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Exploiting π-acceptors for the determination of thyroid hormones (T3 and T4) using a single interface flow system

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ABSTRACT

A fully automated methodology was developed for the determination of the thyroid hormones levothyroxine (T4) and liothyronine (T3). The proposed method exploits the formation of highly coloured charge–transfer (CT) complexes between these compounds, acting as electron donors, and π-acceptors such as chloranilic acid (CLA) and 2,3-dichloro-5,6-dicyano-*p*-benzoquinone (DDQ). For automation of the analytical procedure a simple, fast and versatile single interface flow system (SIFA) was implemented guaranteeing a simplified performance optimisation, low maintenance and a cost-effective operation. Moreover, the single reaction interface assured a convenient and straightforward approach for implementing Job's method of continuous variations used to establish the stoichiometry of the formed CT complexes.

Linear calibration plots for levothyroxine and liothyronine concentrations ranging from 5.0×10^{-5} to 2.5×10^{-4} mol L^{-1} and 1.0×10^{-5} to 1.0×10^{-4} mol L^{-1}, respectively, were obtained, with good precision (R.S.D. <4.6% and <3.9%) and with a determination frequency of 26 h^{-1} for both drugs. The results obtained for pharmaceutical formulations were statistically comparable to the declared hormone amount with relative deviations lower than 2.1%. The accuracy was confirmed by carrying out recovery studies, which furnished recovery values ranging from 96.3% to 103.7% for levothyroxine and 100.1% for liothyronine.

1. Introduction

Charge–transfer (CT) reactions, which involved the formation of intensely coloured complexes between electron donors with a sufficiently low ionization potential, and acceptors exhibiting a sufficiently high electron affinity [1], have been extensively studied and applied in distinct analytical circumstances including the spectrophotometric determination of a variety of pharmaceutical products. Some π-acceptors such as chloranilic acid (CLA), 2,6-dichloroquinone-4-chloramide (DCQ), 2,3-dichloro-5,6-dicyano-*p*-benzoquinone (DDQ) and 7,7,8,8-tetracyano-*p*-quinodimethane (TCNQ) are known to yield CT complexes and radical ions with a variety of electron donors [2].

Thyroid hormones (3,5,3′,5′-tetraiodothyronine, T4 and 3,5,3′-triiodothyronine, T3) are compounds with a noteworthy biological relevance as they play a critical role in the normal development of the central nervous system in infants, skeletal growth and maturation in children, as well as in the normal functioning of multiple organ systems in adults [3]. A deficient or excessive production of thyroid hormones may result in clinical disorders in man known as hypothyroidism and hyperthyroidism, respectively. For the treatment of hypothyroidism, thyroid hormone pharmaceutical preparations are used.

Several analytical methods relying on immunoassays [4,5], electrochemistry [6–8], chromatography [9–14], spectrophotometry [15,16], fluorescence [17] and chemiluminescence [18] have been reported for the determination of thyroid hormones in body fluids and pharmaceuticals. Although some of them are fairly selective they also present important shortcomings, *e.g.* utilization of expensive instrumentation, complex operation and maintenance, narrow working dynamic range, use of hazardous radioactive materials or toxic solvents, etc. In addition, low sample throughput has been often noted, as they may require several minutes per assay cycle for sample incubation or may involve lengthy procedures such as, *e.g.* those requiring column preparation or preparation of working electrodes.

Flow injection (FIA) and sequential injection (SIA) analytical procedures have already been reported for the determination of thyroid hormones with amperometric [8] and UV [15] detection, respectively. The main advantages of flow-based methods include robustness, simplicity and low cost of instrumentation, rapidity of analysis and excellent precision and accuracy.

The recently proposed single interface flow systems (SIFA) [19] presents some additional advantages in relation to FIA and SIA

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systems that rely on the premise that well-defined and compelling sample and reagent volumes no longer have to be optimised because reaction development depends exclusively on the establishment, during system operation, of an unique reaction interface where mutual sample and reagent interpenetration occurs, which facilitates system configuration thus enhancing simplicity and operational versatility.

In this work the formation of CT complexes between levothyroxine and liothyronine, as electron donors, and π-acceptors such as CLA, DCQ, DDQ and TCNQ has been for the first time investigated. The analytical potential of SIFA flow systems was exploited for implementation of fully automated flow-based procedures for the spectrophotometric monitoring of these thyroid hormones in pharmaceutical formulations. The versatility of SIFA also enabled the on-line implementation of the Job's method of continuous variations aiming at establishing the stoichiometry of the formed CT coloured complex.

2. Experimental

2.1. Reagents and solutions

All solutions were prepared with water from a Milli-Q system (conductivity < 0.1 μS cm^{-1}) and chemicals of analytical reagent grade quality. Reagents were then not subject to any further purification.

3,5,3′,5′-tetraiodothyronine (T4, levothyroxine) and 3,5,3′-triiodothyronine (T3, liothyronine) were purchased from Sigma (St. Louis, MO, USA). A 1.0×10^{-3} mol L^{-1} T4 solution was daily prepared by dissolving 22.2 mg in 25 mL of absolute ethanol (Panreac, Barcelona, Spain) and ultrasonicating for 30 s. Working standard solutions were prepared by suitable dilutions with absolute ethanol. A 1.0×10^{-4} mol L^{-1} T3 solution was daily prepared by dissolving 3.36 mg in 0.120 mL of a 0.1 mol L^{-1} NaOH solution, ultrasonicating for 30 s; this solution was diluted up to 50 mL with absolute ethanol. Working standard solutions were prepared by suitable dilutions with absolute ethanol.

CLA (BDH, Poole, UK), DCQ (Merck, Darmstadt, Germany), DDQ (Sigma, Steinheim, Germany) and TCNQ (Fluka, Austria) were used without further purification. The 1.0×10^{-3} mol L^{-1} CLA and DDQ solutions were daily prepared by dissolving 20.9 and 22.7 mg, respectively, in 100 mL of absolute ethanol.

2.2. Sample treatment

Commercial tablets with nominal contents of 25, 50 and 100 μg T4 and 25 μg T3 were analysed. To this end, twenty tablets were weighted and finely grounded. An accurately weighed powder equivalent to about 100 μg T4 or 25 μg T3 was dissolved in absolute ethanol, subject to ultrasonication for 30 s, and then filtered through a 0.20 μm membrane filter.

2.3. Apparatus

The SIFA system comprised two automatic burettes model Micro BU 2031 (Crison Instruments, Allelam, Spain) equipped with 5 mL syringes, two 161 T 031 (NResearch, West Caldwell, USA) two-way solenoid valves, a Jenway 6305 spectrophotometer (Jenway, Dunmow, UK) equipped with a 80 μL internal volume flow cell (10 mm optical path) and reaction coils made of PTFE tubing (0.8 mm i.d.). Home made end-fittings and connectors were also used.

A Pentium-I-based computer was used for system control and for data acquisition and treatment; software was developed in Microsoft Quick-Basic 4.5. The computer was equipped with a PC-LABCard model PCL-711B (Advantech, Taipei, Taiwan) interface card. The automatic burettes were connected to a serial port (RS-232C). A homemade power drive based on the ULN2003 chip or a CoolDrive (NResearch Inc., West Caldwell, USA) was used to operate the solenoid valves.

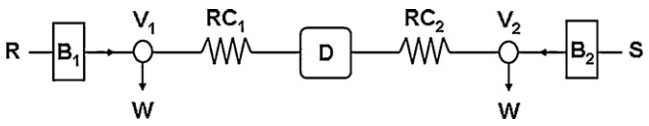

Fig. 1. Single interface flow manifold for the determination of T3 and T4. B_1 and B_2: automatic burettes; V_1 and V_2: solenoid valves; RC_1 and RC_2: reaction coils (65 cm); D: spectrophotometric detector; W: waste; R: reagent, π-acceptor; S: sample or standard.

Analytical signals were also recorded in paper using a Kipp & Zonen (Delft, The Netherlands) BD 111 strip chart recorder.

Spectrophotometric measurements were carried out in a UV–vis Spectrometer model Lambda 45 (Perkin-Elmer Instruments Inc., Norwalk, USA).

2.4. Single interface flow manifold and procedure

The flow manifold (Fig. 1) comprised two automatic burettes (B_1 and B_2) for inserting and propelling the sample and reagent solutions, and two two-way (normally closed) solenoid valves (V_1 and V_2) for directing the flowing streams. The reactions coils, identically lengthened, were placed on both sides of the detector.

The analytical cycle was started by establishing baseline, which was accomplished with the π-acceptor solution. To this end, V_1 was open and B_1 was actuated for propelling the reagent through the reactors as well as through the detector. After reaching V_2 these solutions were discarded. Subsequently, V_2 was opened, B_1 was switched off and the sample solution was inserted into the analytical path by means of actuation of B_2 establishing the single sample/reagent interface that was propelled backwards in order to reach V_1. The reaction products formed as a consequence of the mutual sample/π-acceptor intermingle produced an analytical signal, which was recorded as a peak when the reaction interface passed through the spectrophotometric flow cell. The absorbance of the T4–CLA complex was measured at 538 nm whereas the T3–DDQ complex was monitored at 460 nm.

3. Results and discussion

3.1. Reaction mechanism and spectral characterization

Levothyroxine and liothyronine present high electron density sites, so they may act as powerful electron donors. In the presence of a polar solvent like ethanol, these substances exhibit a maximum absorption band at 300 nm. Upon addition of different π-acceptors to the drug solution, namely CLA, DCQ, DDQ and TCNQ, a bathochromic shift was observed, which could be attributed to the formation of CT complexes. Interaction of T4 with CLA led to a strong absorption band at 538 nm whereas the T3–DDQ complex exhibited maximum absorption at 460 nm (Fig. 2).

The selection of the most suitable solvent for reaction implementation is a very important aspect affecting not only the sample solubility but also the electron transfer and the dissociation of the formed complex into the chromogen free radical anion. In order to select the solvent for CT complex formation, the reaction of CLA, DCQ, DDQ and TCNQ with both T3 and T4 was carried out in different solvents, such as acetonitrile, methanol and ethanol. Acetonitrile did not dissolve both compounds, T4 exhibit similar solubility in methanol and ethanol and T3 was fairly soluble in methanol but only slightly soluble in ethanol. For this reason, T3 was initially solubilised in a minute quantity of sodium hydroxide

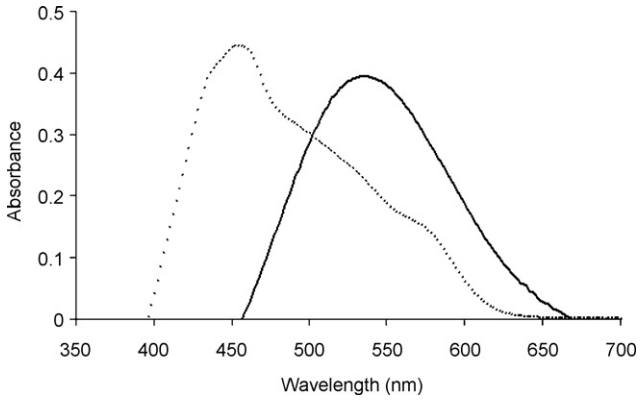

Fig. 2. Absorption spectra of T4–CLA (solid line) and T3–DDQ (dashed line) complexes in ethanol.

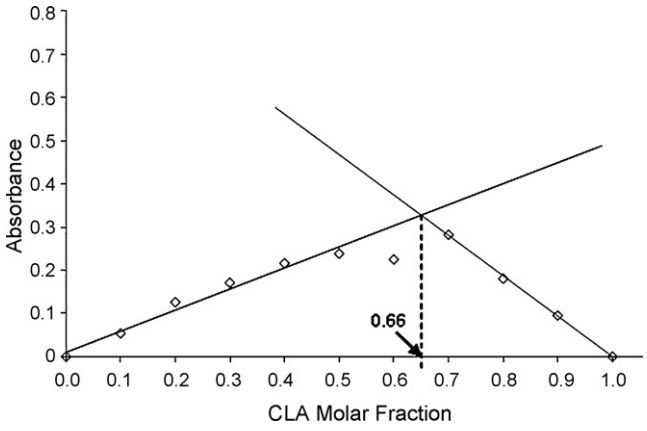

Fig. 3. Continuous variation plot for the reaction of T4 and CLA.

and then diluted with ethanol. When comparing the performance of methanol with ethanol, the later was considered the most adequate solvent for reaction development because it provided an excellent solvating power for CLA, DCQ, DDQ and TCNQ, and enabled the highest yield of the radical anion. As ethanol is a more polar solvent complete transfer of charge from donor to acceptor thus formation of radical anion as the predominant chromogen was more easily accomplished.

The π-acceptor was selected by taking into consideration the highest colour intensity of the formed complex for the same thyroid hormone concentration. For the T4 determination, CLA was chosen as the ideal π-acceptor, whereas DDQ was elected for the T3 determination. These reagents formed the complexes with the highest molar absorptivities and provided fast reaction kinetics even at room temperature.

As it was previously referred, upon reaction of CLA with T4, in ethanol medium, a characteristic large wavelength absorption band at 538 nm was noted, emphasizing the formation of a CT complex through the interaction of CLA as π-acceptor and the studied substance as *n*-donor followed by the formation of radical anion according to the following scheme:

$$\overset{D}{\text{donor}} + \overset{A}{\text{acceptor}} \leftrightarrow \overset{(D-A)}{n-\pi\ \text{complex}} \leftrightarrow \overset{A^{\bullet-}+D^{\bullet-}}{\text{radical ions}}$$

The dissociation of the complex was fostered in view of the ionizing potential of ethanol, which promoted complete electron transfer from the donor to the acceptor, resulting in the formation of the CLA radical anion as the major chromogen. The mechanism for the liothyronine–DDQ complex was similar.

3.2. Chemical parameters

Influence of CLA and DDQ concentration was investigated for concentrations ranging from 1.0×10^{-4} to 5.0×10^{-3} mol L^{-1} and from 2.5×10^{-5} to 1.0×10^{-3} mol L^{-1}, respectively. In both cases the obtained analytical signals showed a pronounced increase up to CLA and DDQ concentration values of 1.0×10^{-3} mol L^{-1} approaching stabilisation for higher values. Therefore, 1.0×10^{-3} mol L^{-1} CLA and DDQ solutions were used in the subsequent experiments.

3.3. Single interface flow methodology

In the development of the SIFA methodology several experiments were carried out in order to improve system performance, namely in terms of sensitivity, accuracy, precision and sampling rate. These features influenced the choices made during the optimisation of the systems, which was carried out using the univariate method. Since no well-defined sample or reagent volumes were used, these parameters were not subject to evaluation, thus simplifying the optimisation process.

The length and configuration of the reaction coils were assessed, for each of the hormones, by using 1.0×10^{-4} mol L^{-1} T4 and 5.0×10^{-4} mol L^{-1} CLA, and 2.5×10^{-4} mol L^{-1} T3 and 2.5×10^{-4} mol L^{-1} DDQ, respectively. The evaluation of the analytical signals obtained with reaction coils ranging from 15 to 115 cm revealed that the analytical signal almost double in both cases up to a coil length of 65 cm, beyond which it approached stabilisation. The shape of the reactor was also assessed by evaluating 65 cm reactors with straight and serpentine configurations. The serpentine configuration provided higher analytical signals probably due to an improved radial mass transport that assures a good sample/reagent mixing while minimising sample broadening and dispersion. Consequently, 65 cm serpentine reactors were selected for further experiments.

Influence of flow rate of the π-acceptor solution used to propel the reaction interface was assessed between 0.1 and 1.0 mL min^{-1}. Increasing flow rate increased the magnitude of the analytical signal up to 0.3 mL min^{-1} and then a slight decrease was observed. A 0.3 mL min^{-1} flow rate was then selected.

After optimization, the SIFA system was used to implement the Job's method of continuous variations in order to estimate the stoichiometry of the formed CT complexes by evaluating the molar ratio of the reactants (drug:π-acceptor) in the formed CT complexes. The results allowed one to infer molar ratios of 1:2 for T4–CLA (Fig. 3) and of 1:3 for T3–DDQ.

3.4. Interferences

After system dimensioning, the interfering effects of the excipients present in the pharmaceutical formulations, such as talc, starch, lactose, sodium citrate and magnesium stearate, were evaluated. A potential interfering species was considered as non-interfering if the analytical signal variation was lower than 3% in relation to the one obtained in its absence. It was found that up to a 100-fold excipient/drug molar ratio (maximum tested value) no interfering effect was observed. These results confirmed the suitability of the proposed method for the determination of T4 and T3 in pharmaceutical formulations.

3.5. Application to pharmaceutical formulations

Under the analytical conditions exhibited in Table 1, linear working concentration ranges between 5.0×10^{-5} to 2.5×10^{-4} mol L^{-1} and 1.0×10^{-5} to 1.0×10^{-4} mol L^{-1} were obtained for T4 and T3,

Table 1
Range of values used in dimensioning the SIFA system, and selected operating conditions for the determination of T4 and T3.

Parameter	Range	Chosen value
Reactor length (cm)	15–115	65
Flow rate (mL min^{-1})	0.1–1.0	0.3
CLA concentration (mol L^{-1})	1.0×10^{-4} to 5.0×10^{-3}	1.0×10^{-3}
T4 concentration (mol L^{-1})	1.0×10^{-5} to 1.0×10^{-3}	5.0×10^{-5} to 2.5×10^{-4}
DDQ concentration (mol L^{-1})	2.5×10^{-5} to 1.0×10^{-3}	1.0×10^{-3}
T3 concentration (mol L^{-1})	1.0×10^{-6} to 1.0×10^{-4}	1.0×10^{-5} to 1.0×10^{-4}

Table 2
Results obtained by SIFA methodology (C_{SIFA}) and declared amounts.

Sample	Declared amount (μg)	C_{SIFA}[a] (μg)	RD (%)
Letequatro (T4)	100	101.4 ± 1.5	1.4
Letter 20 pills (T4)	100	98.4 ± 2.1	−1.6
Letter 60 pills (T4)	100	98.7 ± 1.9	−1.3
Thyrax 25 (T4)	25	24.5 ± 0.1	−2.1
Thyrax 100 (T4)	100	101.4 ± 0.7	1.4
Eutirox 25 (T4)	25	24.8 ± 0.4	−0.8
Eutirox 50 (T4)	50	50.6 ± 0.3	1.2
Eutirox 100 (T4)	100	99.2 ± 3.1	−0.8
Neo-tiroimade (T3)	25	24.9 ± 0.5	0.5

[a]Mean and deviations based on three replications.

respectively. The analytical curves were typically expressed as

$$T4 : A = 1045C + 0.033$$

$$T3 : A = 4025.6C + 0.0163$$

where A is the absorbance and C is the T4 or T3 molar concentration (expressed in mol L^{-1}). The correlation coefficients were 0.9992 and 0.9990 for T4 and T3, respectively.

In order to evaluate the applicability and the accuracy of the proposed method in the analysis of real samples, it was applied to commercially available pharmaceutical formulations. The results (Table 2) were in a fairly good agreement with the amount labelled, with relative deviations between −2.1% and 1.4%. Furthermore, the repeatability was good, with a relative standard deviation lower than 4.6% and 3.9% ($n = 3$) for T4 and T3, respectively, and the sampling rate was about 26 h^{-1} for both analyses.

The accuracy was assessed by spiking levothyroxine and liothyronine formulations with a known concentration of T4 or T3 standard (100 and 25 μg, respectively). Recoveries ranging from 96.3% to 103.7% were found for T4 and a recovery of 100.1% was found for T3.

4. Conclusions

Taking into consideration the chemical structure of levothyroxine and liothyronine, which could act as electron donors, the first time exploitation of π-acceptors as a means to form CT sample/reagent interactions assured a good selectivity and sensitivity as well as an expeditious, simple and accessible chemical pathway for the spectrophotometric determination of these hormones.

The proposed SIFA system allowed fast and reliable analyses of pharmaceutical formulations, with high operational simplicity, good system ruggedness and stability and low maintenance. The SIFA system yields high reproducible and reliable results as confirmed by the low relative deviations between the declared and found amounts as well as the good recovery data obtained in the selectivity studies. Moreover, the single reaction interface assured a convenient and straightforward approach for implementing Job's method of continuous variations used to establish the stoichiometry of the CT complexes formed between T4 or T3 and the π-acceptors (CLA and DDQ, respectively).

When compared with most of the reported methods for the determination of thyroid hormones, the proposed method is simple, versatile and cost-effective. Furthermore, SIFA systems are easier optimized thus potentially more robust than other flow-based analytical procedures. These features make the developed SIFA system suitable for the routine quality control of these drugs in raw material and tablets without interference from the excipients present in the formulations.

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