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PICOGRAM ANALYSIS OF FREE TRIIODOTHYRONINE AND FREE THYROXINE HORMONES IN SERUM BY EQUILIBRIUM DIALYSIS AND ELECTRON CAPTURE GAS CHROMATOGRAPHY

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SUMMARY

A methodology involving equilibrium dialysis and gas chromatography has been developed to measure concurrently the concentration of the dialyzable (*i.e.*, free) fractions of thyroxine and 3,5,3′-triiodothyronine in serum. The electron capture response of the N,O-diheptafluorobutyryl methyl ester derivatives of these hormones allows their detection and quantitation in the picogram range (detection limit approx. 0.2 pg). Purification of the dialysate is achieved using a cation-exchange column which is pretreated with diiodothyronine. Adsorption problems on the glassware are eliminated by silanization. The methodology also resolves 3,3′,5′-triiodothyronine (reverse T_3).

INTRODUCTION

At present, two active thyroid hormones have been found in significant quantities in human serum, thyroxine (T_4) and 3,5,3′-triiodothyronine (T_3). A related substance which has attracted recent interest because of its different serum levels in various normal and diseased states is 3,3′,5′-triiodothyronine or reverse T_3 (RT_3). The major fractions (more than 99%) of each of these substances in serum are reversibly bound to proteins such as thyroxine-binding globulin, albumin, and thyroxine-binding prealbumin. However, it is the free (unbound) forms of T_3 and T_4 (FT_3 and FT_4), present only in trace amounts (generally 1–50 pg/ml), which provide the most indicative index of thyroid function[1].

The analysis of the trace levels of the free thyroid hormones in serum presents a major analytical challenge. Not only is the serum sample itself extremely complex and limited in supply, but the free hormones are engaged in a facile equilibrium with a vastly greater amount of bound hormones. Even a minor perturbation of this equilibrium causes the reported levels of FT_3 and FT_4 to change. Further, the large molecular weights of these hormones (near 1000), the lability of their iodine atoms,

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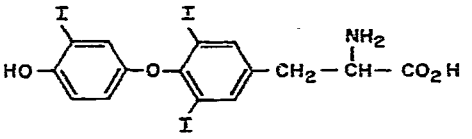
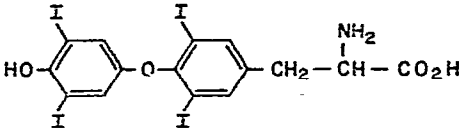
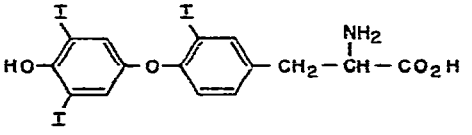


T_3



T_4



REVERSE T_3

their tendency to adsorb onto solid surfaces, and their presence and availability in only trace amounts in serum (in the free form) impose additional constraints.

The problem of isolating free from protein-bound hormone without significantly perturbing the equilibrium involved has been approached most effectively by means of equilibrium dialysis[2], in spite of certain shortcomings, which have been summarized[3]. Ultrafiltration and brief exposure to a binding agent, such as Sephadex, have also been used[3,4].

Due to the trace levels of the free hormones in serum, a variety of methods based on radiochemical techniques have been selected for estimating and monitoring FT_3 and FT_4 (refs. 2–4). While these techniques are currently employed, they suffer certain disadvantages, among which may be the need for accompanying assays of the total amounts of T_3 and T_4, the necessity to work with radioactive materials and their degradation products, the requirement for short half-lived radioisotopes such as ^{125}I because of the need for high specific activities, and the extra work and reagents involved in assaying for both FT_3 and FT_4.

Gas chromatographic (GC) analysis with electron capture detection (GC–ECD) of volatile derivatives of the thyroid hormones has been reported; however, the detection limits to date have only permitted the analysis of the total amounts of T_3 and T_4 in 1 ml of serum[5–12]. Nevertheless, the structure of the hormones, including at least three iodine atoms, would lead one to expect that with the appropriate derivatives and detector noise levels GC–ECD ought to achieve the required detection limits for the analysis of free forms.

This paper reports the successful concurrent analysis of the free thyroid hormones in 1 ml of serum using the GC separation of the N,O-diheptafluorobutyryl methyl ester derivatives with ECD at the required low noise level. Detection limits of 0.2 pg have been achieved. The method utilizes equilibrium dialysis to remove the free thyroid hormone fraction from serum, cation-exchange clean-up of the dialysate, derivatization, and GC–ECD.

EXPERIMENTAL

Apparatus

A Hewlett-Packard Model 5730A gas chromatograph (Avondale, Pa., U.S.A.) equipped with a variable frequency, constant current mode ECD was used. The radioactive source was 15 mCi ^{63}Ni plated directly on the interior of the cell. A 6 ft. × 4 mm I.D. glass column packed with 3% SE-30 on 80–100 mesh Supelcoport (Supelco, Bellefonte, Pa., U.S.A.) was used throughout. The injector and column were maintained at 250° and the detector at 300°, at which the response to the thyroid hormones was found to be optimized. A carrier gas of 5% methane and 95% argon was maintained at 60 ml/min.

Mass spectra of the thyroid hormone derivatives were obtained using a Nuclide 12-90-G single focusing magnetic deflection mass spectrometer (Nuclide, State College, Pa., U.S.A.). The accelerating potential was 3 kV, the electron energy was 70 eV, and the temperature of the ion source was maintained at 250°.

Reactivials (0.3, 1.0, 3.0, and 5.0 ml) from Pierce (Rockford, Ill., U.S.A.) were used to form the derivatives and perform the dialyses. Hamilton microliter syringes (10, 50, and 100 μl) (Reno, Nev., U.S.A.) were employed for all microtransfers and additions. A Cahn 4100 electrobalance (Ventron, Paramount, Calif., U.S.A.) was employed to weigh microgram quantities of the derivative standards.

An IN-V-TRON 2000 (Nuclear Systems, Chicago, Ill., U.S.A.) gamma counter was used to count radioactive T_4 in the experiment dealing with adsorption losses.

The dialysis membranes were Visking precision Nojax cellulose casing (Union Carbide, Chicago, Ill., U.S.A.). Minienert caps equipped with septum injection ports (Supelco) were used to seal the vials which contained solutions to be injected into the gas chromatograph. Polyethylene chromatographic columns (10 cm × 1 cm I.D.) were purchased from Eastman-Kodak (Rochester, N.Y., U.S.A.).

Reagents and chemicals

The esterifying reagent was 25% (w/w) gaseous HCl (Matheson, Gloucester, Mass., U.S.A.) in methanol (Burdick and Jackson, Muskegon, Mich., U.S.A.). The methanol was redistilled and dried over molecular sieve Type 5A. The acetylating agent agent was heptafluorobutyric anhydride (K & K, Plainview, N.Y., U.S.A.) in acetonitrile (Burdick and Jackson). Upon arrival, the heptafluorobutyric anhydride was stored over anhydrous calcium sulfate in a desiccator at —4° and subsequently used without further purification. Acetonitrile was redistilled and dried over molecular sieve Type 5A. Diiodothyronine (T_2), T_3, and T_4 were obtained as the free acids from Sigma (St. Louis, Mo., U.S.A.) and stored in a desiccator at —4°. RT_3 was kindly given to us by Dr. R. I. Meltzer of Warner-Lambert Research Laboratories.

Cation-exchange resin Bio-Rad AG 50W-X2 (H^+), 100–200 mesh, was ob-

tained from Bio-Rad Labs. (Richmond, Calif., U.S.A.). [^{125}I]T_4 was obtained from Abbott Labs. (Chicago, Ill., U.S.A.). The silanizing agent, trimethylchlorosilane, was purchased from Supelco.

Silanization of glassware

Glassware was first cleaned by boiling in concentrated nitric acid for 1 h and rinsing to neutrality with distilled water. The glassware was then boiled in distilled water for 1 h and dried in an oven (140°) overnight. Silanization was conducted using a 4% (v/v) solution of trimethylchlorosilane in dry toluene for 10 min at room temperature. The glassware was returned to the 140° oven for 4 h, and was subsequently allowed to cool in a desiccator. When at room temperature, the vials were sealed with PTFE-coated septa. The silanization procedure was used throughout the entire experiment on all glassware, including reaction vials, volumetric flasks, and pipets, that were to come in contact with the thyroid hormones.

Preparation of 25% (saturated) HCl–methanol (w/w)

A 10-ml volume of dry methanol was pipeted into a dry 25-ml centrifuge tube and weighed. The tube was then placed in a dry ice–methanol bath and a stream of nitrogen was maintained above the solution while a stream of HCl gas was bubbled through the solution for approximately 15 min. After this time, the HCl and nitrogen streams were removed, and the tube was withdrawn, dried, and quickly weighed. This procedure was continued until a 25% increase in weight (±0.1 g) was observed. Once the proper percentage had been obtained (usually after 15 min), the solution was transferred to the esterification samples (*e.g.*, 0.5 ml/serum sample) by decantation. The HCl–methanol solution was always prepared for immediate use.

Derivatization of T_2, T_3, RT_3, and T_4 standards

T_2, T_3, or T_4 (25.0 mg) was placed in a 40-ml centrifuge tube and 25 ml of 25% HCl in methanol were added for esterification. The tube was sealed immediately with a septum and agitated for 3 min on a vortex. Esterification was allowed to continue for 1 h at 60°; subsequently the solution was evaporated to dryness under a stream of dry nitrogen. A freshly prepared solution of 1.5 ml of heptafluorobutyric anhydride in 6 ml of acetonitrile was then added. The tube was kept at 60° for 1 h, cooled to room temperature, and the solution was blown to dryness with dry nitrogen. The resulting solids were recrystallized from acetonitrile.

Since we had only a small quantity of RT_3, 1.00 mg was used to form the derivative. This was done in a 1-ml reaction vial using similar conditions to those mentioned above except that 0.5 ml of HCl–methanol solution and 100 µl of heptafluorobutyric anhydride in 400 µl of acetonitrile were employed. Recrystallization again was from acetonitrile.

In order to determine the percentage yield, the derivatization scheme was conducted on microgram quantities of material, using the appropriate volumes of the esterification and acetylating solutions. After completion of the reaction, the residue was redissolved in acetonitrile and injected into the gas chromatograph. Comparison of the ECD responses with those of standard solutions made from the pure material revealed a 99% yield in each case.

Elemental analyses and melting points of the derivatized thyroid hormone standards were as follows:

Derivatized T_2 — Found: 30.69% C; 1.29% H; 28.45% F; 27.24% I; 1.52% N; 10.81% O; m.p. 103–105°
Calc. for $C_{24}H_{13}F_{14}I_2NO_6$: 30.94% C; 1.41% H; 28.57% F; 27.27% I; 1.50% N; 10.31% O

Derivatized T_3 — Found: 27.18% C; 1.08% H; 25.10% F; 36.01% I; 1.33% N; 1.30% O; m.p. 118–119°
Calc. for $C_{24}H_{12}F_{14}I_3NO_6$: 27.25% C; 1.14% H; 25.17% F; 36.03% I; 1.32% N; 9.05% O

Derivatized T_4 — Found: 24.15% C; 0.86% H; 22.39% F; 42.88% I; 1.20% N; 8.52% O; m.p. 148–150°
Calc. for $C_{24}H_{11}F_{14}I_4NO_6$: 24.35% C; 0.94% H; 22.49% F; 42.92% I; 1.18% N; 8.11% O

Molecular-weight determination was also verified by mass spectrometry. Each of the compounds exhibited easily recognizable molecular ion peaks (M^+) of high relative intensity. The base peak in each spectrum appeared at *m/e* values corresponding to $(M - 213)^+$. Significant peaks in the spectrum for each compound having relative intensities greater than 40% occurred at *m/e* values of $(M - 284)^+$, $(M - 410)^+$ and $(M - 538)^+$. Mechanisms of the fragmentation patterns of these molecules are currently being studied by isotopic labeling and high-resolution techniques.

The thyroid hormone derivatives have proven to possess remarkable stability both in solid form and in solution. Although ECD calibrations of the standards were made immediately after derivatization, solutions stored up to four months revealed no decomposition. The only precaution taken was to store the derivatives in the dark at −4°.

Phosphate buffer

The 0.15 *M* phosphate buffer was composed of 19.70 g dipotassium hydrogen phosphate and 4.92 g potassium dihydrogen phosphate in 1 l of distilled water. Phosphoric acid or sodium hydroxide solutions were used to adjust the pH to 7.40. The solution was stored at 4° in the dark. Before use, the buffer solution was allowed to reach room temperature, and the pH was checked.

Equilibrium dialysis of sera

Dialysis membranes were prepared using the method of Sterling[13], in which membranes were soaked in 0.1 *M* HNO_3 for 24 h and in 0.01 *M* HNO_3 for three days, followed by storage in distilled water at 4°. Before use, the dialysis bags were washed repeatedly with distilled water. During handling of the membranes, plastic disposable surgical gloves were worn. Using forceps, the moist membrane was doubly knotted at one end, filled with 1 ml of phosphate buffer, and inspected visually for pin holes. If satisfactory, the buffer was removed and the bag was rinsed again with distilled water. The pH of the serum was measured and adjusted to 7.40, if necessary. Serum (1.00 ml) was introduced into the bag with a pipet, and the end of the membrane was doubly knotted. The membrane was folded in a "V" and placed in a 5-ml silanized microreaction vial. This vial also contained a triangular stirring bar to provide a gentle stirring of the solution. A 1.50-ml aliquot of phosphate buffer was pipetted into

the vial. The vial was sealed with a PTFE septum, wrapped with aluminum foil (in order to minimize photooxidation) and placed in a water-bath maintained at 37 ± 0.5°. Dialysis with stirring was assumed to be complete after 18 h[14], at which time the vials were removed and allowed to reach room temperature.

Cation-exchange chromatography

The cation-exchange resin (Bio-Rad AG 50W-X2 (H^+), 100–200 mesh) was washed four times in distilled water and the fines were decanted. An aqueous slurry of the resin was poured into a 10 cm × 1 cm I.D. polyethylene column, which contained a silanized glass wool plug, and settled to a packed height of 5 cm with 15 ml of distilled water. The column was then activated with 5 ml of 2 *N* HCl and washed with 5 ml of distilled water. A solution of 15 pg of T_2 in 1.0 ml of phosphate buffer (determined by GC) was injected onto the column, and the column was washed with 15 ml of 0.15 *M* ammonium acetate (pH 8.5), 2 ml of methanol, and 4 ml of methanol–12 *N* ammonium hydroxide (97:3, v/v). The column was treated successively as before with 3 ml of water, 5 ml of 2 *N* HCl, and 5 ml of water. The serum dialysate was pipeted onto the top of the column, and the dialysis vial was rinsed four times onto the column with 0.5-ml volumes of phosphate buffer. The intact dialysis bag was placed over the column and washed with 1 ml of phosphate buffer. Again, the ion-exchange column was washed with 15 ml of 0.15 *M* ammonium acetate (pH 8.5) and then 2 ml of methanol. The thyroid hormone fraction was subsequently eluted from the column with a 4-ml volume of methanol–12 *N* ammonium hydroxide solution (97:3, v/v). This wash procedure followed essentially that used by Tajuddin and Elfbaum[15]. The eluate was then collected in a 5-ml microreaction vial and evaporated to dryness at 40° under a stream of nitrogen.

Derivatization of the free thyroid hormones

The eluent from the cation-exchange column containing the thyroid hormones was dried under a stream of nitrogen at 40° for 20 min. To the residue was added 0.5 ml of the 25% (w/w) HCl–methanol solution, the vial was sealed with a PTFE septum and heated to 60° for 1 h in order to esterify the acids. After this time, the reaction mixture was allowed to reach room temperature before removing the septum. The esterifying solution was evaporated to dryness at 40° with a stream of nitrogen for 15 min. Acetylation was accomplished by adding separately 50 μl of heptafluorobutyric anhydride and 200 μl of acetonitrile to the residue, mixing on a vortex for 3 min and heating to 60° for 30 min. After removing the acetylating solution under a stream of nitrogen at 40°, the reaction vial was sealed with a Minienert cap. This cap was used so that aliquots could be added or removed without exposure of the sample to the atmosphere.

The residue in the vial was taken up in 20 μl of acetonitrile and mixed on a vortex for 5 min. Injections into the gas chromatograph were made by removal of aliquots from the solution via the septum port valve on the Minienert cap. Injection volumes were 5 μl.

RESULTS AND DISCUSSION

As we have already noted, the goal of this research was to develop an

analytical methodology that would permit the simultaneous analysis of the dialyzable (*i.e.*, free) thyroid hormones in sera. The expected amount of the dialyzable hormones in 1 ml of undiluted sera was at the 1–50 pg level[1]. This level places severe demands on detection, and it was clear that beyond incorporation of radioactive species, one of the only possible means of detection was GC–ECD. With such a procedure, severe demands are also placed on the sample clean-up and derivatization steps in order that sample losses are minimized.

For purposes of reference, the overall analytical scheme is shown in Fig. 1. As noted, equilibrium dialysis has been shown to be a valid method for isolating a quantity of unbound thyroid hormone that correlates with disease state[1,13,15–21]. We have simply followed a standard procedure in this step. Originally, derivatization was conducted on a lyophilized serum dialysate. This approach failed to yield a satisfactory sample for GC analysis, and so an additional clean-up step was necessary. A modified cation-exchange procedure proved to be adequate. Derivatization of the eluted fraction, GC separation, and ECD completed the analysis of the thyroid hormones at the picogram level.

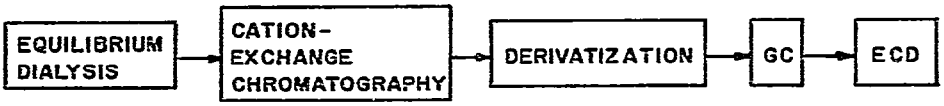


Fig. 1. Block diagram of the procedure for the analysis of free thyroid hormones.

Elimination of adsorption on glassware

Other workers have noted that the thyroid hormones tend to adsorb to glassware[14,22]. At the picogram level this could represent a significant problem in terms of both the accuracy and reproducibility of the analysis. We have found that a satisfactory method for eliminating adsorption on glassware is via silanization with trimethylchlorosilane.

An experiment was performed using $[^{125}I]T_4$ to illustrate the effective elimination of adsorption. Four test tubes each from six sources were selected for study. Two of each set were cleaned and silanized by the previously outlined procedure, and the remaining two were used as received. In each of the test tubes was placed 1.5 ml of phosphate buffer (see Experimental) containing 25,000 cpm of $[^{125}I]T_4$. The tubes were sealed with PTFE tape and placed in a shaker bath at 37° for 18 h (corresponding to the conditions for dialysis). After this time period, the solutions were withdrawn by pipet and each tube rinsed four times with 0.5 ml of phosphate buffer. The tubes were then dried under a stream of nitrogen, resealed and placed in the gamma counter for analysis of the $[^{125}I]T_4$ that adhered to the sides of the tubes. Each tube was counted three times and averaged.

The results of this experiment are shown in Table I. Each value of counts per minute is the average of the two tubes treated identically. (Deviations were generally less than 5%.) As the background cpm is roughly 200, it can be seen that silanization does completely eliminate the adsorption problem. Note that treatment with silicone (tube F) does not reduce adsorption at the low levels of this experiment. In order to standardize the analytical procedure, cleaning and silanization were conducted on all glassware that was used in the work.

TABLE I

COUNTS PER MINUTE (cpm) OF *T_4 ADSORBED ONTO GLASS TUBES

Average background = 203 cpm. Tubes: (A) 16 mm × 150 mm, Rochester Scientific No. 7045; (B) 16 mm × 100 mm, Fisher Scientific No. 14-957-16B; (C) 12 mm × 75 mm, Becton-Dickinson No. 7813; (D) 20 mm × 150 mm, Van-Lab No. 60825-389 (Pyrex); (E) 16 mm × 100 mm, Curtin-Matheson No. 339-309; (F) 16 mm × 100 mm, Venaject No. L-30 (silicone treated). *T_4 = [^{125}I] T_4 (25,000 cpm).

Tube	*No treatment*	*Silanized*
A	1568	207
B	2704	212
C	1338	211
D	1588	198
E	1335	204
F	1382	209

Thyroid hormone standards —separation and calibration

While N,O-dipivalyl methyl esters and other derivatives of the thyroid hormones have been successfully chromatographed, the reported detection limits were not sufficient for our purposes. One of the reasons for the poor detection limits was in part due to detector limitations. With the ECD used in this work, we were routinely able to work at very low noise levels (1×10^{-12} A). In addition, we employed fluorinated derivatives of the thyroid hormones for enhanced volatility and possibly improved detection limits. For this purpose, we selected the N,O-diheptafluorobutyryl methyl ester derivatives of the thyroid hormones.

After successful derivatization of the standards, a GC separation at 250° was developed, as shown in Fig. 2. We have included in this separation reverse T_3, which recently has been evaluated in a variety of normal and diseased states[23]. Note that roughly 10 pg of each standard has been injected.

Calibration plots (peak heights) were found to be identical for each of the three standards, and this calibration is shown in Fig. 2. The standard error in peak height using the data from all three standards was found to be ±1%. Although the plots were found to have a linear dynamic range of 10^5 with correlation coefficients of greater than 0.99, the required linear dynamic range for physiological concentrations in 1 ml of serum is only 10^3. The detection limits for each of these derivatives were found to be 0.2 pg. Accepting the values of free T_3 and T_4 from equilibrium dialysis by Sterling[13], our detection limits indicate that we should be readily able to detect the dialyzable hormones in 1 ml of serum even from hypothyroid individuals (reduced T_3 and T_4 levels), if no losses in sample handling occur. We shall discuss reverse T_3 later in the paper.

Cation-exchange clean-up of dialysate

As mentioned previously, it was found necessary to clean-up the dialysate prior to derivatization. We employed the established method of cation-exchange chromatography[5,6,16,24,25], but in agreement with others[2,5] found recoveries were not quantitative.

In order to assess recoveries at the picogram level, a standard solution of 9 pg/ml T_3 and 30 pg/ml of T_4 in phosphate buffer was prepared. The concentrations

Fig. 2. GC–ECD of the N,O-diheptafluorobutyryl methyl ester derivatives of thyroid hormones. Column, 6 ft. × 4 mm I.D. glass coil packed with 3% SE-30 on 80–100 mesh Supelcoport; temperature, 250°; carrier, methane–argon (5:95); flow-rate, 60 ml/min; injector temperature, 250°; detector temperature, 300°; injection volume, 5 μl; attenuation, ×1.1 = T_3; 2 = reverse T_3; 3 = T_4; 10 pg each.

of the thyroid hormones were ascertained by derivatization and GC analysis. One milliliter of this standard solution was pipetted on top of the cation-exchange column and the column was washed with 0.15 *M* ammonium acetate and then methanol, as outlined in Experimental. The hormones were then collected in an eluent of methanol–12 *N* ammonium hydroxide (97:3), derivatized, and injected into the gas chromatograph. As seen in Table II, recovery of the hormones was found to be only 54% for T_3 and 65% for T_4.

It was assumed that the losses were due in part to adsorption on the polyethylene column, based on observations made by Lee and Pileggi[14]. Following on their work, it was believed that the column might be "deactivated" by the addition of a reagent to the column prior to sample loading. The deactivating agent selected was diiodothyronine (T_2) as its properties of adsorption might be expected to be related to those of T_3 and T_4. Moreover, any T_2 eluting from the resin column with

TABLE II

RECOVERY OF T_3 AND T_4 FROM CATION-EXCHANGE COLUMNS PRETREATED WITH DIIODOTHYRONINE (T_2)

Solution, 9 pg/ml T_3 and 30 pg/ml T_4.

T_2 added (pg)	*T_3 recovered (pg)*	*T_4 recovered (pg)*	*Recovery T_3 (%)*	*Recovery T_4 (%)*
0	5	20	55	55
5	5	24	55	80
8	6	25	67	83
10	6	27	67	90
12	8	29	89	97
15	9	30	100	100
20	9	30	100	100
25	9	30	100	100

the T_3 and T_4 fraction ought to be readily derivatized under the conditions for derivatization of the active hormones. Finally, the T_2 derivative would elute much earlier than the T_3 derivative so that no chromatographic interference would be expected.

Known amounts of T_2 in 1 ml of phosphate buffer were added to the cation-exchange column. Each amount of T_2 was separately determined by derivatization and GC–ECD, using a small-range (1–50 pg) calibration plot for T_2. The column was washed in the same manner as described above, including elution with methanol–ammonium hydroxide. After this elution, the column was washed with 3 ml of distilled water and reactivated with 5 ml of 2 *N* HCl. The column was next loaded with 1 ml of standard solution (9 pg T_3/30 pg T_4), washed, and the fraction containing T_3 and T_4 collected. The thyroid hormones were derivatized and detected by GC–ECD.

The results of this experiment are shown in Table II. As the quantity of T_2 added to the column is increased, the recovered amounts of T_3 and T_4 also increase. It is found that after the addition of 15 pg T_2 in the manner described above, the

TABLE III

REPEATED ANALYSIS OF FREE T_3 AND FREE T_4 IN SERUM

Sample No.	*FT_3 (pg/ml)*	*FT_4 (pg/ml)*
1	6	32
2	4	30
3	6	31
4	6	31
5	6	29
6	5	29
7	6	31
8	5	30
9	7	29
10	6	30
Average	5.7 ± 0.8	30.3 ± 1.9

recovery of T_3 and T_4 is maximized. For all subsequent experiments, the resin columns were first prepared by the addition of approx. 15 pg of T_2, washed, and reactivated before loading of the dialysate.

Serum dialysate analysis

Our next study dealt with the question of losses in the analysis of the dialysate from an actual serum sample. As described in Experimental, 1 ml of serum was dialyzed against 1.50 ml of phosphate buffer, and two 0.50-ml aliquots of dialysate were withdrawn. One aliquot was untreated while to the other was added a 25-μl solution of 15 pg of T_3 and 70 pg of T_4 (as determined from the calibration plot of Fig. 3). Both aliquots were chromatographed by cation exchange, derivatized, and analyzed by GC–ECD.

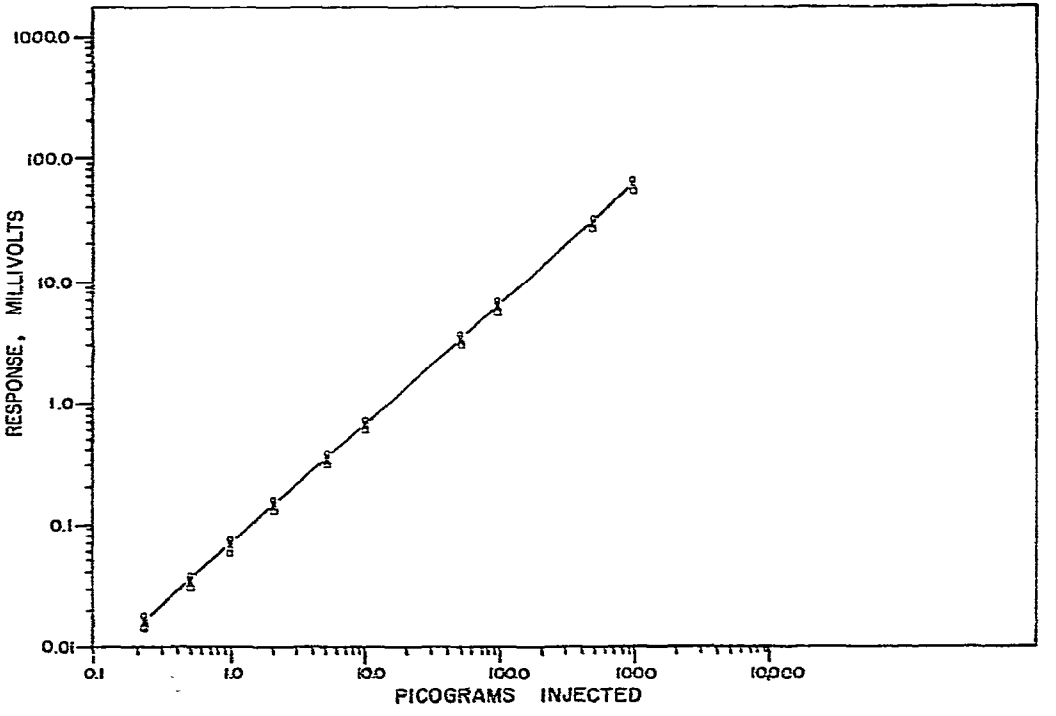


Fig. 3. Calibration plot of the thyroid hormone derivatives. Chromatographic conditions, same as in Fig. 2. ○, T_4; ×, RT_3; □, T_3.

For the untreated dialysate fraction, chromatographic results with 5-μl sample injections indicated 0.5 pg of T_3 and 2.5 pg of T_4. Since the sample had been taken from a total of 20 μl, the amounts of dialyzed hormones were 2 pg of T_3 and 10 pg of T_4. In the same manner the amounts of hormones found in the aliquot containing the added standards were 17 pg of T_3 and 80 pg of T_4. Thus, within the limits of the analytical procedure, no losses after the serum dialysate were observed, indicating quantitative recovery from the dialysate.

In order to determine the overall reproducibility of the method (*i.e.*, dialysis

and analysis) 12 ml of sera were pooled and ten 1-ml samples from this pool were separately dialyzed against 1.5 ml of buffer. Each dialysate was cleaned up by the previously outlined procedure and analyzed by GC–ECD.

The results for each milliliter of serum are shown in Table III. The percentage standard deviations for FT_3 and FT_4 are $\pm 14\%$ and $\pm 7\%$, respectively. Considering the various steps in the procedure and the very low amounts of the thyroid hormones present, the repeatability is good.

An improvement in the reliability of the method can be achieved by the addition of a suitable internal standard to the serum dialysate before cation-exchange chromatography in order to monitor losses and variations. We have tested 5′-chloro-3,5,3′-triiodothyronine ($Cl\text{-}T_3$) as a potential internal standard, as it elutes between T_3 and T_4 in the GC separation of the derivatives and ought to possess similar physical and chemical properties as the hormones. Addition of this compound to the dialysate and subjection to the same analytical steps as described previously for T_3 and T_4 revealed a repeatability of $\pm 1\%$ for FT_3 and FT_4 for the ten sera samples shown in Table III. However, the retention time of the internal standard was found to be identical with that of reverse T_3. We are therefore currently investigating the synthesis of other possible internal standards that maintain the same properties as the thyroid hormones but can be resolved from T_3, T_4, and RT_3.

In Fig. 4 we show a chromatogram of (free) thyroid hormones from the serum of a presumably normal individual. The results correspond to 5 pg of T_3 and 32 pg of T_4 in 1 ml of serum. (Note that only 1/4 of the final sample volume was injected into the gas chromatograph to achieve the chromatogram shown.) Comparable results were obtained on a serum from a second normal person. These values are within the range of concentrations expected for normal FT_3 and FT_4 as reported by Sterling[13] using equilibrium dialysis. As discussed by Lee and Pileggi[14], the dialyzed quantities of T_3 and T_4 are dependent on the conditions of dialysis, *e.g.*, temperature, degree of dilution of serum with buffer prior to dialysis, and relative solution volumes inside and outside the dialysis bag.

Preliminary analyses of pathological sera show the method to be completely applicable without any further modification to such samples. While we have analyzed only a limited number of abnormal samples up to this point, the FT_3 and FT_4 levels were as much as four-fold above and below the levels observed for the two normal sera in our study. Reverse T_3 was also seen in some of these abnormal samples. This was expected, since reverse T_3 is known to be elevated in a number of disease states[23]. We did not observe a peak for reverse T_3 in the two normal sera which we analyzed, however, perhaps because we dialyzed only 1 ml of serum and injected only 1/4 of the final sample volume in each case. A peak for free reverse T_3 might have been seen if the procedure had been scaled up. Chopra *et al.*[23] report a level of free reverse T_3 in euthyroid serum by dialysis–radioimmunoassay to be 1 pg/ml. Work with pathological samples is continuing and will be reported in detail in a future paper.

CONCLUSION

A method has been developed for the analysis of the concentrations of free (*i.e.*, unbound) thyroid hormones in 1 ml of serum. After equilibrium dialysis, a standard procedure, the dialysate is cleaned-up by cation-exchange chromatography,

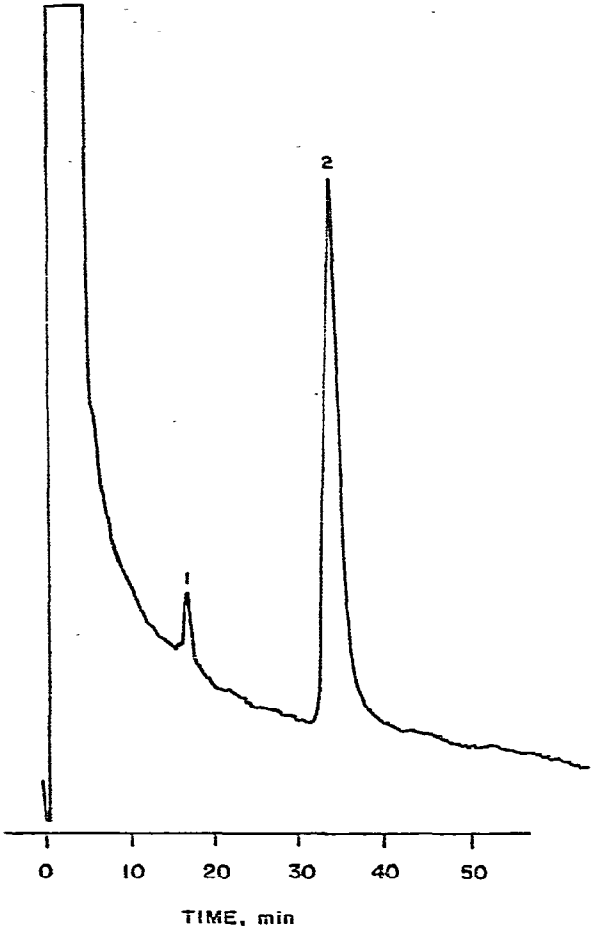


Fig. 4. GC–ECD of the N,O-diheptafluorobutyryl methyl ester derivatives of the dialyzed thyroid hormones. Chromatographic conditions, same as in Fig. 2. 1 = approx. 1 pg T_3; 2 = approx. 8 pg T_4; injection volume, 5 μl; attenuation, $\times 1$.

derivatized to the N,O-diheptafluorobutyryl methyl esters, and subjected to GC–ECD. Detection limits of 0.2 pg are achieved. Reasonable values for FT_3 and FT_4 are obtained from two normal sera, and preliminary work shows free RT_3 in some of the pathological sera examined. Although the repeatability of the method is good and the recovery after dialysis is essentially quantitative, it is expected that introduction of a suitable internal standard will enhance the routine reliability of the procedure based on results with a trial internal standard.

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