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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF IODOAMINO ACIDS FOR TRACER TURNOVER STUDIES OF THYROID HORMONES *IN VIVO*

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SUMMARY

A reversed-phase high-performance liquid chromatographic technique was developed to separate radioiodinated thyroxine (T_4), 3,5,3′-triiodothyronine (T_3), 3,3′,5′-triiodothyronine (rT_3) and two diiodothyronines (3,3′-T_2 and 3′,5′-T_2), in extracts from either serum or urine. Chromatography was performed with 10-μm C_{18} silica gel, packed in a glass column (3 × 300 mm); the mobile phase was methanol–water (55:45) adjusted to pH 3 with H_3PO_4, at a flow-rate of 1.2 ml/min and a pressure of 2800 p.s.i. The results demonstrate the ability of the system to yield a clear-cut separation of the iodothyronines involved in *in vivo* turnover studies, *i.e.*, T_4, T_3, rT_3, and the two T_2 compounds together.

INTRODUCTION

The *in vivo* study of thyroid hormone metabolism with radioactive tracers requires the separation of the intact tracers from their labelled metabolites as a preliminary step to the measurement of radioactivity in biological samples[1]. Separation procedures traditionally used for this purpose include precipitation with trichloroacetic acid, followed by alcohol extraction[2], acidic extraction with ethyl acetate–butanol[3], ion-exchange chromatography[4], specific antibodies[5,6], Sephadex chromatography[1,7,8] and thin-layer chromatography[9]. Most of these methods have significant disadvantages that limit their application to *in vivo* studies: incomplete separation, inconstant and/or low recoveries of the various labelled metabolites[5,10,11] and tracer deiodination in the course of chromatography[3,10].

As an approach to the study of peripheral metabolism of thyroid hormones in man, we have examined the use of high-performance liquid chromatography (HPLC) in the separation of thyroxine and its main metabolites in biological fluids. Since 1974[12], several HPLC procedures for the separation of iodothyronines have been described. These techniques were usually developed to assess the purity of pharmaceutical preparations[12–17], to study the molecular biology of thyroid hormones[12–14,18,19] or to quantitate thyroid hormones and their metabolites in extracts

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of biological samples[14,16,18,20,21]. However, their use for the *in vivo* study of thyroid hormones metabolism, although mentioned by some authors[14,17,21], has not been reported.

Our method has been developed specifically for separating the labelled species generated *in vivo* following the injection of tracer thyroid hormones. In fact, after pulse injection of labelled thyroxine (T_4), the ratio in the serum of newly generated tracer 3,5,3′-triiodothyronine (T_3) and 3,3′,5′-triiodothyronine (rT_3) to parent tracer T_4 at equilibrium may be as low as 0.005–0.01. Therefore, the use of a highly selective separation procedure is mandatory.

MATERIALS AND METHODS

Reagents and instrumentation

Over 99.9% pure thyroxine (T_4), 3,5,3′-triiodothyronine (T_3), 3,3′,5′-triiodothyronine (reverse-T_3, rT_3), 3,3′-diiodothyronine (3,3′-T_2) and 3′,5′-diiodothyronine (3′,5′-T_2) were purchased from Henning-Berlin (Berlin, F.R.G.). These compounds were used for labelling with ^{125}I or ^{131}I, for the preparation of standards and as unlabelled carrier iodothyronines. Radioiodination of the iodothyronines was performed in our laboratory according to a modified chloramine-T procedure described previously[22].

Pre-packed Sephadex G-25 (fine) columns, used for the preliminary extraction of iodothyronines from serum, were supplied by Ames (Division of Miles Laboratories, Elkhart, IN, U.S.A.).

Electrophoretically pure human serum albumin (HSA) was purchased from Behringwerke (Marburg/Lahn, F.R.G.). All other reagents and organic solvents (Chromasol grade) were purchased from Hoechst Riedel-De Haen (Frankfurt am Main, F.R.G.). Double-distilled water was prepared using the Milli-Q apparatus (Millipore, Waters Associates, Milford, MA, U.S.A.). SEP-PAK (C_{18}) cartridges for the extraction of iodothyronines from the HSA solution were purchased from Waters Associates.

The HPLC apparatus was a complete modular system (Model LDC 401) for gradient liquid chromatography, provided with a UV detector operating at 254 nm (UV III Monitor Model 1203) (Laboratory Data Control, Division of Milton Roy, Riviera Beach, FL, U.S.A.). A paper-recorder (Model BD12-743E; Kipp & Zonen, Delft, The Netherlands) was incorporated for continuous recording of the optical absorbance of eluate. The eluate fractions were collected using a Ultrorac 7000 fraction collector (LKB, Bromma, Sweden).

Methods

The samples for the chromatographic separation of iodothyronines were either serum samples from patients into whom ^{125}I-T_4 and ^{131}I-T_3 had been injected for metabolic studies[1], or blank serum samples to which ^{125}I- or ^{131}I-labelled T_4 and/or ^{131}I-T_3 had been added *in vitro* (after incubation at room temperature for at least 1 h). Obviously, the patients' sera contained, in addition to the injected ^{125}I-T_4 and ^{131}I-T_3, a series of labelled metabolites, such as ^{125}I-T_3, ^{125}I-rT_3, two forms of ^{125}I-labelled T_2 (3,3′-T_2 and 3′,5′-T_2) and ^{131}I-3,3′-T_2.

A preliminary to all subsequent procedures was the extraction of all iodo-

thyronines from the serum samples into 1% HSA solution, according to the following method. Sephadex G-25 (fine) columns (0.9 × 1.8 cm) were equilibrated at pH 11.2 with 0.1 *M* sodium hydroxide, and 0.5 ml of serum were applied to each column. At the pH and ionic strength of the columns, all complexes of the iodothyronines with carrier plasma proteins were dissociated, and the iodothyronines were strongly bound to the Sephadex particles. Nine 0.5-ml aliquots of phosphate–EDTA buffer (pH 7.4, 0.07 *M* phosphate, 0.02 *M* EDTA) were then used to elute serum components other than iodothyronines (proteins, iodide, etc.). Quantitative elution of iodothyronines was performed by stepwise addition of six 0.5-ml aliquots of 1% HSA in physiological saline. Fractions 1–6 were pooled, thus yielding a total volume of 3 ml of HSA solution, containing the iodothyronines bound to HSA.

To cleave the iodothyronines from HSA, two extraction procedures were followed.

(A) Extraction with SEP-PAK columns. The HSA solution containing the iodothyronines was brought to pH 4 with 1 *M* HCl, and loaded onto a SEP-PAK column. Prior to applying the 3-ml sample, the SEP-PAK column had been activated by elution in the following sequence: 30 ml methanol, 10 ml of 5% acetic acid and 20 ml water. After loading the sample, the column was washed with 20 ml of 5% acetic acid (thus washing away all the HSA) and with 20 ml water. Residual water was purged by passing 20–30 ml of a nitrogen flow through the column. Elution of the iodothyronines from the column was then performed with 3.5 ml methanol. The eluate was evaporated to dryness under a continuous nitrogen stream. The dry residue was redissolved in 25 μl of methanol–ammonia solution (99:1), in which various unlabelled iodothyronines had been dissolved, as a carrier, at concentrations of 10–30 μg/ml. This final solution was then used for HPLC.

(B) Extraction with ethyl acetate–butanol. Six ml of ethyl acetate–butanol (9:1) were added to the 3 ml HSA solution containing the iodothyronines. After agitationing in a vortex mixer, the mixture was allowed to stand at room temperature for 15–20 min, then centrifuged at 1500 *g* for 20 min. After centrifugation, the contents of the tube were divided into three phases: the aqueous phase (bottom), the albumin phase (a thin layer in the middle) and the phase of organic solvent containing the iodothyronines (top). The upper phase was then transferred to another test-tube and evaporated to dryness under a continuous nitrogen stream. The dry residue was redissolved, as described before, and used for HPLC.

HPLC separation procedure

An HPLC glass tube (3 × 300 mm) was packed with 10-μm C_{18} silica gel by using apparatus obtained from Hoechst Riedel-De Haen (Frankfurt am Main, F.R.G.). A 10-μl aliquot of the methanol–ammonia solution that had been used to redissolve the dry residue from the extraction procedure was loaded for each chromatogram. Isocratic elution was performed with methanol–water (55:45), adjusted at pH 3 with phosphoric acid, at a flow-rate of 1.2 ml/min and a pressure of 2800 p.s.i. The optical absorbance of the eluate was monitored by the continuous-flow UV detector, and the resulting profile was recorded, with the paper speed set at 5 cm/min. The outlet of the column was connected to the fraction collector, and 1.5-ml fractions were collected for later gamma counting.

RESULTS

Recoveries of radioiodothyronines in the extraction procedure

The recovery of labelled iodothyronines in the 1% HSA eluate from the Sephadex G-25 (fine) columns was 92.84 ± 1.87% for ^{125}I-T_4, and 90.75 ± 1.59% for ^{131}I-T_3 (mean ± S.D. from eight separate experiments). A very high recovery was also observed with the two procedures for the extraction of iodothyronines from the 1% HSA solution. The recovery from the SEP-PAK columns was 96.74 ± 0.21% for ^{125}I-T_4 and 97.84 ± 0.12% for ^{131}I-T_3 (six separate experiments). In the case of the ethyl acetate–butanol extraction, the recovery was 92.67 ± 0.78% for ^{125}I-T_4, and 93.45 ± 0.38% for ^{131}I-T_3 (six separate experiments). Since many experiments have demonstrated the virtual absence of loss of labelled compounds during HPLC (100% recovery), the overall recovery of ^{125}I-T_4, from a serum sample containing the radioiodothyronines until completion of HPLC, was 90%, on the average, when using the SEP-PAK cartridges, and 86% with ethyl acetate–butanol. In the case of ^{131}I-T_3, recoveries were 88.8% when using the SEP-PAK columns and about 84.8% with ethyl acetate–butanol.

HPLC separation

The HPLC results, both with standards dissolved in methanol–ammonia and with serum extracts, demonstrated a clear-cut separation of the iodothyronines generated during *in vivo* turnover and conversion studies, namely T_4, T_3, rT_3 and the two T_2 compounds, together. The retention times of these iodothyronines were as follows: 3,3′-T_2 and 3′,5′-T_2, 8 min; T_3, 13 min; rT_3, 20 min; and T_4, 28 min. An example of the separation achieved by the HPLC system is shown in Fig. 1, where the radioactivity profile is superimposed on the optical absorbance profile of the

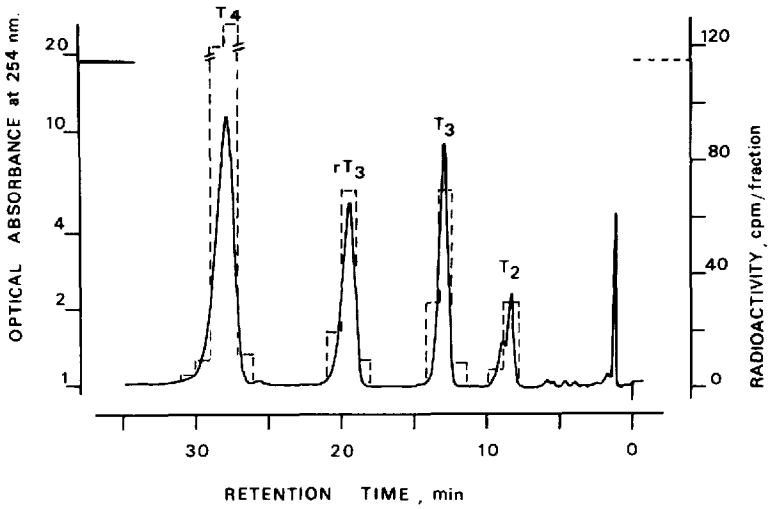

Fig. 1. HPLC chromatogram of iodothyronines. After the injection of ^{125}I-T_4 in a human subject, radioiodothyronines were extracted from serum using the SEP-PAK procedure. Unlabelled iodothyronines were added as a carrier, and as markers of optical absorbance. ——, Optical absorbance profile at 254 nm. —, radioactivity counting profile (cpm per fraction).

various iodothyronines. These profiles were obtained with a serum sample from a subject who had received ^{125}I-T_4 by intravenous injection; the extraction of the iodothyronines from the serum was performed with the SEP-PAK.

Repeated use of the HPLC system over several weeks demonstrated a high reproducibility of the separation profiles.

DISCUSSION

Among the several procedures that have been described previously for the HPLC separation of iodothyronines in samples of various origins[12–21], only a few concern the chromatography of these hormones starting from serum[16,17,21]. Three different procedures for the extraction of thyroxine were described by Lankmayr *et al.*[16]. However, they were time-consuming, and the overall recovery was rather poor. Furthermore, under the conditions described by those authors, the columns become blocked after repeated use, due to the extraction of many other non-polar compounds by the solvent. The method described by Hay *et al.*[17] involves preliminary extraction of iodothyronines with ethyl acetate after precipitation of serum proteins with trichloroacetic acid, followed by adsorption on SEP-PAK (silica). Also in this case, the only compound of interest was thyroxine, in its two stereoisomeric forms (D and L), with an overall recovery as low as 60%. The method described by Burman *et al.*[21], for the extraction of iodothyronines from serum and for the dansyl derivatization of thyroxine, is quite complex and time-consuming. Furthermore, two passes through the HPLC system are required, and a gradient HPLC elution is also necessary, which increases the overall complexity of the method.

In the present report we have demonstrated the feasibility of chromatographic separation of iodothyronines in human serum by using a combination of extraction from serum (either by the SEP-PAK or the ethyl acetate–butanol technique) and HPLC chromatography in a system characterized by an exceedingly high resolution. The main favourable features of this method are: (a) simplicity; (b) high recovery of the iodothyronines; (c) clean resolution of the various iodothyronines involved in T_4, T_3 and rT_3 metabolism and interconversion *in vivo*; (d) perfect reproducibility of the elution profiles; and (e) suitability for application in a system involving gamma-counting of the samples. The advantages of the HPLC method described over classical separation techniques[2–11] are obvious.

When compared with other HPLC separation methods for iodothyronines[12–21] our technique is seen to exhibit a more complete separation and a higher recovery of the iodothyronines. These are important features that make this method most suitable for metabolic studies of the interconversion of thyroid hormones in the peripheral tissues *in vivo*.

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