ENHANCEMENT FLUOROIMMUNOASSAY OF THYROXINE

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1. Introduction

Radioimmunoassay (RIA) has proved to be a highly successful analytical method, but is not without disadvantages. The obligatory physical seperation of antibody-bound and free fractions of labelled antigen or hapten is a practical inconvenience and potential source of imprecision. Radiolabelled materials are often costly, may have limited shelf-life, present a radiation hazard, and necessitate expensive counting equipment. Non-isotopic labelling techniques which can obviate many or all of these shortcomings have accordingly received much attention, which has been largely focussed on enzyme immunoassay methods [1,2]. However, fluorescent-labelling also merits consideration when the extreme sensitivity of RIA is not called for. Fluoroimmunoassay methods based on detection of the extent of antibody binding of fluorescent-labelled hapten in unseparated immunoassay incubation mixtures by measurements of fluorescence polarisation (polarisation fluoroimmunoassay) or fluorescence quenching (quenching fluoroimmunoassay) have recently been applied to assay of gentamicin in serum [3,4].

The present report describes the preparation of a fluorescent derivative of thyroxine (T_4) whose fluorescence is enhanced when bound by anti-T_4 serum, and illustrates in principle the exploitation of the effect in an 'enhancement fluoroimmunoassay' of T_4.

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2. Experimental

Fluorescein-labelled T_4 was prepared by reaction of 1 vol. 20 g/l (51.4 mM) fluorescein isothiocyanate (FITC) (isomer I; Sigma) with 2 vol. 20 g/l (25.7 mM) L-T_4 (free acid; Sigma) in a pyridine/water/triethylamine medium of composition 9 : 1.5 : 0.1 v/v/v. Reaction was complete after 1 h at room temperature (paper chromatography). Crude products were then precipitated with 20 vol. 0.2 M ammonium acetate buffer, pH 4.0, and collected by centrifugation (10 min, 1 000 × g). The supernatant was discarded and the precipitate washed by suspension in 20 vol. distilled water followed by centrifugation as before. The product was re-dissolved in 4–8 vol. 0.05 M NH_4HCO_3 with the aid, if necessary, of a minimal amount of ammonia solution. Aliquots (0.5 ml) were applied to small (0.9 × 4.5 cm) columns of Sephadex G-25 fine grade equilibrated with 0.05 M NH_4HCO_3. The labelled T_4-product became adsorbed to the Sephadex-gel, allowing removal of small amounts of fluorescent impurities by passage of 10 column volumes of 0.05 M NH_4HCO_3. The purified product was then eluted from the columns with distilled water and either stored frozen (−18°C) or freeze-dried to a yellow/orange powder, stored desiccated at 4°C.

Progress of reaction was monitored, and purity of products assessed, by chromatography on Whatman No. 1 paper developed with 0.2 M Na_2HPO_4. Fluorescein fluorophores were visualised under short-wave ultraviolet light, and the iodothyronine function of T_4 with a diazotised sulphanilic acid spray (Pauly reagent).

An Aminco-Bowman SPF fluorimeter was used. In fluoroimmunoassay experiments, excitation and

emission wavelengths were 495 nm and 540 nm, respectively. All fluorescence studies were performed in 0.075 M barbital buffer, pH 8.6. Fluorescein (sodium salt) was from Sigma.

Sheep anti-T_4 serum, previously characterised in an RIA system [5], was used. An antibody dilution curve was constructed by adding 0.75 ml aliquots of labelled T_4 solution (9.9 μg/l) to doubling dilutions (1.25 ml) of anti-T_4 serum. After 15 min incubation (room temperature) to allow for equilibration, the fluorescence intensity of each mixture was measured. The fluorescence background signal contributed by the diluted antiserum was separately measured in absence of labelled-T_4 and the fluorescence intensity of each incubation mixture corrected appropriately. The experiment was repeated using a normal sheep serum in place of the anti-T_4 serum.

For the standard curve, T_4 solutions of known concentration were prepared and to 0.5 ml aliquots were added 0.5 ml labelled-T_4 solution (18.6 μg/l), followed by 0.5 ml of anti-T_4 serum (diluted 1: 1000). After 15 min (room temperature) the fluorescence intensity of each assay mixture was measured. The background fluorescence signal of the diluted antiserum was measured and results corrected as above.

3. Results

Fluorescein-labelled T_4 was reproducibly prepared in yield typically of 60% of the weight of starting products, and appeared at least 99% pure with respect to fluorescein and iodothyronine content by paper chromatography (R_f values: FITC, 0.35; labelled T_4, 0.25; T_4, 0.0). The fluorescence emission maximum (uncorrected) was 520 nm, compared with 517 nm for fluorescein itself. The fluorescence yield at 520 nm of the labelled-T_4 was only 13% of that of fluorescein at the same wavelength.

Figure 1 shows that the fluorescence of labelled T_4 is enhanced to a maximum extent of 3.9-fold upon binding by the anti-T_4 serum. Some non-specific binding to the normal sheep serum is evident, but binding by the specific antiserum is complete at dilutions at which non-specific effects are negligible. The standard curve (fig.2) shows that unlabelled T_4 competes for antiserum binding as indicated by decrease of observed fluorescence with increasing concentration

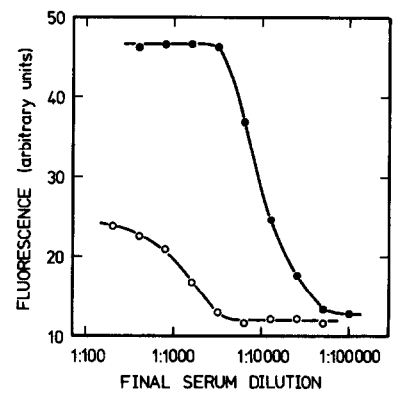

Fig.1. Antibody dilution curves. Closed symbols: anti-T_4 serun open symbols: normal sheep serum.

of added T_4. Unlabelled-T_4 had no effect on the non-specific enhancement in presence of normal serum. A preparation of labelled-T_4 which had been stored for 15 months in frozen solution showed no evidence of deterioration when used in the fluoroimmunoassay system.

4. Discussion

Fluorescein itself and the products of reaction of FITC with common amino acids are very efficient fluorophores in alkaline aqueous solution [6]. Fluorescein-labelled T_4 represents an exception. This

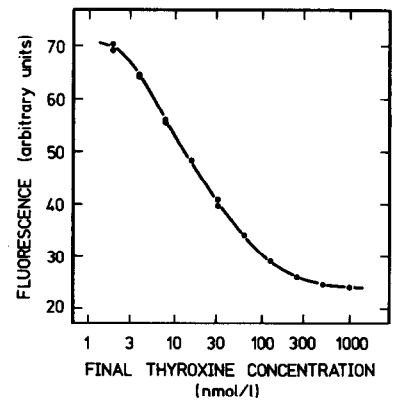

Fig.2. Enhancement fluoroimmunoassay of thyroxine: standard curve.

observation is most likely attributable to intra-molecular quenching of the fluorescein fluorophore by the iodine atoms of the iodothyronine function of T_4. Iodine-containing molecules are well recognised as efficient quenchers of fluorescence by either collisional [7] or external heavy atom [8] mechanisms. The marked enhancement of fluorescence upon binding to antiserum might then be understood in terms of inhibition of quenching when the iodothyronine moiety becomes bound within the combining site of specific anti-T_4 antibody.

The fluorescence enhancement effect provides the basis for a fluoroimmunoassay of T_4 involving only the mixing of three reagents (sample, labelled-T_4 and antiserum), followed after a short incubation by measurement of the fluorescence intensity of the labelled-T_4. Compared with radioassay and RIA methods employing ^{125}I-labelled T_4 which are currently applied in evaluation of thyroid function [9], the technique has several attractions. Fluorescein-labelled T_4 may be easily and safely prepared in any desired quantity from readily available materials, and has practically indefinite shelf-life. The assay end-point is determined by conventional fluorimetry. No seperation step is involved, which simplifies the procedure and should facilitate automation, notably of continuous-flow type. On the other hand, the fluoro-immunoassay is less sensitive than typical RIA methods for T_4 by one to two orders of magnitude, and appreciable and variable levels of intrinsic serum fluorescence present an obstacle to assay of patient samples. However, given a reliable method for removal of such interference, enhancement fluoroimmunoassay might be expected to prove sufficiently sensitive for quantitation of total T_4 levels in routine clinical monitoring of thyroid disorders.

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