Journal of Chromatography, 226 (1981) 383—390
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1037

SIMULTANEOUS DETERMINATION OF D- AND L-THYROXINE IN HUMAN SERUM BY LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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(Received May 12th, 1981)

SUMMARY

A method for the determination of D- and L-thyroxine in human serum is described. The method involves extraction of thyroxine from serum and the separation of thyroxine enantiomers on a reversed-phase, high-performance liquid chromatographic column by use of a chiral eluent containing L-proline and cupric sulfate. Satisfactory resolution of the enantiomers of thyroxine, triiodothyronine, and reverse triiodothyronine can be achieved in 12 min and, employing amperometric detection to monitor the separation, the detection limit for serum thyroxine is in the range of 1—3 ng per injected sample.

INTRODUCTION

The principal naturally occurring thyroid hormone is the levo-enantiomer of thyroxine (3,3′,5,5′-L-tetraiodothyronine, LT_4) [1]. The dextro-isomer, DT_4, considered to have only a fraction of the biological activity of LT_4, has been used extensively to reduce serum cholesterol levels in euthyroid hyperlipidemic subjects [2]. Recently it was shown that either 4 mg of DT_4 or 0.15 mg of LT_4 produced a similar degree of pituitary thyrotrophin suppression and an equal stimulation of basal metabolic rate [3]. Since no adequately sensitive method was available to measure specifically the D and L forms of iodothyronines, it was impossible to define whether (1) LT_4 contaminated the administered DT_4, (2) DT_4 was converted in vivo to LT_4, or (3) DT_4 and/or a dextro-metabolite had true biological activity. In order to investigate these questions we developed a sensitive high-performance liquid chromatographic (HPLC) technique which is capable of rapidly separating the optical isomers of both thyroxine

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and the two triiodothyronines, 3,3',5'-triiodothyronine (T_3) and 3,3',5-triiodothyronine (reverse T_3, rT_3), and can, when combined with an efficient serum extraction method, allow the specific measurement in human serum of both endogenous LT_4 and exogenous DT_4.

In the past, the specific determination of iodothyronine enantiomers was dependent on techniques such as direct measurement of optical rotation [4] or stereospecific oxidation with L-amino acid oxidase [5]. More recently Lankmayr et al. [6] coupled iodothyronines with L-leucine and resolved the resultant diastereomers by reversed-phase HPLC. We have employed a more direct approach by the use of a chiral eluent, adapting the principle first described by Hare and Gil-Av [7], who successfully employed it to separate underivatized amino acid enantiomers on an ion-exchange column.

To permit the application of this technique to our goal of measuring in human serum the thyroxine enantiomers, we required both a detection method capable of measuring iodothyronines in subnanogram quantities and a serum extraction procedure adequately efficient to allow satisfactory HPLC. Recently reported HPLC techniques for detecting iodothyronines by UV light have a sensitivity threshold of 8—10 ng [8, 9]. The post-column catalytic detection system of Nachtmann et al. [10], although adequately sensitive, requires the construction of special equipment and can not be used with a mobile phase containing metallic ions. Electrochemical detection, as reported by Hepler et al. [11], has the potential for measuring T_4 and T_3 down to the 0.12—0.18 ng range. We have successfully employed this detection principle using commercially available amperometric equipment [12]. The serum extraction techniques described by Hepler and Purdy [13] and by Bongiovanni et al. [14] were found to be unsatisfactory for our purposes. Consequently we had to develop a novel extraction method to allow for the first time the specific determination in human serum of the enantiomers of thyroxine.

MATERIALS AND METHODS

Reagents and materials

All solvents were analytical reagent grade. Methanol, ethyl acetate and acetonitrile were supplied by J.T. Baker (Phillipsburg, NJ, U.S.A.). Water was de-ionized and glass-distilled. Sodium acetate trihydrate, cupric sulfate pentahydrate, silver nitrate, L- and D-proline were supplied by Sigma (St. Louis, MO, U.S.A.), who also provided the D- and L-enantiomers of T_3 and T_4. The D- and L-enantiomers of rT_3 were a generous gift from Dr. H. Rokos, Henning Berlin GmbH, Berlin, G.F.R. Sep-Pak silica cartridges were supplied by Waters Assoc. (Milford, MA, U.S.A.).

The mobile phase consisted of an acetonitrile—0.1 *M* sodium acetate solution (30—35:70—65, v/v) containing 0.004 *M* cupric sulfate, 0.008 *M* L- or D-proline and 0.002 *M* silver nitrate. The 2:1 molar ratio of proline to copper was as employed by Hare and Gil-Av [7]. The addition of silver nitrate allowed chromatography at a lower amperometric offset reading. Litre solutions of chiral eluent (without acetonitrile) were routinely prepared by dissolving in water 13.6 g of sodium acetate, 1.0 g of cupric sulfate, 0.92 g of L-proline and 0.34 g of silver nitrate. Prior to chromatography appropriate volumes of ace-

tonitrile were added to the copper—proline solution and the mixture degassed with a vacuum pump for 10 min. Standards for HPLC consisted of DT_3, LT_4 and DT_4 dissolved in methanol at final concentrations of 0.25—1.25 μg/ml for DT_3 and 0.5—2.5 μg/ml for L and DT_4.

Chromatographic instruments

An Altex Model 110A solvent metering pump (Altex Scientific, Berkeley, CA, U.S.A.) combined with a Rheodyne 7120 sample injection valve (100-μl loop) was used for the chromatographic separation. The reversed-phase column (25 cm × 4.6 mm I.D.) employed was an Ultrasphere I.P. (5 μm average particle diameter), a product of Altex. Amperometric detection was performed with electrochemical equipment (TL-5 Kel-F glassy carbon thin-layer cell, LC-4 electronic controller), from Bioanalytical Systems Inc. (West Lafayette, IN, U.S.A.), coupled to a Fisher Series 5000 Recordall pen recorder. Gradient elution and thermostating were not necessary and separations were performed isocratically at room temperature (20—22°C).

Serum extraction

To each 1-ml serum sample are added sequentially 100 μl of ^{125}I-labeled T_4 (20,000 cpm), 3 ml of 5% trichloroacetic acid, and 4 ml of ethyl acetate. Each tube is vortexed vigorously to allow complete mixing of ethyl acetate and precipitated serum proteins. After spinning for 5 min at 1500 *g*, the upper organic layer is transferred with a glass pipette to a 15-ml borosilicate collection tube. Ethyl acetate extraction is then repeated twice using two further 3-ml volumes. The extract is reduced under nitrogen in a water-bath evaporator to a final volume of about 1.5 ml. This 1.5-ml extract is then applied with a glass transfer pipette to a Sep-Pak silica cartridge equilibrated with 5 ml of ethyl acetate. After an 8-ml ethyl acetate wash, the extract is eluted with 4 ml of methanol—ammonium hydroxide (90:10), reduced to dryness under nitrogen and reconstituted in 100 μl of methanol prior to injection on to the column.

Chromatographic procedure

A flow-rate of 1.4—2.0 ml/min was generally used at a pressure of 262—310 bars for the Ultrasphere I.P. column. The potential applied to the detector cell was +0.78 V and detection was routinely performed at a setting on the electronic controller of 2—5 nA/V. Standard solutions of 20—25 μl volume containing 2.5—50 ng of DT_4 and LT_4 and 1.25—25 ng of DT_3 or LT_3 were injected on to the column using Pressure-Lok liquid syringes (0—25 μl, 0—50 μl) Series B110 from Precision Sampling Corporation. Standard curves for DT_4 and LT_4 were constructed from duplicate determinations of standard samples and the peak height (nA) was plotted against the known injected amount of thyroxine (ng).

RESULTS

Optimization of chromatographic conditions

Chromatography was routinely performed with L-proline as the chiral

constituent of the mobile phase. Best results were found to occur when the column and thin-layer cell (with voltage applied) were allowed to equilibrate with the mobile phase for 1—2 h prior to injection of samples. Fig. 1 shows the separation of the enantiomers of T_3 and T_4 obtained within 25 min on isocratic elution with an acetonitrile—copper-proline-acetate (35:65) mobile phase. Since L-proline was used in the chiral eluent, the first peak of each pair represents the L-enantiomer. The use of D-proline would result in reversal of the order of appearance of compounds from the analytical column.

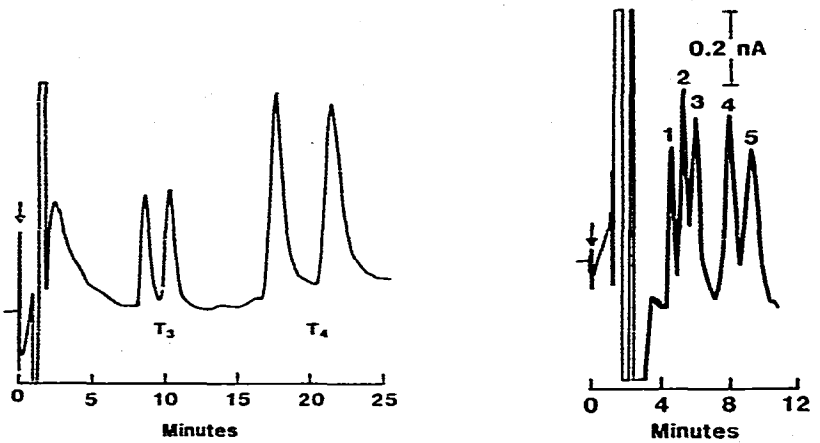

Fig. 1. Chromatogram of the enantiomers of T_3 and T_4. Column was injected with 10 ng each of DT_3 and LT_3 and 20 ng each of DT_4 and LT_4. Mobile phase, 30% acetonitrile; flow-rate, 2 ml/min; detector sensitivity, 5 nA/V.

Fig. 2. Separation of LT_3 (1), LrT_3 (2), DrT_3 (3), LT_4 (4) and DT_4 (5). Column injected with 7.5 ng of each of LT_3, LrT_3 and DrT_3 and 15 ng of each of LT_4 and DT_4. Mobile phase, 35% acetonitrile; flow-rate, 1.5 ml/min; detector sensitivity, 2 nA/V.

To allow a more rapid analysis, the conditions were altered to enable a separation within 12 min of the T_3, rT_3 and T_4 enantiomers. Fig. 2 shows the HPLC pattern obtained when 7.5 ng of LT_3, LrT_3 and DrT_3 and 15 ng of LT_4 and DT_4 were injected onto the column. In this example peak 2 is represented by LrT_3 but it could equally have been DT_3 since these compounds coelute under these conditions. If D-proline was exchanged for L-proline in the mobile phase, then an unknown peak at position 2 with L-proline could be resolved into peaks at either position 1 or 3, representing DT_3 and LrT_3. Judicious choice of the chiral constituent of the mobile phase could therefore permit the chromatographic identification of any of the four triiodothyronine enantiomers.

For quantitative purposes a mobile phase containing 34—36% acetonitrile was employed at a flow-rate of 1.4—1.6 ml/min. Optimal tracings were obtained when conditions (as in Fig. 2) were manipulated to allow the LT_4 peak to appear at about 8 min after injection. Fig. 3 shows a typical calibration curve for the quantitative detection of LT_4 and DT_4. With peak height plotted against concentration, the linearity of the standard curves in the range 2.5—100 ng was determined by calculating the correlation coefficient, which was found to be 0.998 for both the T_4 enantiomers. At a sensitivity setting of 2 nA/V on

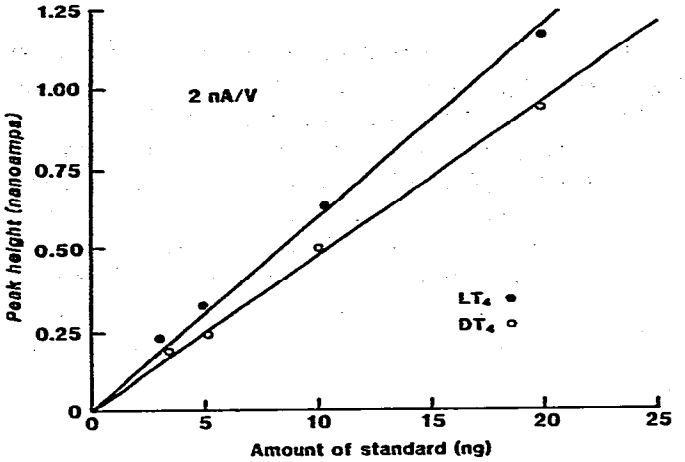

Fig. 3. Calibration curves for assay of LT_4 and DT_4. Conditions as in Fig. 2. Each point represents the average of duplicate determinations which agreed to within 3% of each other.

the electronic controller, the detection limit for either LT_4 or DT_4 was 1—3 ng, while for either of the T_3 or rT_3 enantiomers as little as 0.5—1.5 ng could be detected. Because at 2 nA/V there tended with repeated injections to be increasing fluctuation in the electronic baseline, routine quantitation was performed at the higher setting of 5 nA/V with its consequently higher detection limit of 3—5 ng for T_4.

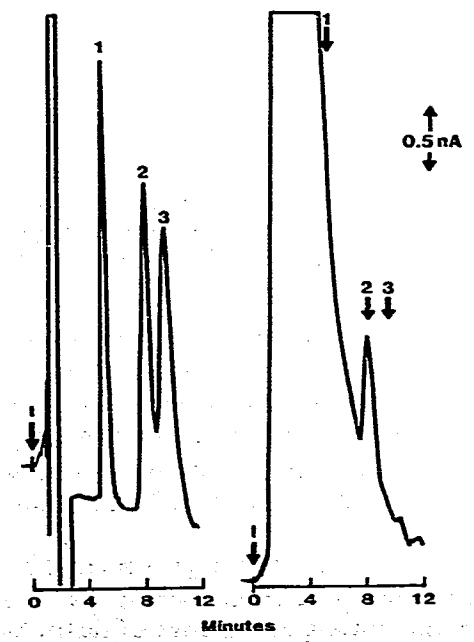

Fig. 4. Chromatogram of a serum extract from a euthyroid patient (right panel) compared with (on left) that derived from a standard solution containing 30 ng of DT_3 (1), 60 ng of LT_4 (2), and 60 ng of DT_4 (3). The single peak seen in the serum extract elutes in the position of LT_4. Mobile phase, 35% acetonitrile; flow-rate, 1.5 ml/min; detector sensitivity, 5 nA/V; injection volumes, 25 μl.

Quantitation of T_4 in serum samples

LT_4, labeled with radioactive iodine (^{125}I), was used to reflect T_4 recovery from serum samples. On average the ethyl acetate extraction recovered 85—95% of the counts while the silica column typically retained 65—70% of the extracted counts which were completely removed by the methanol—ammonium hydroxide (90:10). Using serum samples spiked with known amounts of LT_4, the recoveries were found to be linear over the range 15—200 ng of T_4 (1.5—20.0 μg/dl serum). The overall recovery from 75 consecutive serum extractions was 65 ± 6%. The value of T_4 obtained by electrochemical detection was found to correlate well ($R = 0.84$, $n = 48$, $p < 0.001$) with values obtained by conventional nonstereospecific radioimmunoassay of serum total thyroxine.

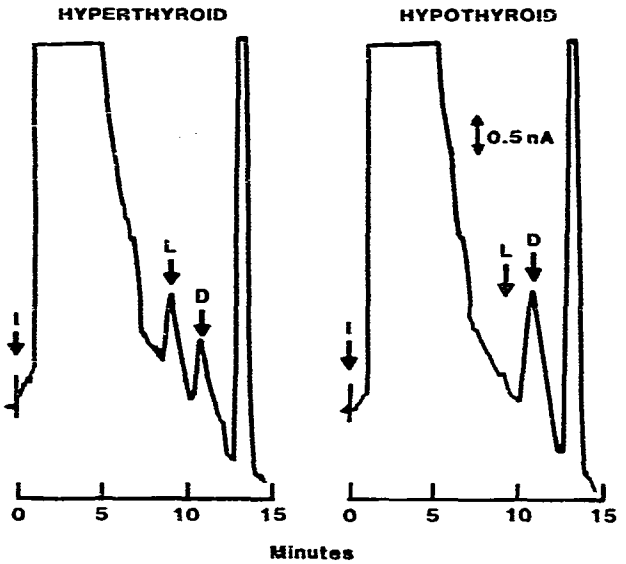

Fig. 5. Chromatograms of serum extracts from hyperthyroid and hypothyroid patients after the addition to the serum of DT_4 (100 ng/ml). Conditions as in Fig. 4, but injection volume for hyperthyroid was 15 μl and for hypothyroid 30 μl. The large peak at 13 min is due to an unknown serum constituent.

The technique was first applied to the determination of the enantiomeric state of circulating T_4 in serum from patients known to be either euthyroid or hyperthyroid as determined by clinical and biochemical assessment. In both these circumstances, a single T_4 peak was seen with the retention time of standard LT_4. Fig. 4 demonstrates the HPLC pattern seen in a euthyroid serum sample and compares the tracing with that using standards of DT_3, LT_4 and DT_4.

When known amounts of DT_4 were added in vitro to either hypothyroid or hyperthyroid human serum, this was reflected in the HPLC tracing by the appearance of a second peak with a retention time identical to that of authentic DT_4 (Fig. 5). Similarly, when a euthyroid patient was orally administered 10 mg of DT_4 and blood drawn 4 h after ingestion, the HPLC pattern obtained from the serum (Fig. 6) showed an identical configuration to that obtained when DT_4 was added in vitro to serum.

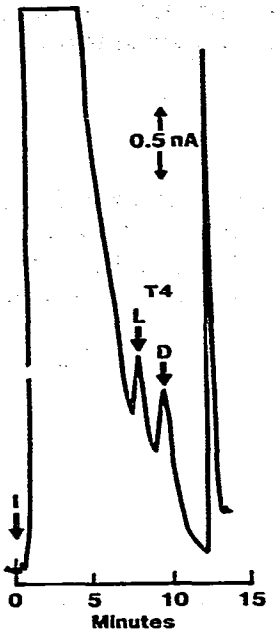

Fig. 6. Chromatogram of serum extract from euthyroid patient given 10 mg of D-thyroxine 4 h prior to venipuncture. Conditions as in Fig. 4. Injection volume, 25 μl.

DISCUSSION

Although multiple HPLC techniques have now been described [6, 8—11, 14] which are capable of separating T_3 from T_4, only the method described by Lankmayr et al. [6] has the stereoselective capability of separating the T_3 and T_4 optical isomers. The technique described in this paper does not require the precolumn synthesis of diastereomers and, by using underivatized samples, it permits the direct quantitation of the iodothyronine enantiomers. The serum extraction we have described is both simple and efficient and has allowed for the first time a stereospecific determination in human serum of circulating thyroxine.

Like the technique of Lankmayr et al. [6] the present method can be used to determine the LT_4 contamination of pharmaceutical preparations of DT_4. Analysis by this technique of currently available U.S. preparations of DT_4 has revealed no evidence of contamination with LT_3 but has demonstrated an LT_4 content of 0.4—0.5%. This minor degree of LT_4 contamination is comparable to that estimated using the classical L-amino acid oxidase method [15] and would not of itself account for the biological effects seen in our recent studies of DT_4 treatment in hypothyroid subjects [3].

The more exciting possibility that ingested DT_4 may in vivo be converted to more bioactive LT_4 has never been investigated, largely because currently available anti-DT_4 antibodies cannot differentiate between DT_4 and LT_4. The presently described technique provides for the first time a methodology capable of verifying or refuting this possibility. Current sensitivity limits allow the detection of serum T_4 levels down into the hypothyroid range. However,

further refinement of the thin-layer electrochemical detection system [11] will be necessary before the technique can be applied to the direct quantitation in human serum of either T_3 or reverse T_3.

ACKNOWLEDGEMENTS

This work was supported in part by a grant from Travenol Laboratories Inc., Deerfield, IL, U.S.A. I.D.H. and T.M.A. were fellows of the Mayo Graduate School of Medicine. Part of the work was presented (by I.D.H.) at the First International Symposium on the Neurochemical and Clinical Applications of LCEC, Indianapolis, IN, May, 1980.

The authors thank Mr. D. Machacek for his highly qualified technical assistance.

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