by 40 mm in length). T_3 was eluted at pH 8.3 and T_4 at pH 6.7.

The separated hormones are present in a comparatively large quantity of eluent (10 and 12 ml, respectively) and must therefore be concentrated. For this, the anion exchange resin AG 1 ×2 is used (column dimension 7 mm in diameter, 10 mm in length). Since the resin bed is not very thick, it needs to be covered with a piece of filter paper. The eluted material from the separation column is passed through these concentration columns where the hormones are held back by the exchange resin. With as little as 0.6 ml of acetic acid (12*M*), 90% of the hormones from the concentration column can be eluted.

Preparation of the Column Reagents. Three different solutions are necessary for preparing the column reagents. Solution 1 is obtained by dissolving 4 grams of NaOH in 1 l. of a mixture of 30% by volume propan-2-ol in H_2O. Solution 2 is prepared by dissolving 12.1 grams of tris(hydroxymethyl)-methylamine and 11.6 grams of maleic acid in 1 l. of H_2O, and Solution 3 by dissolving 12.1 grams of tris(hydroxymethyl)-methylamine and 11.6 grams of maleic acid in 1 l. of a mixture of 30% by volume propan-2-ol in H_2O.

Column-reagent solutions are combined as follows: Column-reagent A, 0.02N NaOH; B, Solution 2 is adjusted to pH 8.3 with 0.1*N* NaOH; C, Solution 3 is adjusted to pH 8.3 with solution 1; D, Solution 3 is adjusted to pH 6.7 with solution 1; E, acetic acid (2.5*M*); and F, acetic acid (12*M*).

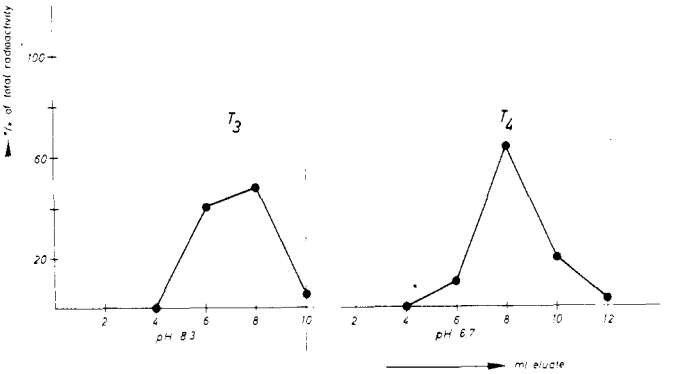

Figure 2. Separate elution of T_3 and T_4 from a combination of isolation and separation columns

Preparation of the Columns. *Column 1.* Columns 7 mm in diameter are filled to a depth of 40 mm with a mixed-bed exchanger (70% SP-Sephadex C-25 and 30% QAE-Sephadex A-25 swelled in 0.02*N* NaOH). *Column 2.* Columns 10 mm in diameter are filled to a depth of 40 mm with a mixed-bed exchanger (40% SP-Sephadex C-25 and 60% QAE-Sephadex A-25 swelled in column-reagent B). *Column 3.* Columns 7 mm in diameter are filled to a depth of 10 mm with an anion exchange resin (AG 1 ×2, 200–400 mesh, eluted with acetic acid 7*M*). A piece of filter paper is placed on top of the exchange resin.

Chromatographic Procedure. a) Two milliliters of blood serum is mixed with 10 ml of column-reagent A and are fed onto column 1 after approximately 20 minutes. T_3 and T_4 are retained on the column, while most of the serum proteins are eluted.

b) The remaining proteins are washed out with 5 ml of column-reagent B, while at the same time column 1 is adjusted to the separation pH value of 8.3.

c) Now columns 3, 2, and 1 are placed one on top of the other. With 2- × 5-ml column reagent C, the T_3 is washed onto column 3, while T_4 is retained on the columns 1 and 2.

d) Immediately after completing this step, a new column 3 is placed under columns 1 and 2 and the T_4 is washed onto this new column 3 with 2- × 6-ml column reagent D. T_3 and T_4 are now present separately, each one on a column 3.

e) These columns are washed with 5 ml of column-reagent E and 0.3 ml of column-reagent F, thereby removing not only the buffer substances, but also any MIT or DIT, respectively, which may be present (*1*).

f) The hormones are now eluted with 0.6 ml of column-reagent F each.

The eluted T_3 is then evaporated in a vacuum at room temperature and afterwards automatically measured after the catalytic reagents have been added.

The T_3 can also be measured without this concentration by evaporation, directly in the acetic acid, if bromine has been added before (*11*). Saturated bromine water is diluted with distilled water in a ratio of 1:50 (vol/vol). One hundred microliters of this diluted solution is then added to the eluted T_3, and, after an incubation period of 5 minutes, the T_3 is catalytically measured.

The T_4 is mixed by a brief shaking. Then aliquot amounts of 0.2 ml are withdrawn and measured after the catalytic reagents have been added.

RESULTS AND DISCUSSION

To obtain useful results for the T_3 analysis, it is of paramount importance that the T_4 content of the eluted T_3 be extremely small. When using the separation method herein described, the T_4 content in the eluted T_3 is always less than 0.05%.

The yield of the chromatographic procedure was analyzed with blood serum from healthy people as well as with serum from pathological cases (hypo- and hyperthyroidism). No significant difference was found. The yield is 80 ± 2% for the T_3 and 85 ± 2% for the T_4 (mean ± std dev, N = 32). Thus a control procedure by means of a radioactive tracer is not necessary.

The measuring range of this method depends on two factors: the amount of eluted material being measured,

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Table I. Normal Ranges of Various Methods of Determining T_3 and T_4

T_3, ng/100 ml blood serum	T_4, μg/100 ml blood serum	Method	Authors
138 ± 23 (N = 82)[a]		Radioimmunoassay	T. Mitsuma *et al.* (*12*)
137 ± 23 (N = 82)		Gas–liquid chromatography	T. Mitsuma *et al.* (*12*)
145 ± 25		Radioimmunoassay	J. Lieblich *et al.* (*13*)
146 ± 20 (N = 23)	7.65 ± 1.07 (N = 23)	Liquid chromatography	K. Horn *et al.* (*6*)
134 ± 21 (N = 76)	7.58 ± 1.28 (N = 76)	Liquid chromatography	G. Knapp *et al.*

[a] (mean ± standard deviation).

and the measuring parameters (measuring period and temperature of reaction) of the digital apparatus for catalytic measurement of the thyroid hormones (*8*).

The most suitable parameters for the catalytic determination of T_3 proved to be 20 °C and 160 sec; for the determination of T_4 they were 20 °C and 40 sec. The measuring range for the T_3 determination extends from 0.02 to 3.00 μg of T_3/100 ml blood serum. The measuring range of the T_4 determination can be varied greatly by measuring any portion of the eluted T_4. If 0.2 ml of the eluted T_4 is measured, then the measuring range extends between 0.2 and 30 μg T_4/100 ml of blood serum.

The precision of the method was tested by means of a pool serum, whose results were within the normal range. Measurement of the T_3 yielded 0.12 ± 0.012 μg/100 ml and of the T_4, 6.6 ± 0.4 μg/100 ml blood serum (mean ± std dev, N = 20).

Furthermore, T_3 and T_4 determinations were also performed for 76 healthy subjects to establish the normal range of the method. Table I shows a comparison of these test values with results obtained by other authors using immunological or chromatographic methods.

The method described has proven to be of great value especially in routine clinical analysis, as it can be quickly and easily performed. The time needed for the complete separation cycle, including concentration by evaporation of the eluted T_3, is approximately 3 hours.

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Isobutane Chemical Ionization Mass Spectra of Lanthanide Perfluorinated β-Diketonates

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The isobutane chemical ionization mass spectra of the lanthanide 2,2,6,6-tetramethylheptane-3,5-dionates [Ln(thd)$_3$] have previously been described (*1*). This work has shown the intrinsic potential of chemical ionization mass spectrometry (CIMS) over electron impact mass spectrometry (EIMS) for the analysis of metals. The CI mass spectra contained no fragmentation in the region of the parent ion as compared to the EI mass spectra which contained extensive fragmentation. In order that metals may be studied by EIMS and CIMS volatile complexes must be prepared, and this earlier study used the non-fluorinated ligand 2,2,7,7-tetramethylheptane-3,5-dione [H(thd)]. This ligand was used as it was felt that it would probably be less susceptible to fragmentation than fluorinated β-diketones for no stable fragments such as hydrogen fluoride could be lost.

In the present study, a fluorinated ligand has been used (1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-4,6-octanedione [H(fod)]) to complex the lanthanide elements. These chelates have been previously studied by gas chromatography (*2*) and electron impact mass spectrometry (*3, 4*).

A representative fragmentation pattern before the mass spectrum becomes too complicated is shown below (*4*):

$[Ln(fod)_3]^+$	(Mol wt = 0)
$[Ln(fod)_3-(CH_3)]^+$	(Mol wt = 15)
$[Ln(fod)_3-(F)]^+$	(Mol wt = 19)
$[Ln(fod)_3-(F_2)]^+$	(Mol wt = 38)
$[Ln(fod)_3-(C_3H_8)]^+$	(Mol wt = 44)
$[Ln(fod)_3-(C_4H_9)]^+$	(Mol wt = 57)
$[Ln(fod)_3-(C_3F_7)]^+$	(Mol wt = 169)
$[Ln(fod)_2]^+$	(Mol wt = 295)

The extensive fragmentation produced by EIMS, although desirable for the elucidation of molecular structure, is a distinct limitation for the ultra-trace analysis of mixtures.

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