CHAPTER-V
INTRODUCTION

Levothyroxine sodium (LVT) is the sodium salt of levo isomer of the thyroid hormone thyroxine which controls the growth and development of protein, lipid and carbohydrate metabolism [1,2]. They stimulate the oxygen consumption of body cells, resulting in increased energy expenditure, heat production and possess a cardiostimulatory effect that may be the result of a direct action on the heart. The production of levothyroxine hormone is regulated by the hypothalamus-pituitary axis through a negative feedback system. When hormone level becomes inadequate, the hypothalamus secretes thyroid stimulating hormone which stimulates the thyroid gland to produce levothyroxine.

Synthetic LVT is primarily used in the treatment of hypothyroidism and as a thyroid stimulating hormone (TSH) suppressant, in the treatment or prevention of various types of euthyroid goiters [3]. It is sensitive to light, temperature, moisture, pH and oxidation [4–6]. It has reported that LVT degradation in solution is pH-dependent, showing less degradation [4] at high
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pH. The proposed degradation pathway in solution follows deiodination, whereas in the solid state, degradation was predominantly by deamination. In fact, it has been shown that in addition to T₄ and T₃, 3,5-diiodothyronine (T₂); 3,3',5,5'-tetraiodothyroacetic acid (TTAA₄); 3,3',5-triiodothyroacetic acid (TTAA₃); 3,5-diiodothyroacetic acid (TTAA₂) (see Fig. 1) can be found in samples as a consequence of levothyroxine degradation [5–7]. Since the first synthetic LVT product was introduced in the United States in 1955 by Flint under the brand name Synthyroid [8], frequent recalls were initiated from the discovery of tablets being sub-potent before the labeled expiration date. This lack of stability and inconsistency in potency causes serious health consequences to patients. Adequate analytical methodologies are therefore required to assure the quality of the commercial samples. The usual methods for the determination of LVT are enzyme immunoassays [9,10] fluorescence [11] time resolved fluorescence [12] radioimmunoassay (RIA) [13] capillary electrophoresis with laser-induced fluorescence [14] HPLC [15] HPLC coupled with plasma mass spectrometry (ICP-MS) [16] chemiluminescence (CL) [17] flow-injection chemiluminescence (FIC) [18] cyclic-voltammetric (CV) [19]. However, these methods are expensive and time consuming.

Recently, RRS technique has aroused a great deal of interest among
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chemists and biochemists due to its high sensitivity, simplicity, rapidity and convenience in performance. To the best of our knowledge the Resonance Rayleigh scattering (RRS) technique has not been used for the determination of LVT. The RRS technique is based on the following phenomenon: when the wavelength of the incident beam of light is close to the absorption band of molecular particles which exist as aggregates, the Rayleigh scattering intensity of some wavelengths will be much higher than normal light scattering [20]. Since its first introduction to the study of the aggregation of porphyrins, using a conventional spectrofluorometer [21], the RRS has become a useful technique for the determination of biological macromolecules like nucleic acids and proteins [22,23]. This technique has also been used for the determination of drugs [24,25], inorganic ions [26] and surfactants [27]. The RRS method is generally based on the interaction of drugs with the probe. The most widely used RRS probes for pharmaceutical analysis include dyes and surfactants.

In this project we have used, for the first time, inorganic oxidant Fe(III) as RRS probe and applied redox reaction for the determination of LVT. Our aim is to study the effect of redox reaction of LVT with Fe(III) and complexation of reduced Fe(II) with \([\text{Fe(CN)}_6]^{3^-}\) which is ascertained from its absorption, RRS, SOS and FDS spectra and to develop highly sensitive, simple, fast and economical methods. We have employed these methods and developed
it particularly for the determination and identification of LVT

The spectral characteristics of absorption and emission (RRS, SOS and FDS0 spectra and the influencing factors were investigated.

**EXPERIMENTAL**

*Materials and method*

Stock solution (100μg/ml^-1) of levothyroxine (Sigma Aldrich) was prepared in water and kept in dark at 5 °C. The working solution (20 μg/ml^-1) was prepared from the stock solution. The Fe(III)solution (3.0×10^{-3}M) was prepared by dissolving ferric ammonium sulfate (NH₄Fe(SO₄)₂·12H₂O) in 1.0 ml concentrated H₂SO₄ and make up to 100 ml with water and [Fe(CN)₆]^{3-} (3.0×10^{-3}M ) was prepared in 250 ml distilled water.

*General procedure*

To a 10.0 ml flask were added certain amount of LVT, 0.2 ml of HCl (0.05M) solution and 1.0 ml (1.6×10^{-4}M) Fe(III). The mixture was heated in a boiling water bath for 25 min and cooled to room temperature, then added 1.0 ml (0.8×10^{-4}M) of [Fe(CN)₆]^{3-}. Finally, it was made up to the mark and mixed thoroughly. The RRS spectra were recorded after 20 min with synchronous scanning at λ_ex = λ_em, and the SOS and FDS spectra were recorded by scanning...
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at $\lambda_{ex} = 1/2\lambda_{em}$ and $\lambda_{ex} = 2\lambda_{em}$, respectively. The scattering intensity $I_{RRS}$, $I_{SOS}$ and $I_{FDS}$ for the reaction product and $I^0_{RRS}$, $I^0_{SOS}$ and $I^0_{FDS}$ for the reagent blank at their $\lambda_{max}$ were measured ($\Delta I = I-I_0$). The absorption spectra were recorded simultaneously.

RESULTS AND DISCUSSION

The absorption spectra

The UV absorption spectrum of LVT (Fig.2A) exhibits a broad peak at 270 nm. When ferric ammonium sulfate is added to it a new peak appears at 310 ascribed to the reduction of Fe(III) to Fe(II). Subsequently $[\text{Fe(CN)}_6]^{3-}$ was added to this mixture, which resulted in the formation of Prussian blue complex $\{\text{Fe}_4[\text{Fe(CN)}_6]_3\}$ [28]. It was verified by the appearance of a strong peak (Fig.2B) in the visible region at 710 nm with molar absorptivity ($\varepsilon$) $9.1 \times 10^4$ L mol$^{-1}$ cm$^{-1}$ which is the characteristic of the Prussian blue complex. Since this method has not been reported so far we have applied it for the determination of LVT.

Resonance Rayleigh scattering spectra

The RRS spectra of the LVT-Fe(III)-$[\text{Fe(CN)}_6]$ $^{3-}$ systems are shown in Fig.3. When Fe(III) was reduced by the LVT to produce Fe(II) in HCl and it further reacted with $[\text{Fe(CN)}_6]$ $^{3-}$ to form $\text{Fe}_4[\text{Fe(CN)}_6]_3$, RRS intensities were
enhanced greatly. It is directly proportional to the concentration of drug.

Second-order scattering (SOS) and frequency double scattering (FDS) spectra

The SOS and FDS spectra of LVT-Fe(III)-[Fe(CN)$_6$]$^{3-}$ systems (Fig. 4.5) show that their intensity changes with the change in incident wavelengths. When $\lambda_{ex}/\lambda_{em}$ was kept at 350/700 nm, the SOS intensity attained a maximum but the intensity of FDS reached maximum when $\lambda_{ex}/\lambda_{em}$ was maintained at 760/380 nm. In this condition, the two kinds of scattering intensities ($\Delta I_{SOS}$ and $\Delta I_{FDS}$) were directly proportional to the concentration of the LVT in a certain range.

Sensitivity of the methods

A plot of $\Delta I$ versus the concentration of LVT, the linear ranges, correlation coefficients and detection limits of RRS, SOS and FDS methods were investigated. The linear range and other parameters are given in Table 1. The sensitivity of RRS method is not only 1–3 orders of magnitude higher than the trivial spectrophotometric method but also higher than those of fluorescence, chemiluminescence and HPLC (Table 2). The RRS method was therefore taken as an example for further studies including optimum conditions, influencing factors, effects of coexisting substances and analytical applications.
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**Optimum conditions for the reaction**

**Effect of acidity**

The effects of HCl concentrations on the RRS of five systems were investigated. An optimum a concentration of HCl (0.05M) was, therefore, maintained (Fig.6).

**Effect of reagent's concentration**

The results of Fe(III) and [Fe(CN)$_6$]$^{3-}$ concentration on the $\Delta I_{\text{RRS}}$ is given in Fig.7-8. The maximum intensities of scattering was obtained when the Fe(III) concentration was kept between 1.2–2.0 $\times 10^{-4}$M. The Fe(III) concentration was, therefore, maintained at 1.6$\times 10^{-4}$M for the LVT systems. The optimum concentration of [Fe(CN)$_6$]$^{3-}$ was about 0.8$\times 10^{-4}$M. The $\Delta I_{\text{RRS}}$ decreased when the reagent concentrations were too low owing to incomplete reaction. The $\Delta I_{\text{RRS}}$ also decreases even when concentration was too high. (Fig.8).

**The complexation and formation of Fe$_4$[Fe(CN)$_6$]$_3$**

The LVT reduces Fe(III) to Fe(II) which further reacts with [Fe(CN)$_6$]$^{3-}$ to form a Prussian blue Fe$_4$[Fe(CN)$_6$]$_3$ complex:

$$\text{LVT}^{(x)} + \text{Fe}^{3+} \rightarrow \text{Fe}^{2+} + \text{LVT}^{(x-1)}$$
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\[ \text{Fe}^{2+} + [\text{Fe(CN)}_6]^{3-} \rightarrow \text{Fe}^{3+} + [\text{Fe(CN)}_6]^{4-} \]

\[ 4\text{Fe}^{3+} + 3[\text{Fe(CN)}_6]^{4-} \rightarrow \text{Fe}_4[\text{Fe(CN)}_6]_3 \]

\( \text{Fe}_4[\text{Fe(CN)}_6]_3 \) as a charge neutralization complex is strongly hydrophobic. Under the extrusion action of water, they drew close to each other and formed aggregate by Van der Waals force:

\[ n\{\text{Fe}_4[\text{Fe(CN)}_6]_3\} \rightarrow \{\text{Fe}_4[\text{Fe(CN)}_6]_3\}_n \]

It may be deduced that the formation of \( \{\text{Fe}_4[\text{Fe(CN)}_6]_3\}_n \) was the main reason for the enhancement in RRS, SOS and FDS intensities.

Selectivity of RRS method and its analytical application

Selectivity

We have investigated the effect of some common metal ions, salt and coexisting substances associated with the LVT in tablet form on the RRS method (Table 3). The results showed that this method does not suffer any interference from commonly associated excipients and additives in the tablets.

Analytical application

The accuracy of the RRS method was checked by performing recovery experiment through standard addition method (Table 4). The result showed that
% recovery was in the range (98.6–103.8) reflecting high accuracy and precision as indicated by the very low values of the %R.S.D (1.261–2.492). The method is, therefore, recommended for the determination of LVT in pharmaceutical formulation (Table 5).

**CONCLUSION**

We have developed a highly sensitive, simple and economical RRS method for the determination of LVT based on its redox reaction with Fe(III). LVT reduces Fe(III) to Fe (II) in acidic medium which further reacted with $[\text{Fe(CN)}_6]^{3-}$ to form a prussian blue complex, $\text{Fe}_4[\text{Fe(CN)}_6]_3$ which resulted in the enhancement of RRS, SOS and FDS intensities. The methods were successfully applied to the determination of trace amounts of LVT in pharmaceutical formulations. It may also be used for its determination in serum and urine samples.
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Fig. 1 Structure of Levothyroxine (LVT)
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Fig. 2(A) Absorption spectrum of LVT (measured against the reagent blank)
(1) Pure LVT (2) LVT-Fe(III)

Fig. 2(B) Absorption spectra of LVT-Fe(III)-Fe(CN)$_6^{3-}$. The concentrations of LVT: 6.0 μg/ml, concentration of Fe(III): 1.6×10$^{-4}$M, and concentration of Fe(CN)$_6^{3-}$: 0.6×10$^{-4}$M
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**Fig. 3** RRS spectra of LVT-Fe(III)-Fe(CN)$_6$$^{3-}$ system: 1. LVT. 2-9 LVT-Fe(III)-Fe(CN)$_6$$^{3-}$ (The concentration of LVT) 2.0 µg/ml$^1$, 3.0 µg/ml$^1$, 4.0 µg/ml$^1$, 5.0 µg/ml$^1$, 6.0 µg/ml$^1$, 7.0 µg/ml$^1$, 8.0 µg/ml$^1$ for spectrum 2 to 9 respectively) The concentrations of Fe(III): 1.6×10$^{-4}$M; concentration of Fe(CN)$_6$$^{3-}$: 0.6×10$^{-4}$M

**Fig. 4** SOS spectra of LVT-Fe(III)-Fe(CN)$_6$$^{3-}$ system: The concentration of LVT 2.5 µg/ml$^1$, 3.5 µg/ml$^1$, 4.5 µg/ml$^1$, 5.5 µg/ml$^1$, 6.5 µg/ml$^1$, 7.0 µg/ml$^1$, 7.5 µg/ml$^1$ for spectrum 2 to 7 respectively) The concentrations of Fe(III): 1.6×10$^{-4}$M; concentration of Fe(CN)$_6$$^{3-}$: 0.6×10$^{-4}$M
Fig. 5 FDS spectra of LVT-Fe(III)-Fe(CN)$_6^{3-}$ system: The concentration of LVT 2.6 $\mu$gml$^{-1}$, 2.8 $\mu$gml$^{-1}$, 3.4 $\mu$gml$^{-1}$, 4.4 $\mu$gml$^{-1}$, 5.5 $\mu$gml$^{-1}$, 6.4 $\mu$gml$^{-1}$, 7.2 $\mu$gml$^{-1}$ for spectrum 2 to 7 respectively) The concentrations of Fe(III): 1.6$\times 10^{-4}$M; concentration of Fe(CN)$_6^{3-}$: 0.6$\times 10^{-4}$M

Fig. 6 Effect of HCl concentration on $\Delta I_{RRS}$, LVT-Fe(III)-Fe(CN)$_6^{3-}$: The concentration of LVT 6.0 $\mu$gml$^{-1}$
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**Fig. 7** Effect of Fe(III) concentration on $\Delta I_{RRS}$, LVT-Fe(III)-Fe(CN)$_6^{3-}$: The concentration of LVT 6.0 $\mu$g ml$^{-1}$

**Fig. 8** Effect of Fe(CN)$_6^{3-}$ concentration on $\Delta I_{RRS}$, LVT-Fe(III)-Fe(CN)$_6^{3-}$: The concentration of LVT 6.0 $\mu$g ml$^{-1}$
<table>
<thead>
<tr>
<th>Method</th>
<th>Wavelength</th>
<th>Linear range</th>
<th>Regression equation $C$, ($\mu g ml^{-1}$)</th>
<th>Correlation coefficient ($r$)</th>
<th>Detection limit ($3\sigma$, $\mu g ml^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RRS</td>
<td>375/375</td>
<td>2.8–8.0</td>
<td>$\Delta I = 119.43 + 891.14C$</td>
<td>0.9970</td>
<td>3.76</td>
</tr>
<tr>
<td>SOS</td>
<td>350/700</td>
<td>2.5–7.5</td>
<td>$\Delta I = -657.73 + 287.57C$</td>
<td>0.9967</td>
<td>13.95</td>
</tr>
<tr>
<td>FDS</td>
<td>760/380</td>
<td>2.6–7.2</td>
<td>$\Delta I = -429.56 + 206.59C$</td>
<td>0.9903</td>
<td>17.77</td>
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</table>
Table 2. Comparison of sensitivities of RRS method with other methods for the determination of LVT

<table>
<thead>
<tr>
<th>Method</th>
<th>Reagent</th>
<th>Medium condition</th>
<th>Linearity</th>
<th>λ&lt;sub&gt;max&lt;/sub&gt; (nm)</th>
<th>LOD</th>
<th>Literature</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC-UV-ICP</td>
<td>-</td>
<td>pH=2.3</td>
<td>-</td>
<td>225</td>
<td>28.9 - 34.5 μgml&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>16</td>
</tr>
<tr>
<td>CL</td>
<td>Luminol-H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>-</td>
<td>15–70 μgml&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>-</td>
<td>23 μgml&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>17</td>
</tr>
<tr>
<td>FIC</td>
<td>KMnO&lt;sub&gt;4&lt;/sub&gt; - Na2SO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Acidic</td>
<td>1×10&lt;sup&gt;-7&lt;/sup&gt;–2×10&lt;sup&gt;-6&lt;/sup&gt; M</td>
<td>-</td>
<td>5×10&lt;sup&gt;-9&lt;/sup&gt; M</td>
<td>18</td>
</tr>
<tr>
<td>RRS</td>
<td>LVT-Fe(III)-Fe(CN)&lt;sub&gt;6&lt;/sub&gt;&lt;sup&gt;3-&lt;/sup&gt;</td>
<td>pH=5.0</td>
<td>2.8–8.0 μgml&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>375/375</td>
<td>3.76×10&lt;sup&gt;3&lt;/sup&gt; ngml&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>SOS</td>
<td>LVT-Fe(III)-Fe(CN)&lt;sub&gt;6&lt;/sub&gt;&lt;sup&gt;3-&lt;/sup&gt;</td>
<td>pH=5.0</td>
<td>2.5–7.5 μgml&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>350/700</td>
<td>13.95×10&lt;sup&gt;3&lt;/sup&gt; ngml&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>FDS</td>
<td>LVT-Fe(III)-Fe(CN)&lt;sub&gt;6&lt;/sub&gt;&lt;sup&gt;3-&lt;/sup&gt;</td>
<td>pH=5.0</td>
<td>2.6–7.2 μgml&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>760/380</td>
<td>17.77×10&lt;sup&gt;3&lt;/sup&gt; ngml&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>This work</td>
</tr>
</tbody>
</table>

FIC: Flow-injection Chemiluminometric, HPLC-UV-ICP: High performance liquid chromatography with inductively coupled plasma mass spectrophometry
<table>
<thead>
<tr>
<th>Concomitant substances</th>
<th>Concentration (µg/mL)</th>
<th>Change in $\Delta I_{RRS}$ (%)</th>
<th>Concomitant substances</th>
<th>Concentration (µg/mL)</th>
<th>Change in $I_{RRS}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$^+$</td>
<td>120</td>
<td>-1.2</td>
<td>Lactose monohydrate</td>
<td>250</td>
<td>-10.5</td>
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<tr>
<td>K$^+$</td>
<td>180</td>
<td>-1.9</td>
<td>Erythrosine</td>
<td>10</td>
<td>2.5</td>
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<tr>
<td>Ca$^+$</td>
<td>200</td>
<td>-3.5</td>
<td>Titanium oxide</td>
<td>90</td>
<td>-5.5</td>
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<tr>
<td>Mg$^+$</td>
<td>200</td>
<td>-3.0</td>
<td>Sucrose</td>
<td>180</td>
<td>-6.1</td>
</tr>
<tr>
<td>Al$^{3+}$</td>
<td>130</td>
<td>-5.8</td>
<td>Silicon oxide</td>
<td>70</td>
<td>-3.7</td>
</tr>
<tr>
<td>Pb$^{2+}$</td>
<td>100</td>
<td>1.4</td>
<td>Magnesium stearate</td>
<td>35</td>
<td>-1.69</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>150</td>
<td>1.9</td>
<td>SDS</td>
<td>40</td>
<td>5.0</td>
</tr>
<tr>
<td>Cu$^{2+}$</td>
<td>150</td>
<td>1.75</td>
<td>β- Alanine</td>
<td>360</td>
<td>-2.35</td>
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<tr>
<td>β- CD</td>
<td>250</td>
<td>-2.8</td>
<td>Glycine</td>
<td>50</td>
<td>-1.2</td>
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</table>
Table 4. Evolution of accuracy and precision of proposed RRS method

<table>
<thead>
<tr>
<th>Added (µgml⁻¹)</th>
<th>Found ± SD² (µgml⁻¹)</th>
<th>(% E_r)</th>
<th>(% RSD)</th>
<th>(% Recovery)</th>
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<tbody>
<tr>
<td>3.0</td>
<td>3.116 ± 0.0776</td>
<td>3.722</td>
<td>2.492</td>
<td>103.8</td>
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<tr>
<td>4.0</td>
<td>3.946 ± 0.0792</td>
<td>1.368</td>
<td>2.008</td>
<td>98.6</td>
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<tr>
<td>5.0</td>
<td>5.148 ± 0.0649</td>
<td>2.874</td>
<td>1.261</td>
<td>102.9</td>
</tr>
<tr>
<td>6.0</td>
<td>6.206 ± 0.0890</td>
<td>3.319</td>
<td>1.434</td>
<td>103.4</td>
</tr>
<tr>
<td>7.0</td>
<td>7.110 ± 0.1392</td>
<td>1.5471</td>
<td>1.958</td>
<td>101.6</td>
</tr>
<tr>
<td>Method</td>
<td>Sample</td>
<td>Label claim</td>
<td>Found (mg)</td>
<td>Mean</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td>-------------</td>
<td>--------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>RRS</td>
<td>Electroxin-50</td>
<td>0.050</td>
<td>0.0511, 0.0498, 0.0512, 0.0499, 0.0501</td>
<td>0.0504</td>
</tr>
<tr>
<td></td>
<td>Electroxin-100</td>
<td>0.100</td>
<td>0.101, 0.103, 0.101, 0.102, 0.101</td>
<td>0.1016</td>
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<tr>
<td></td>
<td>Electroxin-200</td>
<td>0.200</td>
<td>0.201, 0.202, 0.199, 0.197, 0.207</td>
<td>0.2012</td>
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REFERENCES


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