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Note

High-performance liquid chromatographic assays for studying the formation of iodotyrosines and iodothyronines in sheep thyroid peroxidase preparations

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Thyroid peroxidase (EC 1.11.1.7), a membrane bound enzyme in the thyroid gland, is of clinical importance since it is involved in the biosynthesis of the thyroid hormones, triiodothyronine (T_3) and thyroxine (T_4) and their precursors monoiodotyrosine (MIT) and diiodotyrosine (DIT)[1]. The rate of basal metabolism in the body is controlled by the circulatory levels of these hormones. Elevated concentrations of T_3 and T_4 in plasma are symptomatic of hyperthyroidism in humans.

A variety of analytical techniques have been used to study thyroid peroxidase activity. These have included spectrophotometry[2,3], paper chromatography to separate radiolabelled compounds[4] and thin-layer chromatography[5].

This paper describes high-performance liquid chromatographic (HPLC) assays developed for measuring the iodinating and coupling activities of a sheep microsomal/mitochondrial preparation of thyroid peroxidase. The inhibitory action of the thiocarbamide inhibitors, 1-methyl-2-mercaptoimidazole (methimazole) and its metabolite 3-methyl-2-thiohydantoin[6] on the thyroid peroxidase preparation was also studied. Methimazole is an antithyroid drug used for the treatment of hyperthyroidism[1].

EXPERIMENTAL

Chemicals and reagents

Tyrosine, MIT, DIT, T_3, T_4, and glucose oxidase (type V) were obtained from Sigma (London, Great Britain). Methimazole was obtained from Aldrich (Dorset, Great Britain). 3-Methyl-2-thiohydantoin (3-MTH) was prepared according to the method of Scott and Henderson[7]. All other reagents and solvents were AnalaR grade (BDH, Poole, Great Britain).

High-performance liquid chromatography

The iodinated metabolic products from the thyroid preparations were analysed by using a Spectra-Physics Model 3500B liquid chromatograph with a Cecil CE212 variable wavelength ultraviolet monitor and a 10 × 0.46 cm column packed with 5μm ODS-silica (Spherisorb ODS; Phase Separations, Queensferry, Great Britain). The mobile phases were: I, 0.1 *M* sodium dihydrogen phosphate–methanol (70:30) and II, 2.5% acetic acid in water–acetonitrile (25:75).

Calibration standard solutions

Stock solutions of MIT and DIT were 10^{-6} *M* and $2 \cdot 10^{-5}$ *M*, respectively. For calibration appropriate aliquots were injected onto the column by stop-flow injection.

Preparation of sheep thyroid enzyme fraction

Sheep thyroid glands were collected fresh from a local slaughterhouse, and the enzyme preparation prepared according to the method of DeGroot and Davis[8]. The particulate fraction was washed twice with 0.05 *M* Tris–HCl buffer, pH 7.4, containing potassium iodide ($1 \cdot 10^{-4}$ *M*), to remove residual haemoglobin and was stored at −20°. Before use it was thawed and resuspended in Tris buffer to give a protein concentration of 5 mg ml^{-1} as assayed by the biuret method[9].

Iodination of tyrosine and MIT

Incubation mixtures for HPLC analysis contained potassium iodide ($1 \cdot 10^{-5}$ *M*), hydrogen peroxide ($4 \cdot 10^{-4}$ *M*) and either tyrosine ($1 \cdot 10^{-4}$ *M*) and enzyme protein (100 μg), or MIT ($5 \cdot 10^{-4}$ *M*) and enzyme protein (80 μg) in 0.05 *M* phosphate buffer, pH 6.8 (0.2 ml). The mixtures were incubated at room temperature and aliquots (10 or 20 μl) injected onto the HPLC column eluted with solvent system I with UV detection at 280 nm for measuring the iodination of tyrosine and 295 nm for measuring the iodination of MIT. Controls were incubated without enzyme protein.

Formation of T_4

Phosphate buffer, 0.05 *M*, pH 6.8 (5 ml) containing DIT ($1 \cdot 10^{-3}$ *M*), glucose ($1 \cdot 10^{-3}$ *M*), glucose oxidase (5 U ml^{-1}), and enzyme preparation protein (2.5 mg) was incubated for 30 min at 37°, prior to extraction with *n*-butanol (5 ml). The butanol phase was removed and evaporated to dryness under a stream of oxygen-free nitrogen. The extract was reconstituted with the HPLC solvent system II (200 μl) and aliquots (20 μl) were injected onto the HPLC column.

Controls contained enzyme preparation added before the addition of *n*-butanol (5 ml) but after the 30 min incubation.

Inhibition studies

The effect of methimazole and 3-MTH in the range of 10^{-7} to 10^{-4} *M* on the iodination of tyrosine and MIT and the effect of methimazole (10^{-4} *M*) and 3-MTH (10^{-4} *M*) on the formation of T_4 by sheep thyroid enzyme preparations were examined. The incubation and HPLC conditions for tests and controls were the same as described for the iodination of tyrosine and MIT, and the formation of T_4.

RESULTS AND DISCUSSION

The spectrophotometric methods of assay lack specificity for measuring the iodinating and coupling activities of thyroid peroxidase. At the wavelength at which these reactions are usually assayed (330 nm)[2,3] DIT, T_3 and T_4 all have appreciable absorptivities and it is necessary to confirm the identity of the reaction products by

chromatographic methods[2]. The spectrophotometric method is limited to the measurement of the activity of the enzyme in free solution. Comparison of the detection limits of the various analytical techniques used to study thyroid peroxidase (Table I), has shown that HPLC is as sensitive as the spectrophotometric and radiolabelled methods, although the sensitivity of the latter method depends on the specific activity of the radiolabelled material used. However, these methods are time consuming.

TABLE I

COMPARISON OF THE METHODS USED TO DETERMINE THYROID PEROXIDASE ACTIVITY

Technique	*Reaction studied*	*Volume of incubation medium (ml)*	*Limit of detection**	*Time of incubation (min)*	*Total time of analysis (min)*	*Ref. No.*
Paper chromatography using ^{131}I	Iodination of proteins	1.0	$5 \cdot 10^{-9}$	5–60	180–1200	10
Paper chromatography using ^{131}I	Iodination of tyrosine	1.0	$2 \cdot 10^{-11}$	20	Not reported	9
Spectrophotometric	Iodination of MIT	1.0	$4 \cdot 10^{-9}$	5	5	3
HPLC	Iodination of tyrosine or MIT	0.2	$5 \cdot 10^{-9}$	30	5	This paper

* Expressed as the number of moles of I utilised per ml of incubation medium.

An advantage of the developed assays was that incubation volumes were small (0.2 ml), and aliquots could be injected directly onto the ODS-silica column at appropriate time intervals during the incubation.

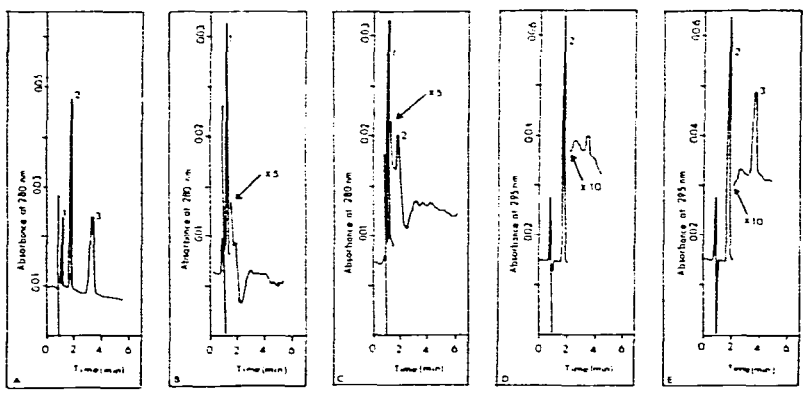

Fig.1. Typical HPLC chromatograms for the iodination of tyrosine and MIT. (A) tyrosine, MIT and DIT standards; (B) control incubation mixture containing tyrosine, KI and H_2O_2; (C) test incubation mixture containing tyrosine, KI, H_2O_2 and enzyme protein; (D) control incubation mixture containing MIT, KI and H_2O_2; (E) test incubation mixture containing MIT, H_2O_2 and enzyme protein. Arrows indicate an increase in detector sensitivity. Conditions: column (10 × 0.46 cm) slurry packed with 5 μm ODS (Spherisorb); mobile phase, solvent system I; flow-rate, 1.2 ml min^{-1}; peaks: 1 = tyrosine; 2 = MIT; 3 = DIT.

MIT and DIT are resolved from tyrosine with solvent system I (Fig. 1A). Over the concentration ranges studied, there was a rectilinear relationship between peak height and concentration.

In all studies there was an excess of substrate, tyrosine, MIT and DIT in the incubation medium, and it was therefore necessary to increase the detector response sensitivity after the substrate peak had been recorded in order to measure MIT (Fig. 1C), DIT (Fig. 1E) and T_4 (Fig. 3C).

The iodination of tyrosine was virtually linear with time for 15 min (Fig. 2). However, since the iodination of MIT to DIT was fast, reaching a plateau in 5 min, a spectrophotometric assay[2] is necessary (unpublished results). The thyroid

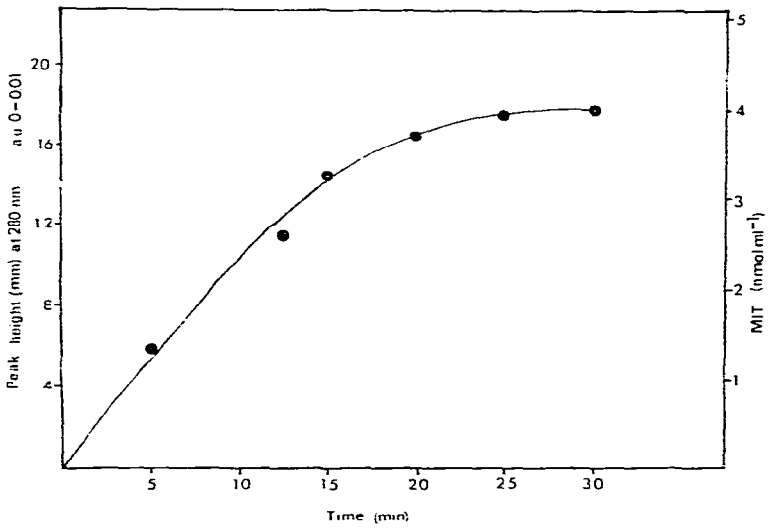

Fig. 2. Kinetics of formation of MIT. The incubation mixtures contained tyrosine ($1 \cdot 10^{-4}$ *M*), KI ($1 \cdot 10^{-4}$ *M*), enzyme preparation protein (100 μg) and H_2O_2 ($4 \cdot 10^{-4}$ *M*).

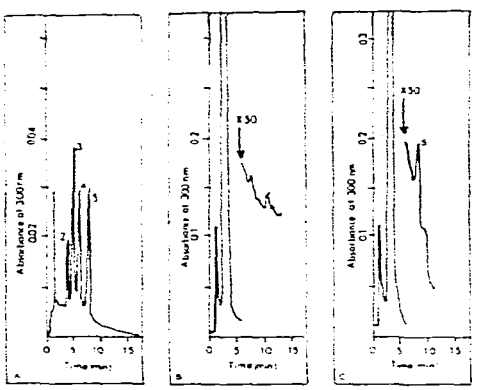

Fig. 3. Typical HPLC chromatograms for the formation of T_4. (A) standards, MIT, DIT, T_3 and T_4; (B) control extract from an incubation mixture containing DIT, glucose and glucose oxidase; (C) test incubation mixture containing DIT, glucose, glucose oxidase and enzyme preparation. Conditions as in Fig. 1, but with solvent system II. Arrows indicate an increase in detector sensitivity. Peaks: 2 = MIT; 3 = DIT; 4 = T_3; 5 = T_4.

hormones, T_3 and T_4 which are formed by the coupling of iodotyrosine molecules, are resolved from MIT and DIT with solvent system II (Fig. 3A). Direct analysis of enzymatically produced T_4 was not possible, since its concentration was below the detection limit. After extraction of the incubation mixture, it was possible to put a more concentrated sample on the column.

The developed HPLC systems are applicable to the study of the effect of the thiocarbamide inhibitors on the enzyme preparation. The inhibitor, methimazole, was well resolved from tyrosine, MIT and DIT in solvent system I (t_R = 80 sec), and it did not interfere with the measurement of MIT, DIT and T_4. 3-Methyl-2-thiohydantoin (3-MTH), another inhibitor and a metabolite of methimazole[6], had a retention time similar to MIT, but since its molar absorptivity is low at 295 nm it did not interfere with the MIT inhibition studies. As shown in Fig. 4, methimazole inhibited the iodination of tyrosine and MIT in a similar manner producing an S-shaped curve comparable to that reported[11]. However, it is noteworthy that 3-MTH inhibition gave a hyperbolic inhibition curve. Methimazole (10^{-4} *M*) completely inhibited the formation of T_4, whereas 3-MTH (10^{-4} *M*) only partially (70%) inhibited T_4 synthesis.

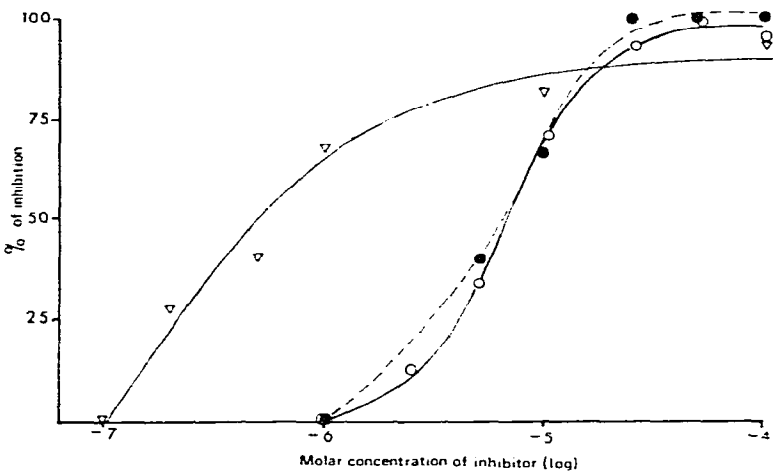

Fig. 4. The effect of methimazole (○) and 3-MTH (▽) on the iodination of MIT, and effect of methimazole (●) on the iodination of tyrosine. Conditions as in Fig. 1.

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