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DETERMINATION OF L-THYROXINE IN REFERENCE SERUM PREPARATIONS AS THE *o*-PHTHALALDEHYDE–N-ACETYLCYSTEINE DERIVATIVE BY REVERSED-PHASE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A simple procedure for the assay of L-thyroxine in serum preparations with D-thyroxine as internal standard is described. The L-thyroxine is extracted with acetonitrile, fractionated on a reversed-phase silica cartridge and analysed by reversed-phase high-performance liquid chromatography of the *o*-phthalaldehyde–N-acetyl-L-cysteine derivative. This derivative is not fluorescent, but may be detected with suitable sensitivity and selectivity with an electrochemical detector.

INTRODUCTION

Radioimmunoassay (RIA) is usually the method of choice for the determination of thyroxine (T4) in serum samples. However, in setting up a reference preparation for use as an immunoassay standard it is advisable to obtain an estimate for T4 content derived by an independent method. There are several published methods for determination of T4 by gas chromatography (reviewed to 1985 by Corkill et al. [1]; see also Refs. 2–6). Most of these have involved elaborate sample preparation and derivatisation before analysis. As an alternative we decided to investigate high-performance liquid chromatography (HPLC), and the method described in this paper was developed as part of a

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study to determine the feasibility of a simple physicochemical assay for T4 in lyophilised preparations of serum intended for reference use.

There have been a number of studies into the separation of T4 and related iodoamino acids by HPLC [7–26]. Most of these have dealt with the analysis of pure standards or pharmaceutical preparations, and others with radioactively labelled preparations. Only a few publications have been concerned with the measurement of T4 in serum [15,17–19]. In principle the levels of T4 in serum (50–100 nmol/l) are within the detection limits of a conventional UV detector, but in practice the high UV absorption background makes this impossible without very extensive clean-up [6]. Alternative detection methods are therefore necessary: these have included the catalytic effect of trace iodine on suitable redox reactions [8,14], fluorescence detection of the dansyl derivative [17] and electrochemical detection [19]. In two of these cases the main intention was to monitor the ratio of L- and D-T4 in serum [15,19], while in the third [17] no use was made of an internal standard, values being based on trial recoveries of ^{125}I-labelled T4. Since we did not expect to encounter D-T4 in the candidate reference preparations under examination we elected to use D-T4 as an internal standard, since preliminary studies showed considerable variations in recovery of T4 during extraction. We first examined reaction with *o*-phthaldialdehyde (OPA)–mercaptoethanol, fluorescamine and dansyl chloride for suitability as fluorescent derivatives. Of these only dansyl chloride gave a fluorescent product, and a scheme based on the analysis of the dansyl derivatives using fluorescence detection and a mobile phase containing proline and Cu^{2+} [27] to resolve D- and L-T4 was adopted. The quenching effect of Cu^{2+} on the fluorescence resulted in physiological concentrations of T4 being at the limit of sensitivity, and we therefore developed an alternative assay using electrochemical detection of the OPA–N-acetylcysteine (NAC) derivatives [28].

EXPERIMENTAL

Apparatus

The HPLC apparatus was assembled from an Altex 110 or Kontron T-414 pump fitted with pulse dampening and, in the latter case, an extra pressure gauge to further reduce flow irregularities. Samples were injected via a Rheodyne 7125 loop injector fitted with a 1-ml loop onto a glass cartridge column of Hypersil ODS (100 mm × 3.0 mm I.D., 5 μm) (Chrompack, London, U.K.) protected by a guard column (5 mm × 3 mm, I.D.) filled with pellicular ODS material (Whatman, Maidstone, U.K.) or, for larger-scale separations, a column (250 mm × 4.6 mm, I.D.) of Alltech C_{18} (10 μm) (Alltech Assoc./Applied Science, Carnforth, U.K.). The main detector used was a Bioanalytical Systems (BAS) Model LC4A electrochemical detector, but a Waters M-460 and an LDC electrochemical detector were used for some analyses (as noted in

Results). All were operated at an oxidation potential of +0.73 V. The BAS detector was normally operated on the 2 nA/V range; the other detectors gave similar results at a sensitivity corresponding to 5 nA/V. The output from the detector was processed on a Hewlett Packard Model 3390A or Spectrophysics Model 4270 integrator. For samples not derived from serum or plasma a UV detector operated at 220 or 280 nm was used. UV spectra of chromatographic peaks were collected with a diode array spectrophotometer (Polychrom, Varian Assoc., Walton-on-Thames, U.K.).

Materials

L-T4, D-T4 and OPA ('phthaldicarboxaldehyde') were obtained from Sigma (Poole, U.K.). NAC was from Boehringer Mannheim (Lewes, U.K.). C_{18} Sep-Pak cartridges were obtained from Waters Assoc. (Watford, U.K.).

Sample preparation

Lyophilised serum or plasma preparations were reconstituted with an accurately weighed volume of water. The vials were capped with sealing film, gently mixed by inversion and allowed to stand at ambient temperature for 2 h. Aliquots of 1 ml of reconstituted serum were dispensed into 7-ml stoppered glass tubes, and 40 μl of D-T4 (2.25 μg/ml) were added as internal standard. The tubes were stoppered and allowed to equilibrate for 1 h. All additions were weighed and final results were corrected accordingly. A 60-μl aliquot of 1 *M* sodium hydroxide was added to each tube and the tubes were mixed and allowed to stand 30 min at ambient temperature. Protein was precipitated by the addition of 3 ml acetonitrile followed by vortex-mixing for 60 s. After standing for 10 min, the tubes were centrifuged at 2000 *g* in a bench centrifuge, and the upper phase was transferred to a fresh glass tube and evaporated to dryness at 60°C in a stream of nitrogen. The extracts were dissolved in 2 ml of 1% (v/v) aqueous trifluoroacetic acid (TFA), mixed by vortex or ultrasonically for 2 min, and the sample and washings (2 ml) were allowed to pass under gravity (about 5 min) through a C_{18} extraction cartridge (which had previously been treated with 2 ml of ethanol followed by 5 ml of 1% TFA). The cartridge was washed under gravity with 2 ml of 50% (v/v) methanol–1% TFA, and the T4 fraction was eluted into a 1.5-ml conical-bottomed plastic tube with 1 ml of 1% (v/v) TFA in methanol forced through with a syringe. The eluate was evaporated to complete dryness at 60°C in a stream of nitrogen and stored at −20°C until ready for analysis.

Derivatisation

Just before analysis the contents of individual tubes were redissolved in 40 μl of water and mixed in a vortex mixer for 2 min. An aliquot of 40 μl of 0.4 *M* sodium borate pH 9.5 was added, the tube mixed, and 10 μl of a (fresh daily) solution of 5 mg OPA and 5 mg NAC in 1 ml borate buffer were added followed

by vigorous mixing. After exactly 1 min, the tube was centrifuged at maximum speed in a 'micro-centaur' centrifuge for exactly 30 s, and an aliquot of approximately 85 μl was injected into the HPLC system within 30 s.

High-performance liquid chromatography

For analysis of serum samples the mobile phase was methanol–phosphoric acid–water (71:0.1:28.9, v/v). The flow-rate was normally 0.6 ml/min. Following the emergence of the T4 derivative peaks (at about 15 min) 1 ml of 0.1% phosphoric acid in methanol was injected to clear retained material from the column. The next sample was injected as soon as the baseline had stabilised (about 10 min). Peak areas were obtained, and the L-T4 was calculated from the known amount of D-T4 added and the peak-area ratios. Duplicate analyses without D-T4 were carried out and values corrected for any D-T4 found. Since in a number of cases examination of diagnostics revealed that the areas calculated by the integrators were not satisfactory, the product of retention time and peak height was used as a measure of area. Where possible figures calculated in this way were compared with those using integrator areas; only minor discrepancies were apparent.

Radioimmunoassay

RIA were performed at the Courtauld Institute of Biochemistry, Middlesex Hospital Medical School using the North East Thames Regional Immunoassay (NETRIA) kit.

Characterisation of product of reaction with OPA–NAC

^{1}H NMR spectrum. L-T4 (1.4 mg in 0.5 ml of 0.005 *M* sodium hydroxide in methanol) was reacted with OPA–NAC reagent (0.5 ml). After 1 min the reaction was terminated by addition of a drop of acetic acid, and the reaction mixture injected onto a column (250 mm × 4.6 mm, I.D.) of ODS silica (10 μm, Alltech). The column was eluted isocratically with 0.2% TFA in 56% acetonitrile, and the UV absorption peak corresponding to the product collected and lyophilised. The preparation gave a single peak corresponding to the L-T4 derivative when analysed in the phosphoric acid–methanol system, and was stable without significant breakdown for several days. The dried sample was reconstituted in deuteroacetone and examined by 500-MHz ^{1}H NMR spectroscopy.

UV absorption spectrum. UV spectra over the range 190–370 nm at the apex of chromatographic peaks of T4 and the OPA–NAC derivatives of leucine and T4 were recorded. The mobile phase used for the leucine derivative was methanol–phosphoric acid–water (60:0.1:39.9, v/v) and for the T4 derivative methanol–phosphoric acid–water (72:0.1:28.9, v/v). Identical spectra for underivatised T4 were obtained in both systems.

RESULTS

Structure of OPA-T4 product

Because of the lack of detectable fluorescence it was thought advisable to confirm the identity of the product of the reaction of T4 with OPA. The UV spectrum of the T4 reaction product was simply the sum of the spectra of the leucine derivative (which is fluorescent and representative of the expected isoindole) and underivatised T4, with maxima at approximately 330 nm at-

TABLE I

CHEMICAL SHIFTS (δ) AND COUPLING CONSTANTS (J) OF 500-MHz ^{1}H NMR SPECTRUM OF T4-OPA-NAC DERIVATIVE

^{1}H[a]	δ[b](ppm)	J[c](Hz)	Comments
a	7.4034	(s)	
b	7.4718	(s)	Wide due to some coupling to Hc and Hd
c, d	3.8397	4.3,13 75	Double doublet due to coupling
	3.4865	11.5,15	to He (4.3 Hz) and Hd (13.75 Hz)
e	6.1743	4.3(dd),11.25	Double doublet coupled to Hc and Hd
f	7.8752	(s)	
g	7.7443	4.45,4.41	Virtual coupling of Hh and Hg to Hi
h	7.0978	4.65(t)	2×4-Hz rather than 8-Hz triplets
i	7.0978	4.65(t)	due to coincidence and virtual coupling
j	7.8482	4.36,4.55	As for g
k	3.1665	7.6,13.7	Double doublet due to coupling with Hl (13.7 Hz) and Hm (7.6 Hz)
l	ca.3.4110	ca.4.1,13.3	Double doublet due to coupling with Hl (13.3 Hz) and Hm (4 1 Hz)
m	4.6400	4.0,7.4	Double doublet due to coupling with Hk (7.4 Hz) and Hl (4 Hz)
n	1.8103	(s)	

[a]See Fig. 1 for labelling.
[b]Referenced to acetone $-CH_3$ at 2.225 ppm.
[c]s = singlet; t = triplet; dd = double doublet.

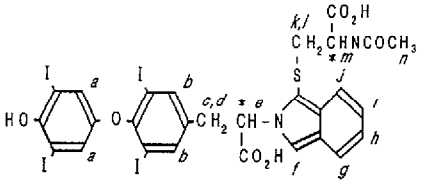

Fig. 1. Assumed structure of the reaction product of T4 with OPA and NAC. The asymmetric carbons are starred and protons are labelled in accordance with the interpretation of the NMR spectrum in Table I.

tributable to the isoindole ring system and at 295 nm contributed by the thyroxine portion. The ^1H NMR resonances could all be assigned on the basis of the expected structure (Table I; Fig. 1). The spectra were consistent with published figures for N-isopropyl-1-isoindole sulphonic acid [29]. We therefore conclude that the expected isoindole derivative is formed (Fig. 1), but that the fluorescence is internally quenched, presumably by the high iodine content.

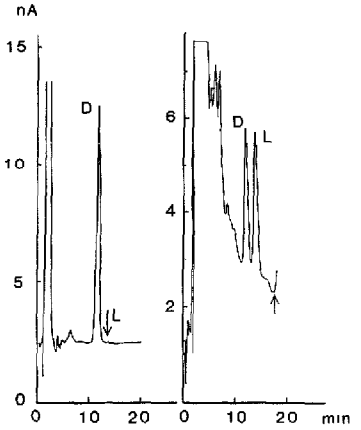

Fig. 2. (Left) Peak due to injection of reaction product corresponding to 90 ng of D-T4; the arrow marks the expected position of elution of the L-T4 derivative. (Right) Extract of 1 ml of reconstituted serum sample C (see Table II). The arrow marks the point at which 1 ml of 1% TFA in methanol was injected. Mobile phase: 71% methanol in 0.1% phosphoric acid; flow-rate: 0.6 ml/min, Waters M-460 detector, with 16 nA f.s.d. (left) and 8 nA f.s.d. (right).

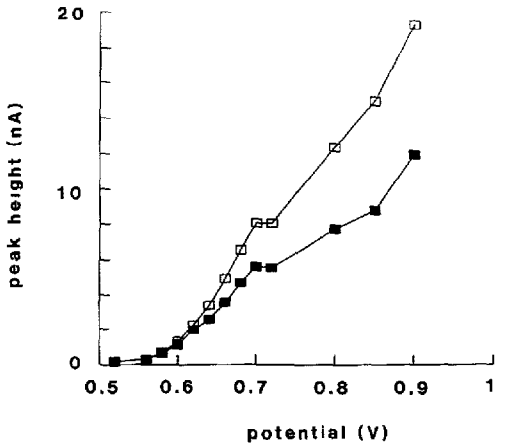

Fig. 3. Voltammogram of peak height versus electrode potential for D-T4 (■) and L-T4 (□)

Separation and stability of derivatives

When reacted with OPA and NAC under the conditions described, L- and D-T4 gave well separated symmetrical peaks. The derivatives showed no sign of racemisation under the conditions and on the time scale used. D-T4 gave a single derivative peak (Fig. 2) free of any significant trace of the L-T4 derivative ($<0.1\%$). By the same criterion there was no significant contamination of the L-NAC with D-isomer. A sample of DL-cysteine was acetylated by published methods [30] and used to derivatise optically pure D-T4. Two equal peaks were obtained which were eluted at times corresponding to the derivatives of L- and D-T4 with pure L-NAC. Racemisation and chiral impurity thus appeared to make insignificant contributions to the assay. For electrochemical detection the effect of voltage on current was examined over the range 0.5–0.9 V (Fig. 3), and a potential of 0.73 V in the plateau region between 0.7 and 0.75 V was chosen for analysis.

TABLE II

COMPARISON BETWEEN T4 VALUES OBTAINED BY RIA AND HPLC

Sample[a]	Concentration (nmol/l)		
	RIA estimate	HPLC estimate	
		Duplicates	Mean
Bio-Rad low	51.5	64.6,70.8	67.7
86/622	60.0	63.7,66.2	64.9
85/644	61.0	62.5,61.9	62 2
A[b]	64.0	61.5,62.2	61.8
85/573	65.0	69.9,68.0	69.0
D	73.0	82.5,89.0	85.8
B	79.0	72.5,71.8	72.1
Bio-Rad medium	82.0	86.5,86.0	86.2
E	108.0	122.4,124.5	123.4
C[c]	120.0	131.8,131.4,136.3,131.7	132.8
85/500[d]	163.5	183.8,174.8,155 3,176 7,184 2	175.0
F	174.0	219.2,196.0	207.6
Bio-Rad high	178.9	184.9,195.9	190.4

[a]Samples A–F were lyophilised serum samples supplied by the EEC Community Bureau of Reference (Brussels, Belgium). Samples 85/500, 85/573, 85/644 and 86/622 were lyophilised plasma samples prepared at this Institute. The three Bio-Rad samples were prepared from standard high and low T4 reference kits (Bio-Rad Labs, Watford, U.K.). A further pair of commercial clinical standard kits were discovered to be positive for HIV antibody and were not used.

[b]LDC amperometric detector.

[c]Waters M-460 detector.

[d]Coefficient of variation for five replicates 6.7%.

Extraction procedure

A number of extraction procedures were assessed [17–20,22]. None appeared in our hands to give recoveries much greater than 30% from serum samples spiked with T4, and the procedure finally adopted was based on the simplest minimum processing. Recoveries from serum (based on the amounts of D-T4 recovered) were 35–40%, but recoveries from T4 samples dissolved in water or 5% human serum albumin were much lower (5–10%). In view of this it was decided to make no attempt to construct standard curves and to base all calculations on the absolute amount of D-T4 added.

Internal standard

For HPLC of biologically derived samples the issue of internal standard has usually either been avoided [22] or solved by the use of radioiodinated T4 [11,17,19]. For many of the studies quantitative recovery has not been important [15,18]. For gas chromatographic work either a deuterated isotope [5] or the 3′,5′-dibromo-3,5-diiodothyronine analogue [6,31] have been synthesised. Rather than synthesize an internal standard we decided to use the D-isomer of T4 which was commercially available in good enantiomeric purity (Fig. 2) and which should behave in a physicochemically indistinguishable manner to the L-isomer during the extraction process.

Analysis of serum samples

The T4 content of thirteen lyophilised serum samples was determined by the method described, and the results were compared with the values obtained by RIA (Table II; Fig. 4). The least-squares best straight line fit to the data

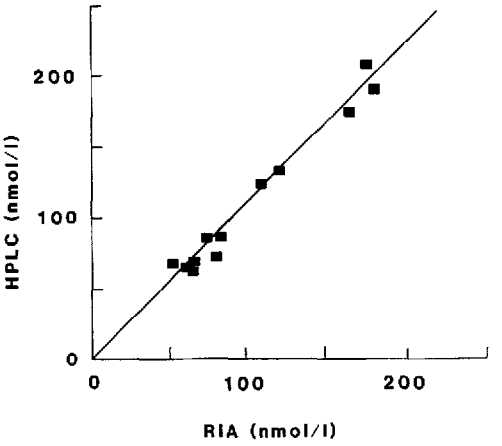

Fig. 4 Comparison of mean values for RIA and HPLC. The line represents the best fit equation: HPLC = 1.1 RIA (see text).

was

$$HPLC = 1.13\ RIA - 2.75; \quad r^2 = 0.976$$

or, when constrained to pass through the origin

$$HPLC = 1.10\ RIA; \quad r^2 = 0.975$$

The Bio-Rad high reference kit values were corrected for a peak corresponding to D-T4 approximately 10% of the area of L-T4 in the sample processed without internal standard.

DISCUSSION

The observation that the OPA–NAC derivative of T4 did not give a fluorescent peak in systems used for the analysis of the OPA derivatives of the common amino acids was unexpected, especially in view of statements to the contrary [32]. The derivative was clearly visible with UV detection and had the UV and NMR spectrum expected. Reaction with OPA did not significantly improve the sensitivity of electrochemical detection, but derivatisation with either dansyl chloride or OPA led to markedly better peak shapes than for underivatised T4. The slightly increased retention of the NAC–OPA derivatives also helped to shift the T4 peaks beyond the region of major electrochemically active impurities. The results obtained show good agreement between the method described and RIA: although the slope is not exactly 1 the number of samples available is too few to assess the significance, but it would not be unusual for RIA and chemical figures to show such a discrepancy. Our conclusions are that the method described provides a suitable basis for the rapid determination of T4 in lyophilised serum samples, and we are in the process of applying it to a larger-scale study of reference T4 serum preparations. The amounts of T4 involved are well within the sensitivity limits of modern electrochemical detectors, and a reduction in sample size seems feasible. Nevertheless the recovery figures show room for improvement, and the extraction process is under reinvestigation. There appears to be no reason why the method should not be equally applicable to non-lyophilised serum samples.

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