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HIGH-PRESSURE LIQUID CHROMATOGRAPHY OF AMINO ACIDS, PEPTIDES AND PROTEINS

V. SEPARATION OF THYROIDAL IODO-AMINO ACIDS BY HYDROPHILIC ION-PAIRED REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The separation of thyroidal iodoamino acids has been carried out by high-performance liquid chromatography in phase systems consisting of chemically bonded C_{18}-hydrophobic supports as the stationary phase and water–organic solvent mixtures containing phosphoric acid or other ion-pairing reagents as the mobile phase. Under conditions of hydrophilic ion-pair formation, excellent resolution of the iodoamino acids is observed. This method permits the rapid separation and, hence, analysis of mixtures containing thyroxine, 3,3′,5-triiodothyronine and 3,3′,5′-triiodothyronine and related compounds in *ca.* 30 min with sensitivity, using a UV monitor at 210 nm, at the 1–10-pmole level.

INTRODUCTION

During the past few years, much attention has been focused on new methods for the quantitative determination of drugs, drug metabolites and endogenous compounds present in biological media. The application of high-pressure (performance) liquid chromatographic (HPLC) techniques has proved particularly useful for the separation and analysis of those biogenic compounds which exist in aqueous solution only in ionized states, *e.g.*, quarternary ammonium compounds or as amphoteric substances. Early studies using reversed-phase liquid–liquid chromatography were limited in their application due to the low efficiency and stability of the columns. However, recent developments in chromatographic methodology have made available highly efficient reversed-phase columns with chemically bonded stationary phases

coupled to silica micro-particles clearly proving the more versatile type of column support. Ion-pair partition combined with reversed-phase liquid–liquid chromatography has been found to give separation systems of high efficiency and selectivity for a large variety of compounds. One obvious application of these methods is the analysis of the thyroidal iodoamino acids both in the pharmaceutical and clinical situation. Karger *et al.*[1] have reported an ion partion liquid–liquid separation of thyroxine (T_4) and triiodothyronine (T_3) using perchlorate ion pairs with a mobile phase consisting of methylene chloride–butanol (17:3). A variety of other procedures has been described[2–6] for the separation of mixtures of these iodo compounds by thin-layer (TLC) and paper chromatography, Sephadex gel or controlled-pore glass exclusion chromatography, ion-exchange column chromatography and gas–liquid chromatography (GLC). Many of these earlier methods have special requirements in terms of derivatization of the sample or require large amounts of material.

Recently we reported[7,8] the use of phosphoric acid and other ion-pairing reagents in reversed-phase HPLC for the analysis of underivatized peptides. One advantage of this rapid procedure was that aqueous samples can be applied directly to a chromatographic support and subsequently eluted with an aqueous mobile phase. We illustrated the selectivity of this method by the resolution of small peptides differing only in one amino acid residue. Utilising a similar approach we now report in this paper a new method for the rapid chromatographic analysis of the thyroidal iodoamino acids by reversed-phase partition HPLC.

EXPERIMENTAL

Chromatographic equipment

A Waters Assoc. (Milford, Mass., U.S.A.) HPLC system was used which consisted of two M-6000A solvent-delivery pumps, a U6K universal liquid chromatograph injector and a M-660 solvent programmer, coupled either to a Series 440 UV absorbance detector and a Rikadenki double channel recorder or to a Cecil 515 or Cecil 212 variable-wavelength UV monitor with 10-μl or 8-μl flow-through cells, respectively, and a Linear Instruments Corp. double-channel chart recorder. Unless otherwise indicated the detector operated at a wavelength of 254 nm.

The Bondapak C_{18}-Corasil (37–50 μm) and μBondapak C_{18} (10 μm) columns, which had normal column dimensions of 61 cm $\times$ 2 mm I.D. and 30 cm $\times$ 4 mm I.D., resp., were purchased from Waters Assoc.

Samples injections were made with Pressure-Lok liquid syringes (0–10 μl, 0–25 μl) Series B110 from Precision Sampling. Solvents were filtered using a Pyrex filter holder (Millipore) while iodoamino acid samples were filtered using a Swinney Filter assembly (Millipore) with AP2500 filters.

Reagents

All solvents were AnalaR grade. The methanol was supplied by Mallinckrodt (St. Louis, Mo., U.S.A.) or Fisher Scientific (Pittsburgh, Pa., U.S.A.) and distilled from 0.1% metaphenylene diamine. The acetonitrile, supplied by Fisher Scientific, was further purified by the method of Walter and Ramaley[9]. Orthophosphoric acid was from May and Baker (Dagenham, Great Britain), potassium dihydrogen phosphate from BDH (Poole, Great Britain) and the ion-pairing chromatographic

reagents, PIC Reagent A and PIC Reagent B-5 and B-7 were from Waters Assoc. Water was de-ionised and glass distilled. The iodoamino acids were either obtained from Sigma (St. Louis, Mo., U.S.A.) or prepared by well established literature methods; rT_3 was a generous gift from Dr. Steinmaus, Henning, Berlin, G.F.R. Stock solutions of thyroxine (T-4), 3,3',5-triiodothyronine (T-3), 3,3',5'-triiodothyronine (rT_3), 3,5-diodothyronine (T-2), 3,5-diiodotyrosine (DIT), 3-iodotyrosine (MIT), thyronine (T_0) and tyrosine (Tyr) were prepared by dissolving the compounds in 1% methanolic NH_4OH (methanol–concentrated ammonium hydroxide, 99:1) at a concentration of *ca.* 10 mg/ml.

Methods

A flow-rate of 2 ml/min was generally used, which was maintained at a pressure of *ca.* 1500 p.s.i. for the Bondapak C_{18}-Corasil and 2200 p.s.i. for the μBondapak C_{18} column. The solvent reservoirs, pre-column delivery systems and columns were maintained at a temperature of *ca.* 20°. Samples of the iodoamino acids were prepared from the stock solutions by serial dilution using Eppendorf micro-syringes and diluted with the first eluting solvent. Sample size varied between 10 ng and 10 μg of material injected in volumes 1–25 μl. Detection was at a fixed wavelength of 254 nm using the Series 440 detector or in the range of 190–280 nm, depending on the nature of the sample and mobile phase, using the variable-wavelength detectors. All bulk solvents were degassed separately; water and 100 m*M* KH_2PO_4 for at least 30 min, organic solvents for 1.5 min. The solvents were mixed to the required volumes, degassed for 1.5 min and equilibrated at room temperature. Orthophosphoric acid was added, where indicated, to the aqueous solvents at a concentration of 0.1%. All solvents were stirred magnetically during equilibration and elution. All columns were equilibrated to new solvents or re-equilibrated with the starting solvent after a gradient run for at least 30 min. The mobile-phase solutions were routinely filtered using 0.5 μm Millipore filters.

RESULTS AND DISCUSSION

In contrast to adsorption or polar-phase ion-pair partition chromatography where the order of elution follows the increase in polarity, in the reversed-phase mode for a given mobile phase composition the more polar compounds elute earlier. In preliminary experiments using C_{18}-Corasil and μBondapak C_{18} columns and mobile phases of water–methanol (5–75%) or water–acetonitrile (5–50%), good separations of Tyr, MIT and DIT could be achieved, although these elution systems did not resolve adequately mixtures of the higher thyronine analogues due to their long retention times and poor peak shapes. Previously, we had observed[7,8] with underivatized peptides that the addition of sodium or potassium dihydrogen phosphate (up to 100 m*M*) or orthophosphoric acid (0.1%) to the mobile phase dramatically reduced retention times of non-polar compounds, increased resolution and allowed the use of eluants which contain significantly lower concentrations of organic solvents. Shown in Fig. 1 is the separation of MIT and DIT on a C_{18}-Corasil column eluted with 5% methanol–100 m*M* NaH_2PO_4. Under these conditions Tyr emerges at the solvent front, whilst the thyronine analogues T_3 and T_4, again have very long retention times and consequently are poorly resolved. However these materials can be completely resolved by increasing the methanol concentration to 50% (Fig. 2). This order

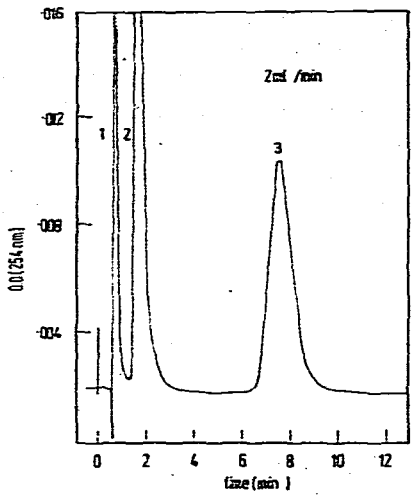

Fig. 1. Separation of tyrosine (1), 3-iodotyrosine (2) and 3,5-diiodotyrosine (3) on a C_{18}-Corasil column with a 5% methanol–100 m*M* sodium dihydrogen phosphate–0.1% H_3PO_4 mobile phase: 2 ml/min; injection volume 10 μl, 20 μg each.

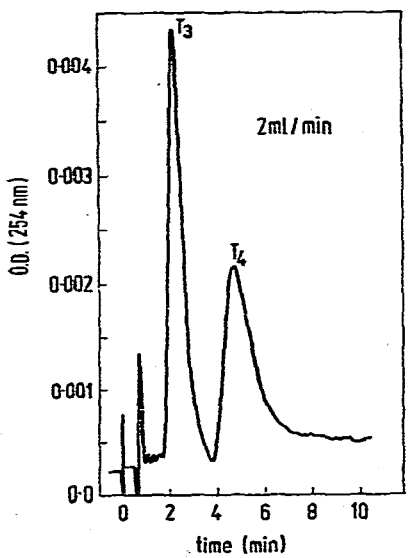

Fig. 2. Separation of the thyroid hormones R_3 and T_4 on a C_{18}-Corasil column using a mobile phase containing 50% methanol–100 m*M* sodium dihydrogen phosphate–0.1% H_3PO_4. Other conditions as for Fig. 1.

of elution is in accordance with the anticipated increasing hydrophobicity of the solvated iodoamino acids taking into account the known hydrophobic nature of the iodo-group[10]. Furthermore, this observed order parallels earlier reports[1,5] of the elution order of T_3 and T_4 on controlled-pore glass and silica particles coated with a stationary phase of 0.2 *M* $HClO_4$–0.8 *M* $NaClO_4$. It is interesting to note that T_0 and

DIT are poorly separated under these conditions. Further studies rapidly confirmed that the remaining thyroidal amino acids also segregated into the two loose chromatographic groupings: tyrosine and its iodo derivatives and thyronine and its iodo analogues, on chemically bonded reversed-phase columns. Although it is possible to carry out a two-stage elution to achieve the complete separation of the compounds MIT, DIT, T_0, T_1, T_2, T_3, rT_3 and T_4 on C_{18}-Corasil columns, two alternatives were examined. Firstly, continuous gradients can now be readily achieved under programmed solvent-delivery conditions. A chromatographic profile of the thyroid hormones under conditions of gradient elution using the elution system 100 m*M* NaH_2PO_4–0.1% H_3PO_4 with a 5–80% methanol gradient is shown in Fig. 3. As can be seen, this method permits the resolution of iodo-amino acids with widely differing polarities. However, due to the change in the methanol concentration during the gradient, the baseline optical density drifts with inherent loss of sensitivity. These difficulties can however be overcome by using reversed-phase packings of smaller particle diameter *e.g.*, 10 μm for the fully porous μBondapak C_{18} supports compared to 37–50 μm for the pellicular C_{18}-Corasil. As can be seen in Fig. 4, the complete separation of many of the thyroidal amino acids can be achieved on μBondapak C_{18} columns by an isocratic elution with methanol–water–H_3PO_4 (50:50:0.1) in less than 30 min. Similar elution profiles were observed using acetonitrile (20%) instead of methanol.

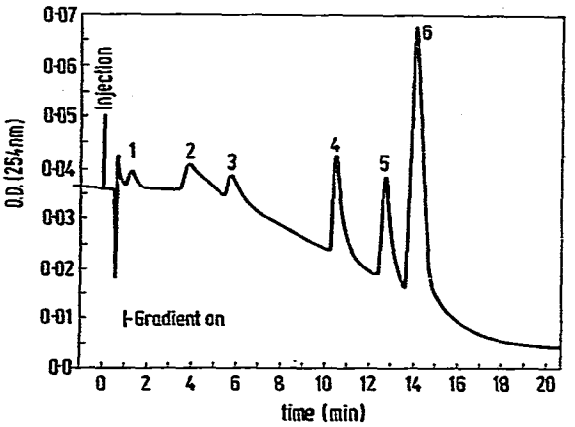

Fig. 3. Gradient elution of iodo-amino acids on a C_{18}-Corasil column using a 5–80% methanol–100 m*M* sodium dihydrogen phosphate–0.1% H_3PO_4 gradient; 2 ml/min; injection volume 10 μl, 1 μg each spiked with thyroxine; 1 = MIT, 2 = DIT, 3 = T_0, 4 = T_2, 5 = T_3 and 6 = T_4. The gradient was generated using programme 5 on the M-660 solvent programmer.

It is noteworthy that the two triiodothyronine isomers, T_3 and rT_3 and thyroxine are completely resolved under these conditions (Fig. 5). The simplicity of the present method has considerable advantages over the other existing methods which usually require prior derivatization[2] or lengthy TLC or GLC techniques for the chromatographic separation and analysis of these closely related compounds. In view of their high extinction coefficients, reproducible analysis of the thyroid hormones could be carried out at *ca.* a 10-pmole level at 254 nm while further improve-

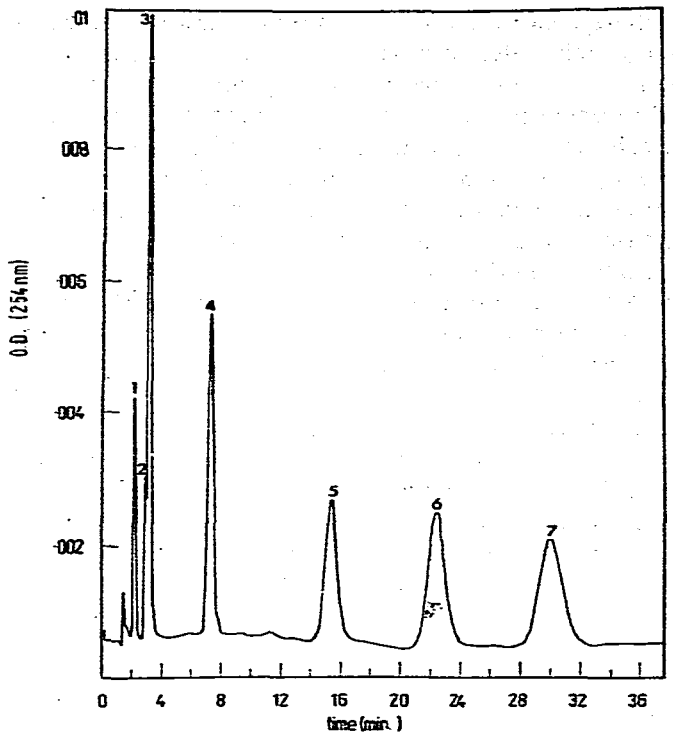

Fig. 4. Isocratic elution of iodo-amino acids using 50% methanol–water–0.1% H_3PO_4; 2 ml/min, on a μBondapak C_{18} column; injection volume 10 μl, 0.2 μg each; 1 = MIT, 2 = DIT, 3 = T_0, 4 = T_2, 5 = T_3, 6 = rT_3 and 7 = T_4.

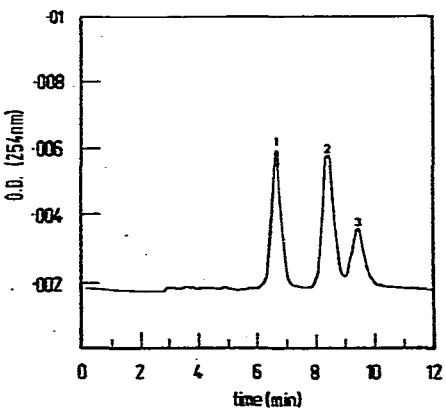

Fig. 5. Separation of the thyroid hormones T_3, rT_3 and T_4 using 62.5% methanol–water–0.1% H_3PO_4, 1 ml/min, on a μBondapak C_{18} column.

ment in sensitivity (*ca.* a factor of 10 depending on the iodoamino acid) can be achieved by using variable-wavelength monitors operating at 210 nm under the elution conditions.

As is evident from Figs. 4 and 6, the compounds DIT and T_0 are poorly

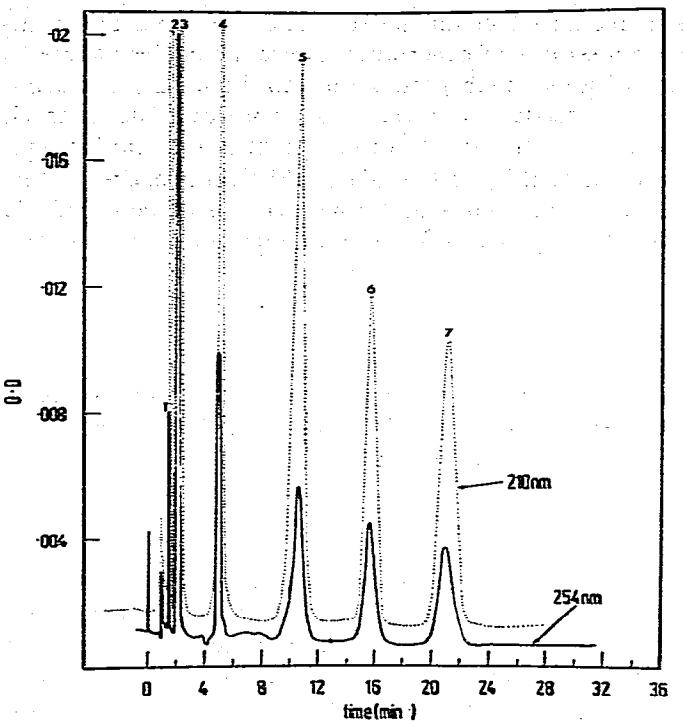

Fig. 6. Comparative elution profiles of the iodo-amino acids detected at 210 and 254 nm, chromatographic conditions as given in Fig. 4, injection volume 20 μl.

resolved on a μBondapak column using the elution system methanol–water–H_3PO_4 (50:50:0.1). However adequate separation of these two compounds can be achieved (at the expense of longer retention times for the less polar iodothyronines) using a lower methanol concentration or acetonitrile–water–H_3PO_4 (15:85:0.1). Alternatively, purified hydrophobic ion-pairing reagents, *e.g.*, the PIC reagents, can be used to improve the resolution of DIT and T_0 although the PIC reagents themselves

TABLE I

RETENTION TIMES OF 3,5-DIIODOTYROSINE (DIT) AND THYRONINE (T_0)

Eluents	*Retention time (min)*	
	3,5-Diiodotyrosine	*Thyronine*
Acetonitrile–water (15:85) + H_3PO_4*	5.6	6.7
Methanol–water (40:60) + H_3PO_4*	1.4	1.6
Methanol–Pic A (40:60)*	3.1	4.2
Methanol–Pic A (40:60) + H_3PO_4*	3.1	3.5
Methanol–Pic A (40:60) + Pic B_7*	3.2	3.4
Methanol–water (40:60) + H_3PO_4**	2.8	3.7
Methanol–water (50:50) + H_3PO_4**	2.4	2.7

* 2 ml/min, on C_{18}-Corasil column.

** 2 ml/min, on μBondapak column.

do not appear to have as significant an effect on the resolution of these two compounds as has been observed with mixtures of other amphoteric molecules (Table I).

In conclusion, a set of conditions which permit the rapid analysis and separation of the thyroid hormones and related compounds is reported. The method, which employs underivatized samples, is amenable to direct quantitation and is rapid. It is logical to expect that application of this, and similar, HPLC approaches to the rapid detection of thyroid hormones and their metabolites will be of considerable value for the routine pharmaceutical analysis and for both *in vitro* and *in vivo* studies involving these compounds.

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NOTE ADDED IN PROOF

Nachtmann *et al.*[11] have recently reported a catalytic detection system for post-chromatographic analysis of iodine-substituted molecules. This elegant method, based on the Sandell–Kolthoff reaction, permits trace determinations of iodinated molecules with the limit of detection for tetraiodothyronine below the 1-ng level. Associated with an efficient chromatographic separation, this new detection method should have sufficient sensitivity for the sequential determination of the thyroid hormones and their metabolic products in plasma and tissues. The ability to rapidly carry at these analyses contrasts with other methods presently used including those based on radio immunoassay techniques.

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