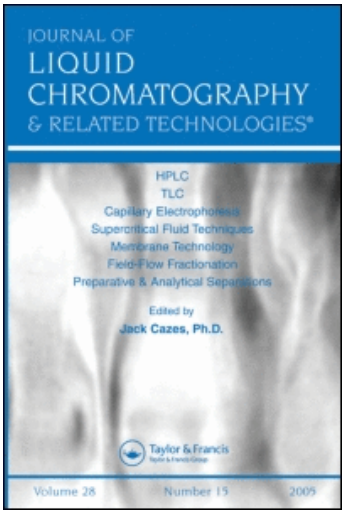

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Rapid Separation and Determination of Thyromimetic Iodoamino Acids by Gradient Elution Reverse Phase Liquid Chromatography with Electrochemical Detection

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RAPID SEPARATION AND DETERMINATION OF THYROMIMETIC IODOAMINO ACIDS BY GRADIENT ELUTION REVERSE PHASE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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ABSTRACT

The usefulness and capabilities of gradient elution techniques with electrochemical detection are shown with a rapid separation of D,L-tyrosine (Tyr), 3-iodo-L-tyrosine (MIT), 3,5-diiodo-L-tyrosine (DIT), D,L-thyronine (T_0), 3,5-diiodo-D,L-thyronine (T_2), 3,3',5-triiodo-L-thyronine (T_3), and L-thyroxine (T_4) by reverse phase liquid chromatography. A rapid (five minute) isocratic separation of T_0, T_2, T_3, and T_4 is also reported. Limits of detection are in the sub-nanogram range with a linear dynamic range to 500 ng for T_2 and T_3, and 1000 ng for T_4. Analysis of levothyroxine sodium tablets and injectable intravenous samples is described.

INTRODUCTION

The mechanism of action of the thyroid hormones, T_3 and T_4, is of considerable interest in part because of the amazing diversity of thyroid hormone effects. These agents influence the metabolism of almost every class of food stuff. They exert profound effects on many enzymes and on almost all organ systems, and they play an integral role in the complex biological processes involved in growth and differentiation (1). The time consuming procedures in

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USP monographs have been used for analysis of levothyroxine sodium tablets (2) and liothyronine sodium tablets (3). The assay of the major thyroid hormones T_3 and T_4 also is done by wet analysis (4-6), radioimmunoassay (7-10), chemical derivatization followed by gas chromatography with electron capture detection (11,12), thin layer chromatography (13-15), paper chromatography (16,17), electrophoresis (18), and gas chromatography/mass spectrometry (19). The thin layer and paper chromatography as well as the electrophoretic procedures do not have good limits of detection for this kind of analysis. Although the gas chromatographic procedures are sensitive, they require the isolation of the iodoamino acids in a pure form, which must then be converted to a volatile derivative for chromatographic analysis. The radioimmunoassay procedures are impractical for a small number of samples and have the added problem of disposal of the radioactive wastes.

Liquid chromatography would normally be the method of choice for these analyses because of the poor volatility of the compounds. In fact, HPLC methods for separation of pure iodoamino acids have appeared in the literature (20-24). Recently T_3 and T_4 tablets have been analyzed by reverse phase LC using UV detection (25) and a gradient elution separation of sixteen thyromimetic iodoamino acids has been reported (26). However, the low molar absorptivity of these compounds at 254 nm precludes their determination at trace levels. Detection at 220 nm improves this situation somewhat (26) and a clever catalytic post-column detection scheme has also been shown (27). Most recently, application of amperometric electrochemical detection to these compounds has been shown to give excellent limits of detection (28), as has dansyl derivatization and subsequent fluorescence detection (29).

The purpose of this study was to demonstrate the usefulness of gradient elution techniques with electrochemical detection for the separation of seven thyromimetic iodoamino acids. A rapid isocratic separation of T_0, T_2, T_3, and T_4, as well as analysis of T_4 both in tablets and injectable intravenous samples is presented.

EXPERIMENTAL

Cyclic Voltammetry System

To find an approximate active potential for the thyromimetic iodoamino acids, a CV-1A cyclic voltammetry instrument and an electrochemical cell made by BioAnalytical Systems, Inc. (West Lafayette, IN) were used. The working and reference electrodes were glassy carbon and Ag/AgCl, respectively. Before running the CV experiments, the sample solutions were purged for 20 minutes with helium. Cyclic voltammetry was then carried out in an inert He atmosphere. A Plotamatic MFE-715 (MFE, Salem, NH) X-Y recorder and digital voltmeter were used to record the cyclic voltammograms.

Liquid Chromatography System

Either a Waters 6000A (Waters Associates, Milford, MA) isocratic solvent delivery unit with an Altex 210 (Altex Scientific, Berkeley, CA) injection valve with 5, 10 and 20 μL loops, or an Altex model 322 gradient liquid chromatograph with two model 100A pumps were used in conjunction with an Altex Ultrasphere octyl column, 250 x 4.6 mm, or an Altex Ultrasphere ODS, 150 x 4.6 mm, both having 5 μm particle diameters. For isocratic elution, the mobile phase was CH_3OH:H_2O:H_3PO_4 (70:30:0.2), while for gradient elution, solutions of 0.15% H_3PO_4 in H_2O (solvent A) and 0.20% H_3PO_4 in CH_3OH (solvent B) were used. The mobile phase was degassed with He during chromatographic runs. Phosphoric acid was used as background electrolyte and also to suppress the ionization of the thyromimetic iodoamino acids (30).

Electrochemical Detector

An LC-4 amperometric controller and electrochemical cell from BioAnalytical Systems, Inc., were used. The working and reference electrodes were glassy carbon and Ag/AgCl, respectively.

Standard Solutions

The compounds Tyr, MIT, DIT, T_0, T_2, T_3, and T_4 were purchased from Sigma Chemical (St. Louis, MO) and were stored in a freezer. Standard solutions were prepared by dissolving appropri-

ate amounts of each compound in methanol containing 1% ammonium hydroxide and were stored in a refrigerator.

Preparation of T_4 Tablet Solution and Injectable T_4 Sample

Twelve tablets (1.5676 g) containing levothyroxine sodium were dissolved in 20 mL of 0.01 M sodium hydroxide using an ultrasonic bath. The sample solution was heated at 60°C for 3 minutes, shaken for 3 minutes, and then filtered through F2406-9 (S/P) filter paper. Before chromatographic injection this solution was again filtered with a Rainin (Rainin Instruments, Woburn, MA) HPLC sample filter syringe using a 0.45 μm nylon-66 membrane filter. The injectable sample was present as a powder and was prepared by dissolving in 5 mL 0.9% sodium chloride solution. This resulted in a clear solution which was then filtered with the sample filter syringe.

RESULTS AND DISCUSSION

Gradient Elution LC/EC

Amperometric electrochemical detectors are generally considered incompatible with gradient elution techniques (31,32). The necessity of the presence of a background electrolyte and the dependence of the charging or residual current on the exact composition of the mobile phase has discouraged attempts to use this powerful liquid chromatographic technique. Changes in the polarity and dielectric constant of the mobile phase during a gradient program yield steeply sloping baselines from the ever changing charging current. Indeed, to our knowledge the only published report of gradient elution LC/EC used a gradient of only 36-60% methanol (33).

Initial attempts were made using equal concentrations of background electrolyte in both the water and methanol reservoirs. During a gradient from 0 to 100% methanol a large negative shift in background current was noted, so the background electrolyte concentration was increased in the methanol reservoir. This then increases the concentration of the background electrolyte in the

mobile phase as the gradient progresses and somewhat lessens the effect of the decreasing polarity and dielectric constant on the residual current. Figure 1 shows the baseline change during a blank injection and a gradient from 0 to 90% methanol with a background electrolyte concentration of 0.15% H_3PO_4 in water and 0.20% H_3PO_4 in methanol. It should be stressed that the potential of the working electrode during this gradient program was +1.4 V and that lower working potentials should show even less baseline shift. Also, no extraordinary efforts were made to purify the water used, and some of the peaks observed are undoubtedly from trace organic compounds which had adsorbed at the top of the column. Other common background electrolytes have not yet been tried but should behave in a similar manner.

Figure 2 shows the rapid gradient elution separation of the 7 thyromimetic iodoamino acids which are shown in figure 3. The peak at 3.5 minutes is from the ammoniacal methanol used to dissolve the sample. This chromatogram demonstrates the potentially powerful applications of gradient elution LC/EC.

Isocratic Separations

To maximize the signal-to-noise ratio of an electrochemical detector, the applied potential should be held at the minimum value at which the current reaches the limiting current plateau of the analyte ($E_{plateau}$). This potential can be quickly estimated from cyclic voltammetry (34) and can then be determined precisely from an electrohydrodynamic voltammogram (EHDV) in which the current is measured vs. applied potential point by point. An EHDV for T_2, T_3, and T_4 is shown in figure 4. Each point is the average signal from two, 5 µL injections of a 50 ppm solution (0.25 µg/injection) at a flow rate of 1 mL/min. As seen in figure 4, a potential of +1.2 V is a reasonable potential for measurement of these compounds, and the other thyromimetic iodoamino acids were also found to produce large signals at this potential.

Analytical calibration curves, current vs. concentration, for T_2, T_3, and T_4 are shown in figure 5. Each point is the average

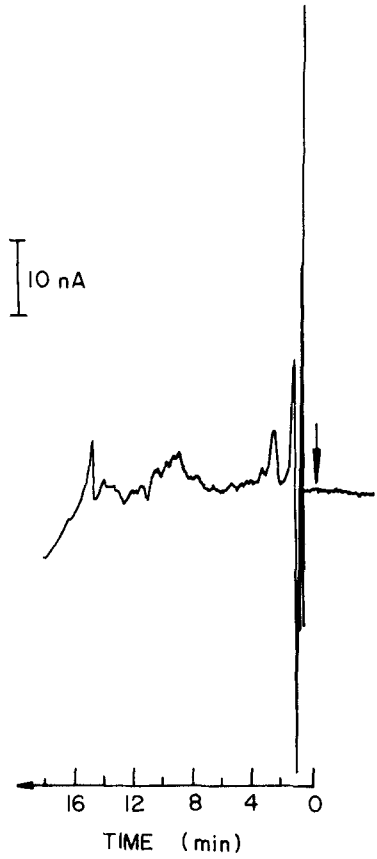


FIGURE 1. Baseline during gradient program with no sample injection. E = +1.4V vs. Ag/AgCl. Solvent A: 0.15% H_3PO_4 in H_2O. Solvent B: 0.20% H_3PO_4 in methanol. Flow rate 2.0 mL/min. Gradient program: Initially 100% A, then immediate linear ramp to 40% B over 8 min, to 60% B over 3 min, and to 90% B over 7 min.

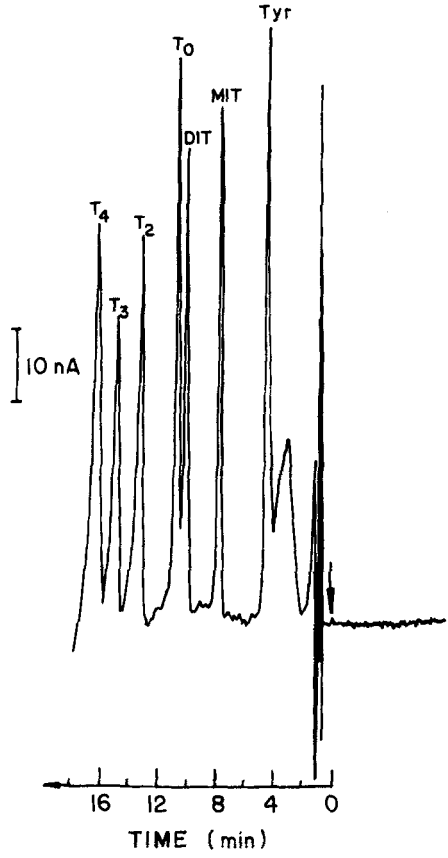
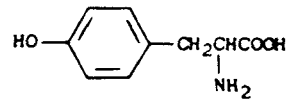


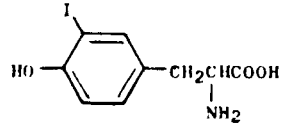
FIGURE 2. Separation of seven thyromimetic iodoamino acids. Column: Altex Ultrasphere ODS, 150 x 4.6 mm. 10 µL injection of 20 ppm Tyr, 40 ppm MIT, 70 ppm DIT, 25 ppm T_0, 60 ppm T_2, 60 ppm T_3, and 200 ppm T_4, other conditions as in figure 1.

Thyromimetic Iodoamino Acids and Related Compounds

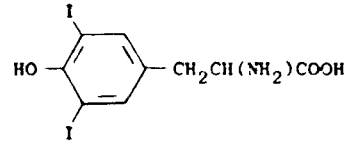
D,L-Tyrosine (Tyr)



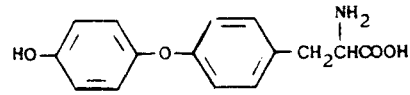
3-Iodo-L-Tyrosine (MIT)



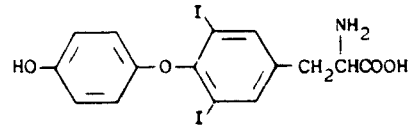
3,5-Diiodo-L-Tyrosine (DIT)



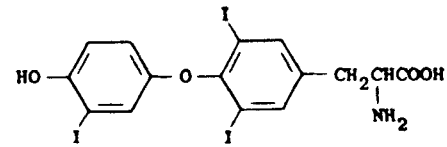
D,L-Thyronine (T_0)



3,5-Diiodo-D,L-Thyronine (T_2)



3,3',5-Triiodo-L-Thyronine (T_3)



L-Thyroxine (T_4)

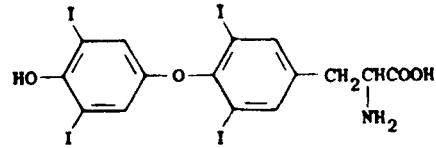


FIGURE 3. Thyromimetic iodoamino acids used in this study.

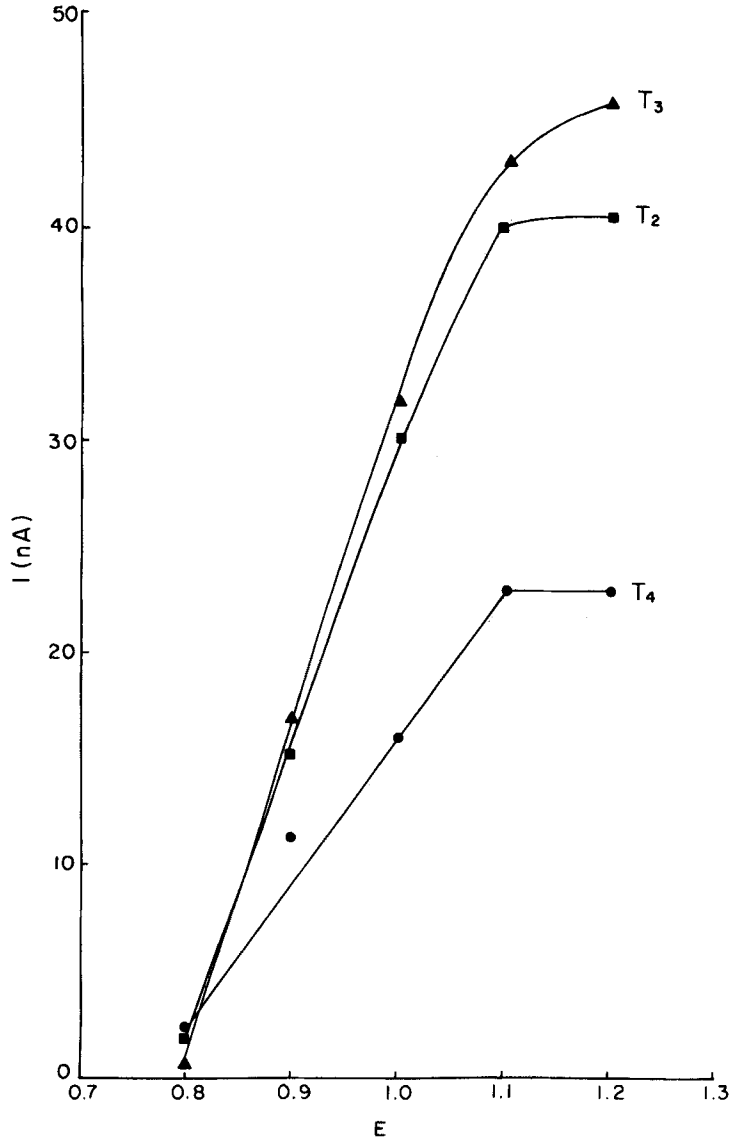


FIGURE 4. Electrohydrodynamic voltammogram for T_2, T_3, and T_4. Column: Altex Ultrasphere Octyl, 250 x 4.6 mm; mobile phase: 70:30:0.2 $CH_3OH:H_2O:H_3PO_4$; flow rate: 1.0 mL/min; 5 µL injection of 50 ppm solution.

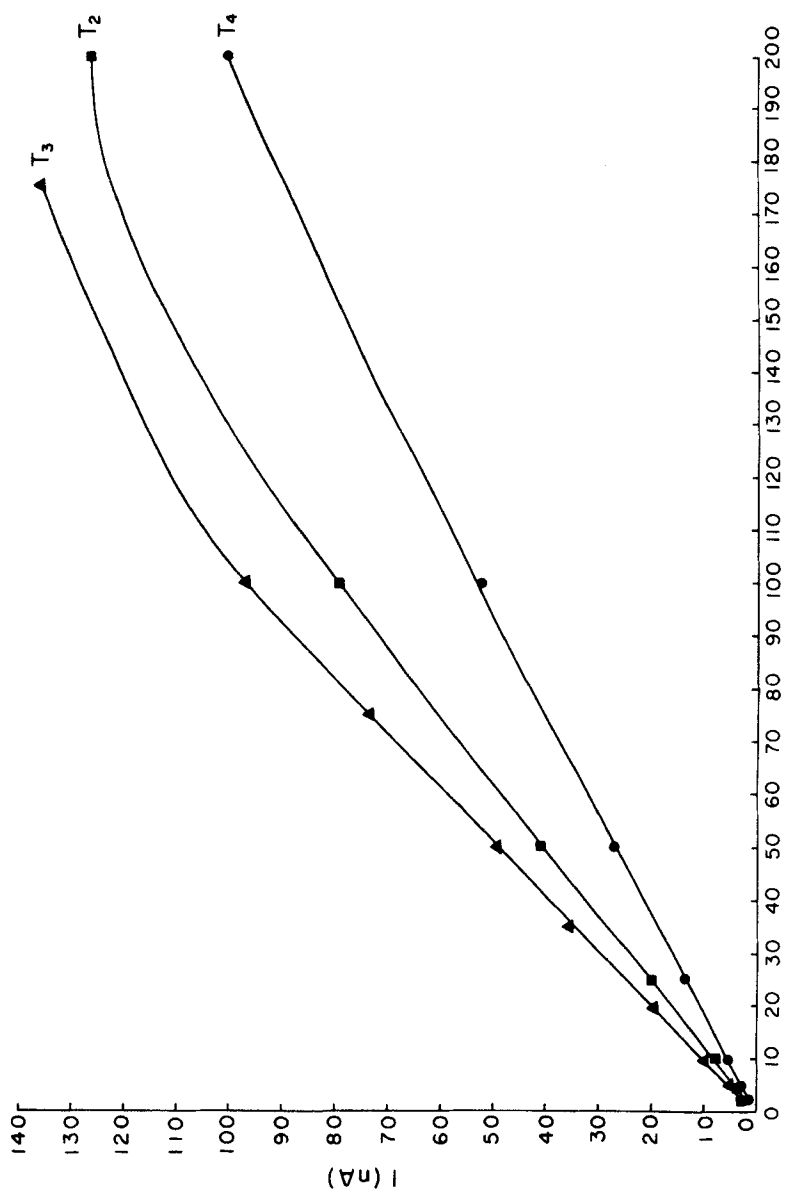


FIGURE 5. Analytical curves for T_2, T_3, and T_4. E = +1.2V. Other conditions as in figure 4.

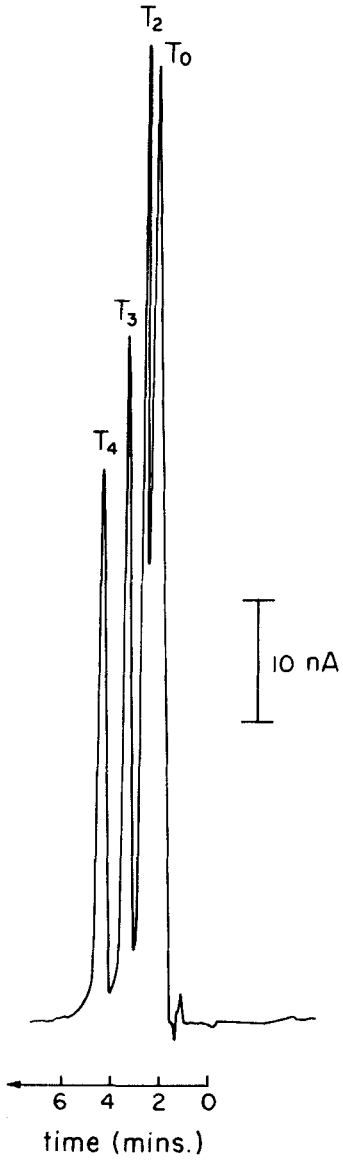


FIGURE 6. Isocratic separation of T_2, T_3, and T_4. Flow rate: 2.0 mL/min. E = +1.2V. Other conditions as in figure 4.

TABLE 1

Analytical Figures of Merit for T_2, T_3, and T_4. E = +1.2V, Other Conditions as in Figure 4.

Compound	Limit of Detection (ppm)	(ng)	Upper Limit of LDR (ppm)	(ng)	Sensitivity (nA/ppm)	(nA/ng)	Log-log Slope	t_R (min)
T_4	0.130	0.65	200	1000	0.460	2.3	0.97	9.0
T_3	0.061	0.31	100	500	0.984	4.9	0.98	7.0
T_2	0.074	0.37	100	500	0.805	4.0	1.00	6.0

signal of two, 5 µL injections of standard solutions with an applied potential of +1.2 V. The limit of detection (LOD), defined as three times the peak-to-peak noise/sensitivity, the maximum concentration of the linear range, the sensitivity (slope), and the log-log slope are given in Table 1. As can be seen, the limits of detection are in the sub-nanogram range, and the LODs for Tyr, MIT, DIT, and T_0 should be even lower, as the signal for these compounds is greater than for an equal concentration of T_2, T_3, or T_4. Figure 6 shows a rapid isocratic separation of T_0, T_2, T_3, and T_4.

Assay of T_4 Preparations

To demonstrate the usefulness of electrochemical detection for these compounds, both T_4 tablets and intravenous solutions were analyzed. For the determination of T_4, a calibration curve was prepared using standard solutions. Each standard was measured two times and the average peak height signal of these two measurements was used for the calibration curve. The average signal of 5 measurements was used for the unknowns. The average amount of L-thyroxine per tablet was found to be 24.3 µg (25 µg/tablet claimed), and the injectable solution was found to contain 704 µg (500 µg claimed). The reason for this large excess is unknown.

As amperometric detectors, particularly with glassy carbon working electrodes, are known to undergo changes in sensitivity with time, it is necessary to run two or more standards daily to

reestablish the slope of the working curve. A study of reactivation methods for solid electrodes used in LC/EC and flow injection analysis has recently been made (35). It is also necessary to prepare fresh standards daily, as the compounds were found to slowly decompose, with old standards showing a small peak eluting before the T_4 peak.

CONCLUSIONS

A method for utilizing gradient elution techniques with electrochemical detectors is described. This advance should greatly increase the usefulness of this detection method and should serve to shorten analysis times where electrochemical detection is the method of choice.

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