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Simultaneous determination of L-thyroxine (L-T_4), D-thyroxine (D-T_4), and L-triiodothyronine (L-T_3) using a sensors/sequential injection analysis system

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Abstract

Requirements of high purity and enantiopurity for the raw materials of active substances used for the pharmaceutical formulations involved utilization of high reliable analytical techniques for the analysis of the active compound. Sequential injection analysis system with electrochemical sensors as detectors proved to be a very good alternative for the chromatographic methods, as it is more reliable, not expensive, and faster. Drugs containing only L-thyroxine (L-T_4) or both L-T_4 and L-triiodothyronine (L-T_3) are formulated for the dysfunctions of thyroid. A sequential injection analysis system that can use two amperometric immunosensors (for the assay of L-T_3 and L-T_4) and an amperometric biosensor (for the assay of D-thyroxine, D-T_4) as detectors is proposed for the purity and enantiopurity tests of the raw materials used for the formulation of the drugs for thyroid. The system proved to be very reliable. The three compounds can be determined on-line in synthesis process control with a frequency of 20 samples per hour.

Keywords: L(D)-T_4, L-T_3; Amperometric biosensor; Amperometric immunosensor; D-Amino acid oxidase; Sequential injection analysis; Simultaneous detection

1. Introduction

The high purity and enantiopurity (for the substances with a chiral centre) are essential for the active compounds utilized in pharmaceutical formulations. Chromatographic methods especially if coupled with a MS detector have a big role on identifying the impurities, but unfortunately they are not always enough accurate and precise for quantifying the purity and enantiopurity of active compounds [1]. Radioimmunoassay is very efficient in the analysis based on an antigen–antibody reaction, but at the same time very expensive [1].

The electrochemical techniques are a good alternative to these techniques, being able to be used for enantiopurity tests (simultaneous detection of enantiomers) and purity tests of active compounds [2]. They can be used as detectors in a sequential injection analysis for on-line purity and enantiopurity tests assuring a better reliability of the analytical information and a faster time of analysis.

L-T_4 ((+)-3,3′,5,5′-tetraiodo-L-thyronine) is a drug utilized for the treatment of thyroid dysfunctions. It can be present as unique active compound or combined with L-triiodothyronine (L-T_3) in the pharmaceutical formulation. D-Thyroxine (D-T_4) is a by-product in the synthesis of L-T_4 and is not active in the thyroid. Therefore, enantiopurity and purity tests are required. The following techniques were proposed for the assay of L-T_3: HPLC [3], radioimmunoassay [4,5], and direct amperometry [6,7] and for the assay of L-T_4: HPLC [3], radioimmunoassay [8,9], fluorescence immunoassay [10], electrochemiluminescence [11,12], and direct amperometry [6,13].

The emphasis of this paper is on the SIA system designed for the simultaneous assay of L-T_4, D-T_4, and L-T_3. The electrochemical sensors used as detectors are amperometric immunosensors for the assay of L-T_4 and L-T_3 and amperometric biosensor for the assay of D-T_4. A phosphate buffer

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(pH 7.4) was used for the assay of L-T_4 and L-T_3 at 450 and 650 mV versus Ag/AgCl, respectively, and a phosphate buffer (pH 7.0) was used for the assay of D-T_4 at 650 mV versus Ag/AgCl.

2. Experimental

2.1. Electrodes design

2.1.1. Amperometric immunosensors for the assay of L-T_4 and L-T_3

The antiserums were diluted to a working dilution of 1:30 in 0.01 mol l^{-1} phosphate buffered saline, pH 7.4, containing 0.1% sodium azide [6,13]. 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 or *anti*-L-T_3 to obtain a final composition of 0.9% (w/w) in *anti*-L-T_4 or *anti*-L-T_3. The carbon paste (graphite powder and paraffin oil) was filled into a plastic pipette tip leaving about 3–4 mm empty in the top to be filled with the chemical modified carbon paste that contains *anti*-L-T_4 or *anti*-L-T_3. 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 each immunosensor was wetted with double distilled water and then polished with an alumina paper (polishing strips 30144-001, Orion). When not in use, the amperometric immunosensors were stored in a dry state at 4 °C.

2.1.2. Amperometric biosensor for the assay of D-T_4

Paraffin oil and graphite powder were mixed in a ratio 1:4 (w/w) to form a graphite paste [7]. Hundred microliters from the enzymatic solution (1 mg enzyme ml^{-1} of 0.1 mol l^{-1} phosphate buffer, pH 7.0; Merck, Darmstadt, Germany) of L-amino acid oxidase (L-AAOD) (EC 1.4.3.2, Type I: crude dried venom from *Crotalus adamanteus*, Sigma, St. Louis, MO, USA) were added to the carbon paste. A plastic tip was filled with the corresponding graphite–paraffin oil paste leaving an empty space of 3–4 mm in the top part filled with carbon paste containing the enzyme. The diameter of the sensor was 3 mm. Electric contact was obtained by inserting a silver wire into the carbon paste. The electrode tip was gently rubbed on fine paper to produce a flat surface. The surface of the electrode was wetted with de-ionized water and then polished with an alumina paper (polished strips 30144-001, Orion) before use. The biosensors were stored dry at 4 °C.

2.1.3. Apparatus

A 663 VA Stand (Metrohm, Herisau, Switzerland) in connection with a PGSTAT 20, a Multiplexer module SCNR16 and software (Eco Chemie version 4.9) was used for all chronoamperometric measurements. A glassy carbon electrode and a Ag/AgCl electrode served as the counter and reference electrodes in the cell.

2.1.4. Sequential injection system

The immunosensors and the biosensor were incorporated into the conduits of a 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, USA). Tygon tubing (0.76 mm i.d. for holding coil and 0.89 mm i.d. for the three reaction coils) was used to construct the manifold; coils were wound round suitable lengths of glass tubing (15 mm o.d.). A 0.1 mol l^{-1} NaCl solution was used as carrier. The capacity of the system is about 20 samples per hour. The device 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 [14] software package (obtainable from MINTEK) for computer-aided flow analysis was used throughout for device control.

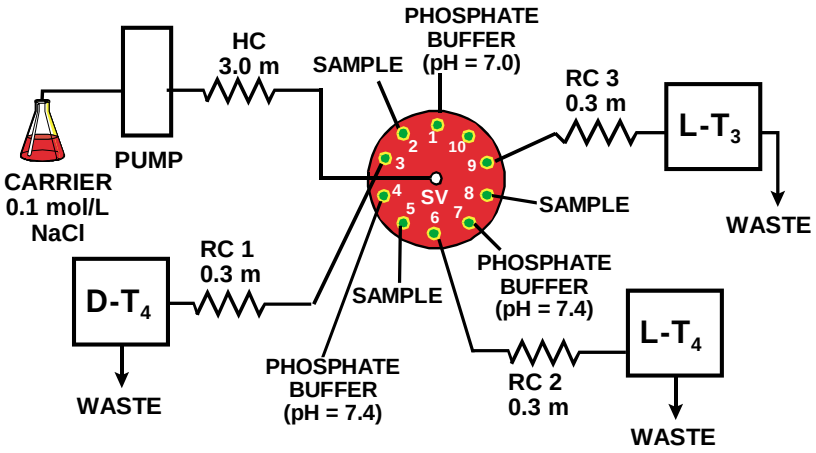

Fig. 1. Schematic flow diagram of SIA system used for the simultaneous determination of L-T_4, D-T_4, and L-T_3.

Table 1
Device sequence for one cycle of the SIA system

Time (s)	Pump	Valve	Description
0	Off	Buffer pH 7.0	Pump stop, select buffer stream (valve position 1)
5	Reverse	Buffer pH 7.0	Draw up buffer solution
9.5	Off		Pump stop
10.5		Sample	Select sample stream (valve position 2)
11.5	Reverse	Sample	Draw up sample solution
16	Off		Pump stop
17		D-T_4 cell	Select D-T_4 cell line (valve position 3)
18	Forward		Pump stack of zones to D-T_4 cell
48	Off		Pump stop
49		Buffer pH 7.4	Select buffer stream (valve position 4)
50	Reverse	Buffer pH 7.4	Draw up buffer solution
54.5	Off		Pump stop
55.5		Sample	Select sample stream (valve position 5)
56.5	Reverse	Sample	Draw up sample solution
61	Off		Pump stop
62		L-T_4 cell	Select L-T_4 cell line (valve position 6)
63	Forward		Pump stack of zones to L-T_4 cell
93	Off		Pump stop
94		Buffer pH 7.4	Select buffer stream (valve position 7)
95	Reverse	Buffer pH 7.4	Draw up buffer solution
99.5	Off		Pump stop
100.5		Sample	Select sample stream (valve position 8)
101.5	Reverse	Sample	Draw up sample solution
106	Off		Pump stop
107		L-T_3 cell	Select L-T_3 cell line (valve position 9)
108	Forward		Pump stack of zones to L-T_3 cell
138	Off	Home	Pump stop, return valve to starting position (valve position 1)

2.2. Reagents and materials

The immunological systems composed from L-T_4 and monoclonal *anti*-L-T_4 and L-T_3 and monoclonal *anti*-L-T_3 were supplied by Sigma. Synthroid® (Levothyroxine Sodium, USP) (injection containing 200 g L-T_4 ml^{-1}) was supplied by Bots Pharmaceuticals (Nottingham, UK) and Eltroxin® (tablets containing 50 g L-T_4 per tablet) was supplied by Glaxo Laboratories, Ltd. (Greenford, UK). Graphite powder with a particle size of 50 μm was supplied by Merck. Paraffin oil was supplied by Fluka (Buchs, Switzerland). Phosphate buffer (pH 7.0 and 7.4) was supplied by Merck. All other reagents were of the highest analytical grade. All the solutions were prepared using de-ionized water.

De-ionized water from a Modulab system (Continental Water Systems, San Antonio, TX, USA) was used for all solutions. The L-T_4 and D-T_4, and L-T_3 solutions were prepared from standard L-T_4, D-T_4, and L-T_3 solutions (10^{-2} mol l^{-1}), respectively, by serial dilutions.

3. Results and discussion

The optimization of the SIA system was described elsewhere [15]. An optimum flow rate of 3.61 ml min^{-1} was used to propel the solutions in the SIA system [15]. In the SIA system, the sample and buffer consumption is only 270 l each per measurement of L- and D-enantiomer of each substance, which is very economical. The SIA/sensors system is working at non-equilibrium conditions. Therefore, a major advantage of its utilization is the absolute repeatable handling of sampling due to the control of the flow pattern.

3.1. Sensors response

The response of the three sensors was determined using a chronoamperometric technique (E = 450 and 650 mV versus Ag/AgCl for the assay of L-T_4, L-T_3, and D-T_4, respectively [6,7,13]), at a pH 7.4 and 7.0 (phosphate buffer) when the immunosensors and the biosensor, respectively, are used as detectors. The pH values are optimum for the immunoreaction and for the enzymatic reaction, respectively [6,7,13]. The calibration equations obtained for the amperometric sensors are as follows:

$$\text{L}-\text{T}_4 : H = 0.92 + 0.57C; \quad r = 0.9998; \quad \langle H \rangle = \text{pA}; \quad \langle c \rangle = \text{ng ml}^{-1}$$

$$\text{D}-\text{T}_4 : H = 0.08 + 33.6C; \quad r = 0.9996; \quad \langle H \rangle = \text{nA}; \quad \langle c \rangle = \text{nmol l}^{-1}$$

$$\text{L}-\text{T}_3 : H = 0.02 + 0.47C; \quad r = 0.9999; \quad \langle H \rangle = \text{pA}; \quad \langle c \rangle = \text{ng ml}^{-1}$$

Table 2
Selectivity coefficients (pK_{amp}) for the amperometric sensors utilized as detectors in SIA system

Interfering species (J)	D-T_4	L-T_4	L-T_3	PVP
L-T_4	11.82	–		4.02
D-T_4	–	3.60		4.42
L-T_3	10.92	9.82	–	4.05

All values are the average of 10 determinations.

where H is the peak height and C, concentration of L-T_4, D-T_4, and L-T_3.

Linear concentration ranges between 10 and 780 ng ml^{-1}, and between 50 and 500 nmol l^{-1} and between 15 and 380 ng ml^{-1} for L-T_4, D-T_4, and L-T_3, respectively, with limits of detection of 8.52 ng l^{-1}, 20 nmol l^{-1}, and 12 ng ml^{-1}, respectively, were obtained for the amperometric immunosensors and amperometric biosensor. The working concentration ranges as well as the limit of detection demonstrated the suitability of the proposed sensors for the on-line purity and enantiopurity tests.

The response obtained for the sensors revealed a good stability and reproducibility over the tests performed for 2 weeks. (The R.S.D. values obtained for the response of the proposed electrodes during this period were less than 0.1%.) The high precision in possible due to the complete automation of the system and because a memory effect is not present as we let the buffer brush the surface of the sensors for a period, which is sufficient to keep the sensor surface clean.

3.1.1. Selectivity of the sensors

The selectivity of the sensors was checked using the mixed solutions method, over L(D)-T_4, L-T_3, and polyvinylpyrolidone (PVP). Amperometric selectivity coefficients were determined following the method proposed by Wang [16]. PVP is very often used as compression compound for tablets. In the evaluation, the concentration of the interferent, was selected to be 10 times higher than for the enantiomer of interest. As is shown in Table 2, the proposed biosensors are enantioselective when used as detectors in a SIA system. The results obtained, revealed that both sensors also have a good selectivity over PVP. Inorganic cations such as Na^+, K^+, Ca^{2+} do not interfere in the analysis of enantiomers.

3.2. Analytical applications

The flow systems obtained by incorporation of the amperometric immunosensors and amperometric biosensor in the conduits of a SIA system, proved to be useful for the simultaneous assay of L-T_4, D-T_4, and L-T_3 in L-T_4 raw material, with average recoveries of 99.98 ± 0.02% (n = 10), 99.92±0.02% (n = 10), and 99.93±0.03% (n = 10) which are in concordance with the results obtained using the standard methods proposed by US Pharmacopoeia.

Table 3
Determination of L-T_4 from pharmaceutical products

Sample	Numbers	Recovery (%)	
		L-T_4	D-T_4
Eltroxin®	1	99.87 ± 0.01	0.09 ± 0.02
	2	99.90 ± 0.01	0.08 ± 0.02
	3	99.92 ± 0.03	0.08 ± 0.03
Synthroid®	1	99.53 ± 0.02	0.21 ± 0.03
	2	99.49 ± 0.02	0.19 ± 0.02
	3	99.60 ± 0.01	0.24 ± 0.03

Content uniformity assay. All values are the average of three determinations.

The results obtained for the uniformity content test of Eltroxin® tablets and Synthroid® injection are presented in Table 3. L-T_4 can be reliably assayed from the tablets and injection with a high average recovery and low R.S.D.% values. The results are in good agreement with those obtained using the US Pharmacopoeia methods: for L-T_4: 99.90 ± 1.02% (proposed method) and 99.55 ± 1.48% (standard method), and for D-T_4: 0.07±0.02% (proposed method) and 0.18±0.04% (standard method) for the pharmaceutical formulations: Eltroxin® and Synthroid®, respectively [17]. No presence of L-T_3 was identified in these final pharmaceutical formulations of L-T_4. The advantage of the proposed method versus the one recommended by the US Pharmacopoeia is the simplicity and higher precision due to the lower values of the R.S.D. (%).

4. Conclusions

The paper opens a new and very important field in the utilization of different sensors as detectors in a SIA system for multiple determinations. The main advantages of the proposed system are: simplicity of construction and operation—that involved its introduction for on-line monitoring of enantiomers in the synthesis of enantiomers stream, high reliability of analytical information, rapidity, and low cost of the analysis. The high precision of the flow-based systems is due to the fact that all the measurements are done after the same interval of time, the surface of the sensors being continuously polished by the sodium chloride solution.

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