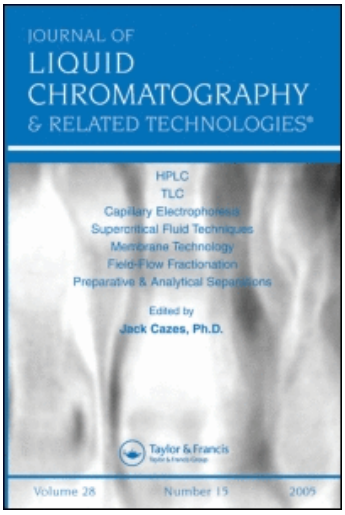

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High Pressure Liquid Chromatography of Thyromimetic Iodoamino Acids

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HIGH PRESSURE LIQUID CHROMATOGRAPHY OF THYROMIMETIC IODOAMINO ACIDS*.

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ABSTRACT

The chromatographic properties of 16 thyromimetic iodoamino acids and related compounds on microparticulate non-polar stationary phases have been examined and conditions determined which allow optimised resolution with analysis time ca.60 minutes. These compounds elute in order of increasing hydrophobicity which correlates with the progressive increase in the number of iodo-groups present in the tyrosine or thyronine aromatic nucleus. The reverse isomers, e.g. rT_3, have consistently greater k' values than their corresponding analogues, e.g. T_3. Conditions for the direct application of the rapid HPLC analyses of the iodoamino acids in biological or pharmaceutical samples have been examined.

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* HIGH PRESSURE LIQUID CHROMATOGRAPHY OF AMINO ACIDS, PEPTIDES AND PROTEINS XIV. PART XIII REF.20.

INTRODUCTION

In view of their pronounced hormonal properties, the naturally occurring thyromimetic iodoamino acids have attracted considerable attention since thyroxine (3,3',5,5'-tetraiodothyronine) was first isolated[1] in 1919. Compelling evidence has been found for the biological interrelationship of many of these compounds by pathways involving enzymatic deiodination, side chain deamination, oxidation or decarboxylation reactions both *in vivo* and *in vitro*. The known vertebrate iodoamino acids predominantly belong to two structurally related groups - either iodinated tyrosines or iodinated thyronines. Because of their biological importance, many studies have been addressed to the problem of separation and analysis of these compounds. A variety of chromatographic methods have been reported based mainly on open column or flat bed chromatography, and include Sephadex gel[2-4] or controlled pore glass exclusion chromatography[5], ion exchange chromatography[6], thin layer chromatography on silicagel[7] or cellulose layers[8], paper chromatography[9] and gas-liquid[10] and liquid-liquid chromatography[11]. Generally only small α value differences are observed for the individual compounds using these methods and the consequent resolution obtained with complex mixtures of the iodoamino acids is often unsatisfactory. In addition, many of these earlier methods suffer from long analysis times, large amounts of materials needed or special requirements in terms of sample derivatisation. Spontaneous loss of iodine from the iodoamino acids leading to degradative products and other artefacts have also been found to occur with several of these chromatographic techniques. Recently, we reported[12] a rapid chromatographic method, based on ion-pair partition reversed-phase high performance

liquid chromatography (HPLC), which circumvented many of the difficulties found with alternative techniques. A feature of this method was the complete resolution of the underivatised thyroid hormones T_4, T_3 and rT_3 with analysis times under 30 minutes.

The purpose of the present study was to examine further the chromatographic properties of the thyroidal iodoamino acids on chemically bonded hydrocarbonaceous stationary phases as a prelude to the development of HPLC method suitable for the direct quantitative analysis of the iodoamino acids in biological or pharmaceutical samples. In this paper we report simple HPLC techniques which allow the separation, and direct quantitation, of 15 naturally occurring iodoamino acids and related metabolites and demonstrate the potential usefulness of this approach for biological analyses.

MATERIALS AND METHODS

Chromatographic Equipment

The apparatus consisted of a Waters Associates Liquid Chromatography system comprising two M6000A pumps, a U6K injector and a M660 solvent programmer, coupled either with a Series 440 or Series 450 UV detector and a Rikadenki double channel chart recorder. The Bondapak C_{18}-Corasil (37-50μm) and μ-Bondapak C_{18} (10μm) columns, which had norminal column dimensions of 61cm x 2mm I.D. and 30cm x 4mm I.D. respectively, were purchased from Waters Associates.

Solvents were filtered using a pyrex filter holder (Millipore Corporation) whilst iodoamino acid samples were filtered using a Swinney Filter assembly (Millipore Corporation) with AP2500 filters. Sample injections were

made with Pressure-Lok Liquid Syringes (0-10µl, 0-25µl) Series B110 from Precision Sampling Corporation.

Reagents.

All mobile phases used organic solvents of AnalaR grade which were purified as reported previously[12]. The iodoamino acids were either synthesised by well established literature methods or obtained from Henning, Berlin GMBH. Stock solutions of the iodoamino acids and related compounds (see Table) were prepared by dissolving the compounds in 1% methanolic NH_4OH (methanol-concentrated ammonium hydroxide 99:1) at a concentration of ca. 5mg/ml. Iodoamino acids presents in the sera of healthy euthyroid subjects were extracted by the methods of Nauman et.al.[13] and Taurog and Chaikoff[14].

Methods.

The solvent reservoirs, pre-column delivery systems and columns were maintained at a temperature of ca. 20°. All bulk solvents were degassed separately, water for at least 30min. and organic solvents for 1.5min. Mobile phases were prepared by mixing the required volumes of bulk solvents and degassed for 30sec. All columns were equilibrated to new mobile phases or re-equilibrated to the initial buffer after a gradient run for at least 30min.

RESULTS AND DISCUSSION

Recently we discussed[15] the role of ion-pairing phenomena in separation of underivatised amino acids, peptides and proteins on chemically bonded reversed phase packings. Studies reported[16-18] from several

TABLE

Structures of Iodoamino Acids and Related Compounds.

No.	Abbreviation	Structure
1.	Tyr	Tyrosine
2.	MIT	3-Iodotyrosine
3.	DIT	3,5-Diiodotyrosine
4.	T_0	Thyronine
5.	T_1	3-Monoiodothyronine
6.	rT_1	3′-Monoiodothyronine
7.	T_2	3,5,-Diiodothyronine
8.	rT_2	3′,5′-Diiodothyronine
9.	T_2'	3,3′-Diiodothyronine
10.	T_3	3,3′,5 - Triodothyronine
11.	rT_3	3,3′,5′-Triiodothyronine
12.	T_4	3,3′,5,5′-Tetraiodothyronine
13.	Diac	3,5-Diiodothyroacetic acid
14.	Triac	3,3′,5-Triiodothyroacetic acid
15.	Tetrac	3,3′,5,5′-Tetraiodothyroacetic acid
16.	Diprop	3,5-Diiodothyropropionic acid

groups have clearly shown that ion-pairing principles can be effectively exploited for the separation of amino

acids by appropriate choice of counter-ion. Mobile phases with low pHs, e.g. pH3, will cause protonation of free amino-groups with concomittant ionic suppression of the carboxyl groups. Small, polar, highly solvated counter-ions which ion-pair to ammonium groups will result in an apparent increased polarity (decreased retention to hydrocarbonaceous stationary phases) of the eluate molecules, whilst hydrophobic counter-ions or ionic suppression will result in decreased polarity (increased retention). The use of phosphoric acid and phosphates, in particular has proved[15,16,18,19] very useful for the resolution of non-polar amino acids and peptides. Under these conditions, the elution characteristics of amphoteric compounds are consistent with hydrophilic ion-pair formation.

In previous studies, we reported[12] conditions for the resolution of the thyroid hormones T_2, T_3, rT_3 and T_4 on hydrophobic stationary phases. The observed elution order, $k'(T_2)<k'(T_3)<k'(rT_3)<k'(T_4)$, appeared to reflect the relative hydrophobicity of the amino acid side chains. Listed in the Table, are the iodo-amino acids used in the present study, together with tyrosine and a group of related iodothyro-acetic and -propionic acids. This latter group of compounds, which lack a free amino group, can only undergo ionic suppressior under elution conditions employing phosphoric acid. Based on our previous findings, it was anticipated that these compounds would have substantially longer retention times than the corresponding iodoamino acid.

For multicomponent samples, such as those obtained as biological extracts of the thyroid hormones and their metabolites, consideration has to be given to the choice of elution mode. Isocratic and gradient elution methods

have been the two most commonly utilised elution modes for multicomponent samples of this type. The convenience of a single mobile phase and excellent reproducibility obtained with isocratic elution of chromatographically similar compounds is offset with samples of widely differing k´ values because long retention times will result in dilution of the solutes with consequent decrease in sensitivity. Gradient elution modes, on the other hand, are capable of optimising a separation involving solutes with a wide range of k´ values, thus minimising the dilution. Despite the advantages which gradient elution offers, the interpretation of most chromatographic data obtained with substances of biological importance has been restricted to applications involving isocratic elution conditions. From chromatographic data obtained with amino acids and peptides, attempts[18,20] have however been made to correlate retention behaviour, chemical structure in terms of the hydrophobicity of the solute and apparent eluant strength of the gradient mobile phase. However, general predictive rules based on theoretical considerations have yet to be rigorously developed. To be effective, such rules would have to be capable of generating optimal gradient shape and mobile phase composition whilst ensuring a minimal analysis time and adequate selectivity. A semi-emperical optimisation strategy based on window diagrams has been employed[21] to estimate the optimum pH of a buffer for the separation of simple ionisable molecules, e.g. weak organic acids. However, a systematic approach to provide optimum chromatographic performance by adjusting other partition factors eg. counterion concentration, ionic strength, mobile phase solvent polarity or flow rate - still remains elusive. Such an

approach would be most pertinent to biological extracts of molecules like the iodoamino acids where gradient optimisation could be required for the resolution of minor components present in a complex mixture.

Isocratic elution conditions using methanol-water-orthophosphoric acid (50:50:0.1%) were found in our earlier study[12] suitable for the complete resolution of T_2, T_3, rT_3 and T_4. Preliminary experiments rapidly demonstrated that these conditions were unsuited for the separation of mixtures containing compounds (1-16) in reasonable analysis times. Consequently, a series of gradient elutions was examined. Shown in Figures 1,2, and 3 are the concave, linear and convex gradient elution profiles for the separation of the iodoamino acids (4-12) on a μ-Bondapak C_{18} column using mobile phases generated from water-phosphoric acid (100:0.1%) and methanol-water-phosphoric acid (80:20:0.1%) with programmes 3,6 and 9 of a Waters 660 solvent programmer respectively. The linear gradient shape clearly represents the best compromise of local optima for the separation of these compounds under the above conditions. Isocratic elution using methanol-water-phosphoric acid (50:50:0.1%) gives satisfactory resultion of most of the thyronine analogues. However, under these conditions the thyroacetic acids have very long retention times ($k' > 50$).

Although excellent separation for most of the iodoamino acids is obtained using isocratic or gradient elution conditions the only exception is the pair 3,3′- and 3′,5′-diiodothyronine which co-elute. In view of the greater column efficiencies found with acetonitrile compared to methanol with unprotected amino acids and peptides, a linear gradient of acetonitrile-water-phosphoric acid (10:90:0.1%) to acetonitrile-water-phosphoric acid (75:25:0.1%) was examined in an attempt to resolve this pair.

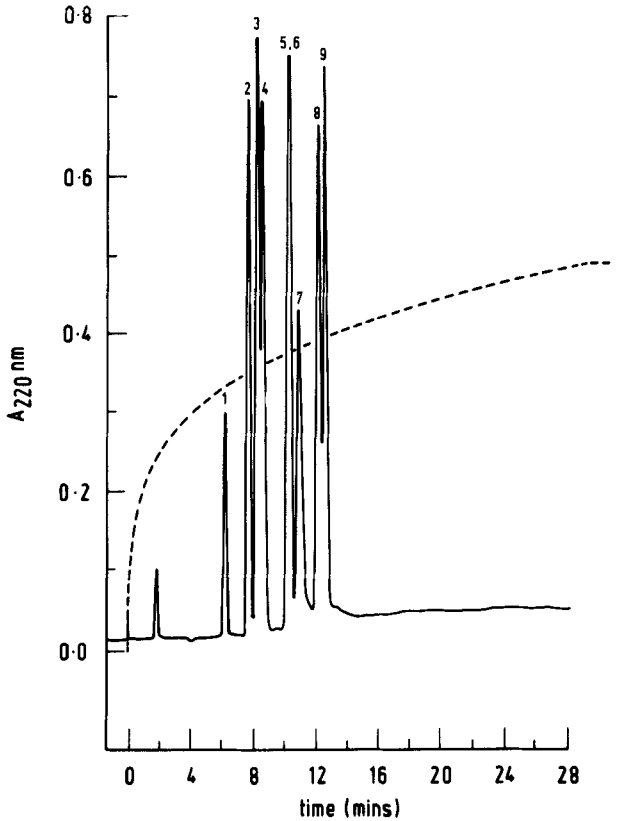


Figure 1. Separation of a series of iodothyronines on a μ-Bondapak-C_{18} column using a 60 minute concave gradient (#3-WAters 660 solvent programmer) at a flow rate of 2ml/min. The gradient was started at the time of injection of the sample and generated from water-phosphoric acid (100:0.1%) to methanol-water-phosphoric acid (80:20:0.1%). Compounds: 1,T_0;2,T_1;3,rT_1;4,T_2;5,rT_2;6,T_2';7,T_3;8,rT_3;9,T_4.

This was not achieved although, as can be seen (Figure 4) these conditions otherwise give excellent resolution of the compounds (1-15).

The thyroacetic acids (13-15) were the last three compounds to elute using acetonitrile or methanol gradients.

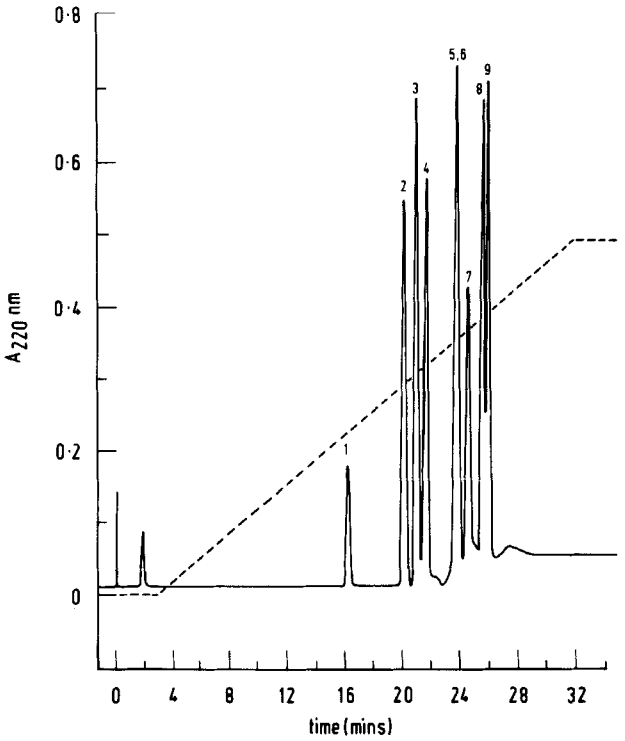


Figure 2. Separation of iodothyronines (4-12) using a 60 minute linear gradient (#6-Waters 660 solvent programmer). Other conditions as in Figure 1.

As anticipated, these compounds are retained more on the non-polar stationary phase than the corresponding iodo-amino acid but are, never-the-less, completely separated. The longer retention of the thyroacetic acids under these elution conditions is in accord with ionic suppression of the carboxyl groups, resulting in a net decrease in polarity. It is noteworthy, that the 3,5-diiodothyro-acetic (13) and 3,5-diiodothyropropionic (16) acids are also well resolved with either isocratic or gradient elution modes (Figures 5 and 6). This observation is

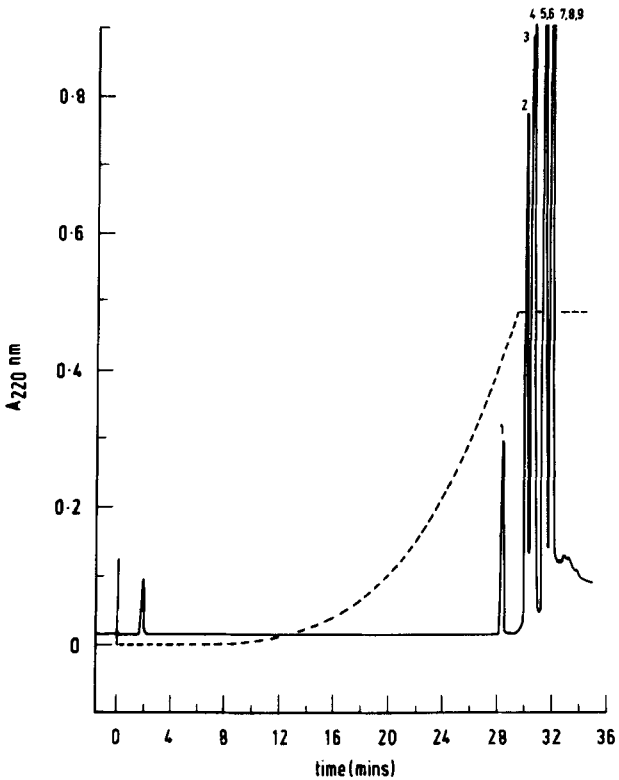


Figure 3. Separation of iodothyronines (4-12) using a 60 minute convex gradient (#9-Waters 660 solvent programmer). Other conditions as in Figure 1.

consistent with other reports[18,22] on the separation of homologous acids differing by as little as a single methylene group. The ability to separate and quantitate these two compounds should considerably simplify the analysis of extracts following the biological α-deaminative and α-oxidative conversion of T_2 to compounds (13) or (16).

The results obtained by isocratic elution of the thyronine analogues at three different methanol concen-

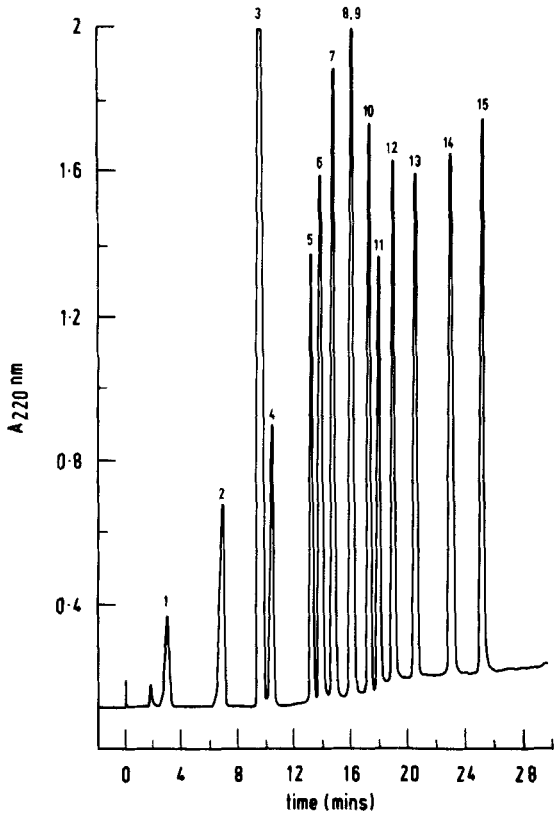


Figure 4. Gradient elution profile of the thyroidal iodoamino acids (1-12) and thyroacetic acids (13-15)(see Table for symbols) on a μ-Bondapak-C18 column at a flow rate of 2ml/min. A 60 minute linear gradient from acetonitrile-water-phosphoric acid (10:90:0.1%) to acetonitrile-water-phosphoric acid (75:25:0.1%) was started at the time of injection of the sample.

trations are shown in Figure 7, which illustrates the apparent linear relationship between the number of iodine atoms present in the thyronine nucleus and the log k′ values. A similar trend is apparent for the thyroacetic acids as well as the tyrosine analogues (Figure 8). The only compound to significantly diverge from this relation-

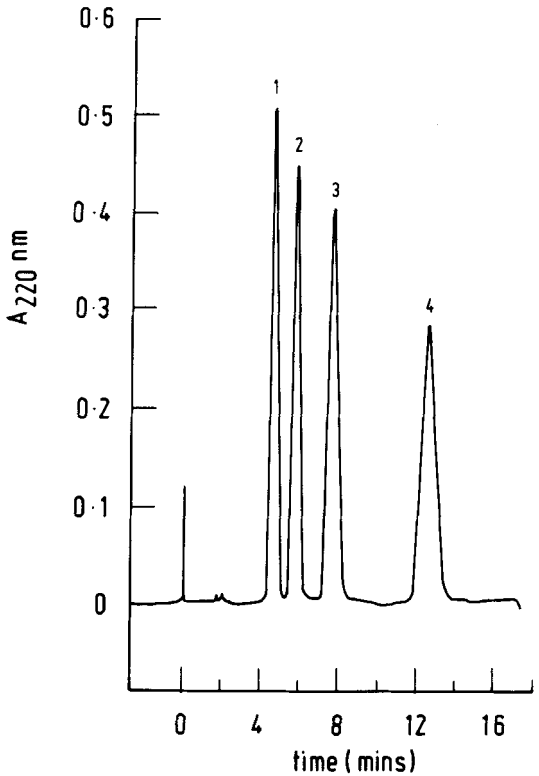


Figure 5. Isocratic elution profile of the thyroacetic and thyropropionic acids (compounds 13-16 in Table) on a μ-Bondapak-C_{18} column at a flow rate of 2ml/min. The mobile phase composition was methanol-water-phosphoric acid (60:40:0.1%). Compounds: 1,Diac;2,Diprop;3,Triac; 4,Tetrac.

ship was T_2 which elutes more rapidly than predicted. The hydrophobic nature of the iodo-group has been recognised in previous studies[12,23] and the greater k′ for MIT (2) compared to Tyr(1) and T_1(5) compared to T_0(4) is consistent with the introduction of a large non-polar group into the aromatic nucleus. The progressive increase in retention for the α-amino acid series Tyr→

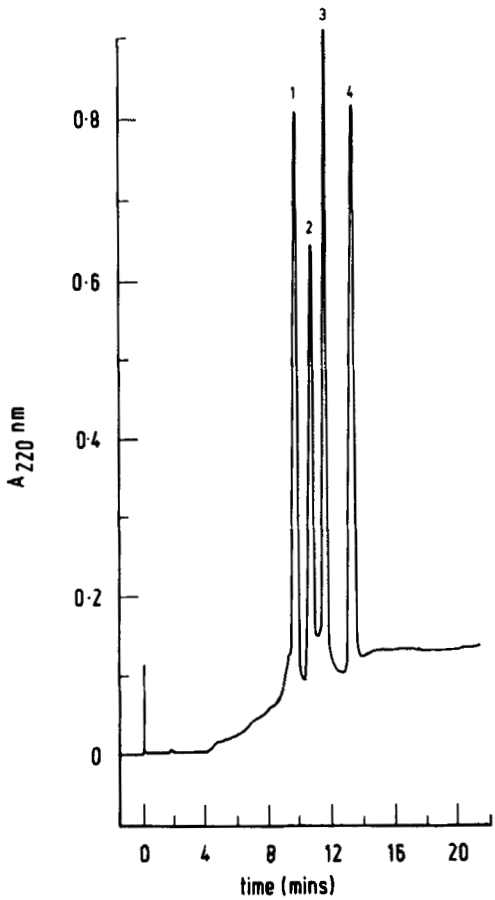


Figure 6. Separation of the thyroacetic and thyropropionic acids (compounds 13-16 in Table) on a μ-Bondapak-C_{18} column using gradient elution at a flow rate of 2ml/min. A 10 minute linear gradient was formed from methanol-water-phosphoric acid (40:60:0.1%) and methanol-water-phosphoric acid (80:20:0.1%) and started at the time of injection of the sample. Other symbols as in Figure 5.

T_4 (1→12) and desamino acid series Diac→Tetrac (13→15) noted in the present study can be related to composite free energy changes arising from both decreasing inter-

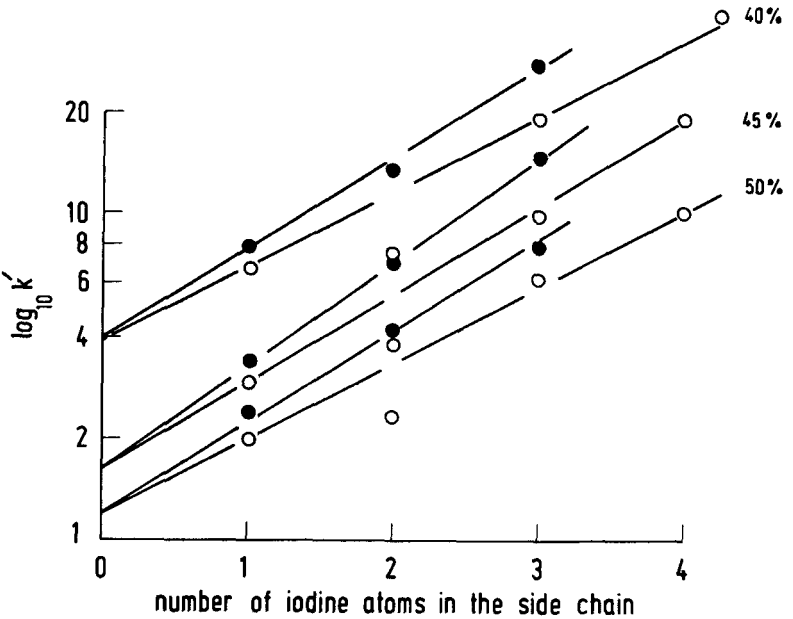


Figure 7. Plots of the logarithm of the capacity factor k', against the number of iodine atoms in thyronine analogues (compounds 4-12), ring A isomers (-O-O-), ring B isomers (-●-●-). The chromatographic conditions were: column, μ-Bondapak C_{18}; flow rate, 2ml/min; mobile phase, methanol: water:0.1% phosphoric acid at 40,45 and 50% methanol concentration.

action of the solute complex with the solvent molecules and reduction in the effective contact surface area of the solute complex exposed to the mobile phase due to progressively greater binding of the compounds, in each series, to the non-polar stationary phase. The latter effect is clearly dominant with ionisable molecules and should manifest itself by an apparent linear relationship between log k' and molecular size of the type found for the iodo-compounds. It is interesting that the so-called reverse isomers (6,8,11), with predominantly ring B iodination patterns, have consistently greater k'

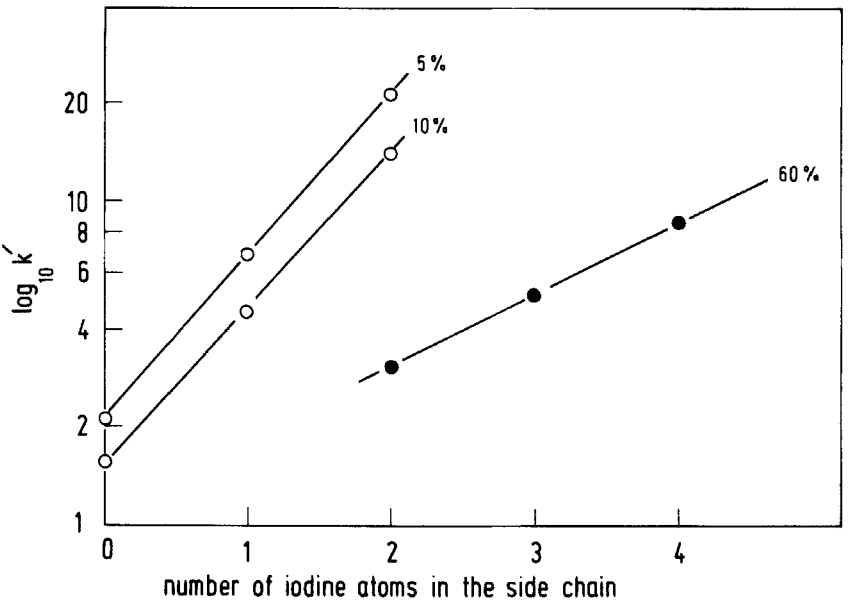
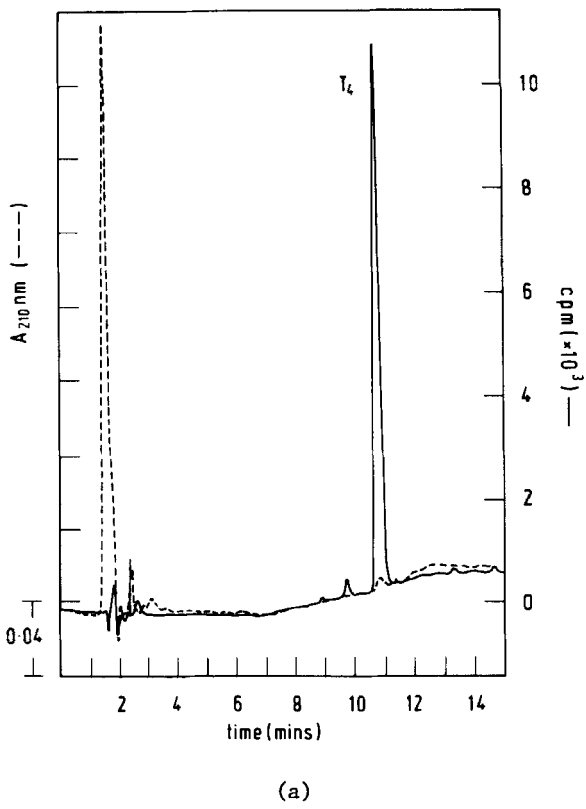


Figure 8. Plots of the logarithm of the capacity factor k′, against the number of iodine atoms in the thyroacetic acids (compounds 13-15)(-●-●-) and tyrosine derivations (compounds 1-3) (-o-o-). The chromatographic conditions were: column, μ-Bondapak-C_{18},; flow rate, 2ml/min; mobile phase, methanol-water-0.1% phosphoric acid at 60% methanol concentration for the thyroacetic acids and 5% and 10% methanol concentration for the tyrosine derivatives respectively.

values than the corresponding ring A type isomers (5,7, 10). These k′ difference between isomeric compounds must reflect relative decreases in polarity of the reverse isomers and may be attributable to modification of the polar effects of the 4′-hydroxyl group due to the B-ring iodo-groups, analoguous to the known[24] lowering of the

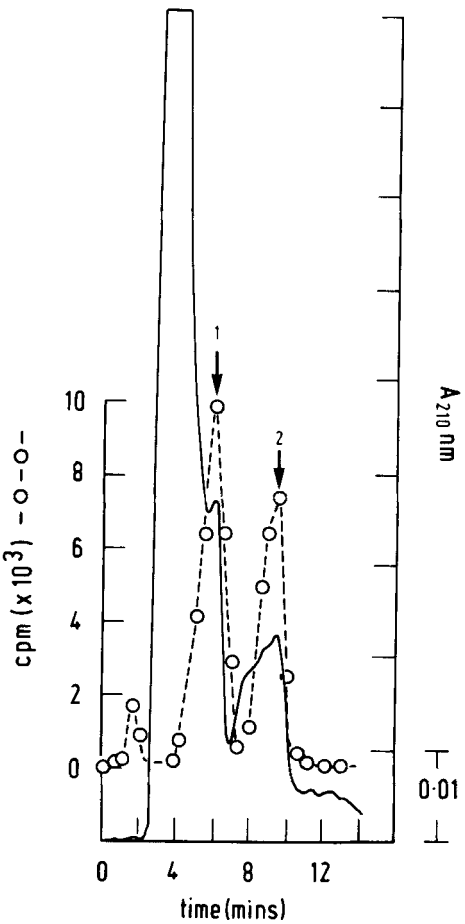
phenolic pK values. The divergence of T_2 from the predicted linear relationship manifested as shorter retention times and greater polarity than expected on the basis of molecular size, could also arise from a related effect.

We have found the techniques described in this paper useful for the detection of thyroid hormones and their metabolites in the nanogram range in biological extracts as well as in pharmaceutical preparations (Figure 9). Improved sensitivity for quantitative studies in some of the areas should be feasible using detectors[25] based on the Sandell-Kolthoff reaction. This reaction relies on the catalytic activity of iodine to promote the pseudomonomolecular redox reaction between Ce(IV) and As(III). Because the catalytic activity of some of the iodo-amino acids eg. T_3, in this reaction is weak, the usefulness of this method for routine determinations of the low concentrations present in normal serum remains doubtful. Until catalytic detectors are firmly established, variable wavelength UV monitors coupled with larger sample sizes remain more convenient to use for the detection of the unlabelled molecules. The excellent resolution of these closely related iodo-amino acids and analogues as well as the rapid analysis time which can be achieved using these HPLC methods should also be of considerable assistance with iodine metabolism studies employing isotopically-labelled substrates eg. ^{125}I-thyroxine. The procedures report in this paper have been applied for checking the purity of radio-active thyroid hormones and for following deiodination mechanisms in mammalian tissues. The results of these studies will be described in detail elsewhere[26].



(a)

Figure 9. Chromatographic analysis of iodoamino acid products extracted from human serum using (a) gradient or (b) isocratic elution modes. Chromatographic conditions as in Figure 2 and 7. The extracts, after removal of the solvent, were taken up in methanolic NH_4OH (methanol-conc. ammonium hydroxide 99:1) (1ml) containing a 10µl aliquot of labelled ^{125}I-T_4 (gradient) or ^{125}I-T_3 and ^{125}I-T_4 (isocratic), (1) and (2) respectively.



(b)

ACKNOWLEDGEMENTS

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