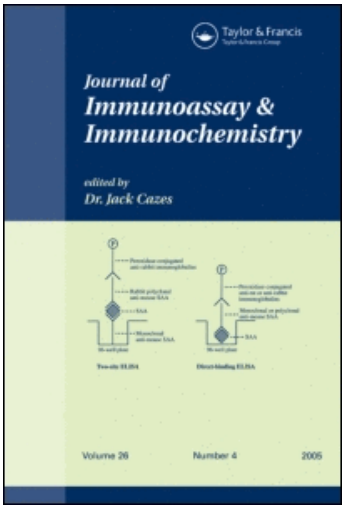

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Determination of (+)-3,3′,5,5′-Tetraiodo-L-thyronine (L-T_4) in Serum and Pharmaceutical Formulations using a Sequential Injection Analysis/Immunosensor System

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Abstract: A sequential injection analysis/immunosensor system is proposed for the analysis of thyroxine (L-T_4) in serum and in pharmaceutical formulations with a rate of 75 samples/hour. The immunosensor design is based on the physical immobilization of anti-L-T_4 in carbon paste. The working concentration range of the immunosensor in the sequential injection analysis system is between 36 and 1080 ng/mL with a limit of detection of 24.6 ng/mL. The system is very reliable and very easy to design and operate.

Keywords: Amperometric immunosensor, L-T_4, Sequential injection analysis, Thyroid hormone

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INTRODUCTION

Thyroxine (L-T_4, (+)-3,3′,5,5′-tetraiodo-L-thyronine) is a thyroid hormone. It plays the role of prohormone for L-T_3 and also inhibits glutamate dehydrogenase. Its quantification in serum samples is very important because deviations from the normal limits can cause thyroid dysfunction and many diseases. Enantiopurity tests of L-T_4 and purity tests for the pharmaceutical formulations of L-T_4 are essential for quality control and quality assurance. The methods proposed up to now for the determination of L-T_4 are: HPLC,[1] radioimmunoassay,[2,3] fluorescence immunoassay,[4] electrochemiluminescence,[5,6] and direct amperometry.[7,8]

The fast and reliable quantification of L-T_4 in biological and pharmaceutical samples is necessary. Accordingly, a reliable flow system should be coupled with a reliable detector. The best flow system that can be used in these analyses is sequential injection analysis (SIA), as it is very reliable, the cost of analysis is low, and the consumption of the sample and buffer is very low. As detector, nothing can compare as simplicity and reliability, with the amperometric immunosensors. That is why, the emphasis of this paper is on the SIA/immunosensor system utilized for the determination of L-T_4 in serum and pharmaceutical samples. The consumption of the sample and buffer is very low (270 μL of each).

EXPERIMENTAL

Amperometric Immunosensor Design

The antiserum was diluted to a working dilution of 1:30 in 0.01 mol/L phosphate buffered saline, pH = 7.4, containing 0.1% sodium azide. The graphite powder was heated at 700°C for 15 s in a muffle furnace and cooled to ambient temperature in a dessicator. The paraffin oil and graphite powder were mixed in a ratio of 1:4 (w/w) and then it was added to the diluted anti-L-T_4 to obtain a final composition of 0.9% (w/w) in anti-L-T_4. The carbon paste (graphite powder and paraffin oil) was filled into a plastic pipette tip, leaving about 3 to 4 mm empty in the top to be filled with the chemically modified carbon paste that contains anti-L-T_4. The diameter of the immunosensor was 3 mm. Electric contact was made by inserting a silver wire in the carbon paste.

Before each use, the surface of the electrode was wetted with double distilled water and then polished with an alumina paper (polishing strips 30144-001, Orion). When not in use, the amperometric immunosensor was stored in a dry state at 5°C.

Apparatus

A VoltaLab 40 (Radiometer Copenhagen) was used for all amperometric measurements. A platinum electrode and a Ag/AgCl (0.1 mol/L KCl) electrode served as counter and reference electrodes in the cell.

Reagents and Materials

The immunological system composed from L-T_4 and monoclonal anti-L-T_4 was supplied by Sigma (St. Louis, MO, USA). Synthroid® (Levothyroxine Sodium, USP) (injection) was supplied by Bots Pharmaceuticals (Nottingham, UK) and Eltroxin® (tablets) was supplied by Glaxo Laboratories, Ltd. (Greenford, UK). Graphite powder with a particle size of 50 μm was supplied by Merck (Darmstadt, Germany). Paraffin oil was supplied by Fluka (Buchs, Switzerland). All other reagents were of the highest analytical grade. All the solutions were prepared using de-ionized water from a Modulab system (Continental Water Systems, San Antonio, TX, USA).

Recommended Procedures

Sequential Injection System

The biosensors were incorporated into the conduits of the SIA system (Fig. 1) constructed from a Gilson Minipuls peristaltic pump and a 10-port electrically actuated selection valve (Model ECSD10P, Valco Instruments, Houston, TX). Tygon tubing (0.76 mm i.d. for the holding

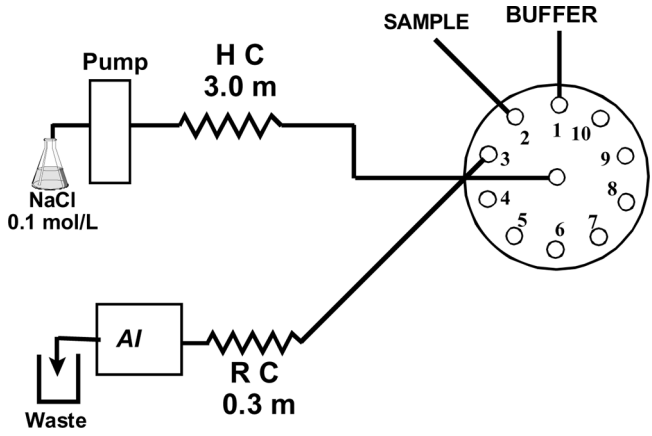

Figure 1. Schematic diagram of the system.

Table 1. Device sequence for one cycle of the SIA system

Time (s)	Pump	Valve	Description
0	Off	Buffer	Pump stops, select buffer stream (valve position 1)
5	Reverse	Buffer	Draw up buffer solution
9.5	Off		Pump stops
10.5		Sample	Select sample stream (valve position 2)
11.5	Reverse	Sample	Draw up sample solution
16	Off		Pump stops
17		Amperometric immunosensor cell	Select amperometric immunosensor cell line (valve position 3)
18	Forward		Pump stack of zones to amperometric immunosensor cell
48	Off		Pump stops, return valve to starting position (valve position 1)

coil and 0.89 mm i.d for the mixing coil) was used to construct the manifold; coils were wound round suitable lengths of glass tubing (15 mm o.d.); 0.1mol/L NaCl was used as carrier. The capacity of the system is about 75 samples per hour. The device operating sequence is shown in Table 1. The device control was achieved using a PC30-B interface board (Eagle Electric, Cape Town, South Africa). The FlowTEK[9] software package (obtained from Mintek) for computer-aided flow analysis was used thoughout for device control.

Content uniformity assay of Eltroxin® Tablets and Synthroid® Injections

Ten Eltroxin® tablets are individually placed in ten 100 mL calibrated flasks, and dissolved in distilled water. These solutions served as samples in the SIA/immunosensor system, and the peak height was measured. The unknown concentration was determined from the calibration graph.

Synthroid® injections are diluted with 0.19% NaCl solution to 5 mL. Ten aliquots of 0.2 μL solution were diluted to 10 mL using distilled water, and the peak height was measured. The unknown concentration was determined from the calibration graph.

RESULTS AND DISCUSSION

An optimum flow rate of 3.61 mL/min was used to propel the solutions. In the SIA system, the sample and buffer (phosphate buffer, pH = 7.4) consumption is only 270 μL each per measurement of the concentration

of L-T_3, which is very economical. All measurements were performed at +450 mV vs Ag/AgCl.

Amperometic Immunosensor Response in SIA System

The equation of calibration for L-T_4 is:

$$H = 0.08 + 0.033C$$

where H (pA) is the peak height and C is the concentration (ng/mL) of L-T_4. The correlation coefficient, r, is 0.9999. The linear concentration range for the immunosensor is between 36 and 1080 ng/mL.

Selectivity of the Amperometric Immunosensor in SIA System

The selectivity of the amperometric immunosensor was checked using both: separate and mixed solutions methods, versus L-T_3 ((+)-3,3',5-triiodo-L-thyronine), D-T_4 (the pair enantiomer of L-T_4), and polyvinylpyrolidine (PVP). The selection of L-T_3 for the selectivity test is to prove the suitability of the method to be used for the assay of L-T_4 in the presence of L-T_3 in blood samples. It is also essential to determine L-T_4 in the presence of D-T_4 for the enantiopurity tests of the raw materials of drugs containing L-T_4 as active substance. Polyvinylpyrolidine is one of the most used compression compounds for tablets; therefore, it may influence the current intensity during the uniformity content test of tablets containing L-T_4 as active substance.

Amperometric selectivity coefficients were determined following the method proposed by Wang.[10] The amperometric selectivity coefficients values obtained for L-T_3 ($pK_{amp} = 6.82$), D-T_4 ($pK_{amp} = 4.55$), and PVP ($pK_{amp} = 5.12$) demonstrate the specificity of the immunosensor for the assay of L-T_4. The inorganic cations, like Na^+, K^+, Ca^{2+}, do not interfere in the assay of L-T_4.

Analytical Applications

The response characteristics, as well as the specificity of the immunosensor, made it suitable for both clinical and drug analysis. The recovery

Table 2. Determination of L-T_4 in the presence of D-T_4

L-T_4, average recovery*, (%) L:D (mol:mol)				
2:1	1:1	1:2	1:9	1:9
99.42 ± 0.01	99.82 ± 0.02	99.86 ± 0.02	99.61 ± 0.01	99.62 ± 0.02

*n = 10.

Table 3. Determination of L-T_4 from pharmaceutical products. (Content uniformity assay) all values are the average of three determinations

Sample	No.	Recovery, %
Eltroxin®	1	99.72 ± 0.04
	2	99.98 ± 0.03
	3	99.80 ± 0.03
Synthroid®	1	99.53 ± 0.04
	2	99.49 ± 0.03
	3	99.60 ± 0.04

tests were performed in the presence of D-T_4. As can be seen from Table 2, the SIA/immunosensor system is suitable for enantioanalysis of the raw material. No significant differences in the recovery values were recorded for the ratios between L:D enantiomers varying from 1:9 to 1:99.99.

The results obtained for the uniformity content test are presented in Table 3. L-T_4 can be reliably assayed from the tablets and injection with a high average recovery and low RSD% values. The results are in good agreement with those obtained using the U.S. Pharmacopoeia method: 99.82 ± 1.00% and 99.40 ± 1.50% for the pharmaceutical formulations: Eltroxin® and Synthroid®, respectively.[11] The advantage of the proposed method versus the one recommended by the U.S. Pharmacopoeia is the simplicity and higher precision due to the lower values of the RSD (%).

The results obtained (Table 4) for the determination of L-T_4 in serum demonstrated the suitability of the proposed immunosensors/sequential injection analysis system for on-line determination of the thyroid

Table 4. Determination of L-T_4 in serum using immunosensor/sequential injection analysis (SIA) system and the standard method[12]

L-T_4 (pmol/L) immunosensors/SIA	Standard method[12]
8.20	8.18
8.59	8.70
9.00	9.02
12.95	12.80
17.00	17.04
19.82	19.80
21.45	21.40
25.20	25.21
27.80	27.69

hormone in blood samples. Furthermore, they correlate very well with those obtained using the standard method.[12]

CONCLUSIONS

The sequential injection analysis/amperometric immunosensor system provides excellent features for the immunoassay of L-T_4 in pharmaceutical formulations, as well as in the blood samples. The design of the system is simple, fast, and reproducible. The reliability of the analytical information is assured by the low RSD values obtained in the recovery tests and by the rate of the sample analysed per hour (75 samples per h).

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